Original Paper

Retinoic Acid Increases Fatty Acid Oxidation and Irisin Expression in Skeletal Muscle Cells and Impacts Irisin *In Vivo*

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Key Words

Fatty acid/oxidation • Obesity • Muscle • Retinoids/vitamin A • Nuclear receptors • Intramyocellular lipids • Myokines • PPAR β/δ • AMPK

Abstract

Background/Aims: All-trans retinoic acid (ATRA) has protective effects against obesity and metabolic syndrome. We here aimed to gain further insight into the interaction of ATRA with skeletal muscle metabolism and secretory activity as important players in metabolic health.

Methods: Cultured murine C2C12 myocytes were used to study direct effects of ATRA on cellular fatty acid oxidation (FAO) rate (using radioactively-labelled palmitate), glucose uptake (using radioactively-labelled 2-deoxy-D-glucose), triacylglycerol levels (by an enzymatic method), and the expression of genes related to FAO and glucose utilization (by RT-real time PCR). We also studied selected myokine production (using ELISA and immunohistochemistry) in ATRA-treated myocytes and intact mice. **Results:** Exposure of C2C12 myocytes to ATRA led to increased fatty acid consumption and decreased cellular triacylglycerol levels without affecting glucose uptake, and induced the expression of the myokine irisin at the mRNA and secreted protein level in a dose-response manner. ATRA stimulatory effects on FAO-related genes and the *Fndc5* gene (encoding irisin) were reproduced by agonists of peroxisome proliferator-activated receptor β/δ and retinoid X receptors, but not of retinoic acid receptors, and were partially blocked by an AMP-dependent protein kinase inhibitor. Circulating irisin levels were increased by 5-fold in ATRA-treated mice, linked to increased *Fndc5* transcription in liver and adipose tissues, rather than skeletal muscle. Immunohistochemistry analysis of FND5 suggested that ATRA treatment enhances the release of FND5/irisin from skeletal muscle and the liver and its accumulation in interscapular brown and inguinal white adipose depots. **Conclusion:** These results provide new mechanistic insights on how ATRA globally stimulates FAO and enhances irisin secretion, thereby contributing to leaning effects and improved metabolic status.

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Introduction

Skeletal muscle accounts for ~40% of total body weight and 50% of total energy expenditure in mammals and is a highly adaptable tissue that plays a key role in whole body lipid and glucose metabolism. Alterations in skeletal muscle metabolism are involved in insulin resistance, the metabolic syndrome and type 2 diabetes mellitus [1]. Further, skeletal muscle dynamically secretes signaling molecules that impact muscle metabolism and allow inter-organ communication to favor the orchestration of systemic functions, adaptative responses, and homeostasis. Signaling proteins produced and released by muscle fibers, collectively named myokines, exert their effects locally within the muscle and in a hormone-like fashion on distant organs of the body, such as adipose tissue, liver, pancreas, bones and brain [2]. Among them, irisin was discovered in 2012 in mice as a regulated myokine that induces the acquisition of brown adipose tissue (BAT) features in white adipose tissue (WAT) depots [3]. The latter phenomenon, known as WAT browning, similar to BAT activation can favor leanness and metabolic health [4]. In fact, although conflicting results are not lacking, a beneficial role of irisin in relation to metabolic diseases have repeatedly been described both in animals and humans [5, 6]. Irisin is encoded by the fibronectin type III domain containing five gene (*Fndc5*) and was originally proposed to result from proteolytic cleavage of the membrane-bound FNDC5 protein [3].

Retinoic acid, the carboxylic acid form of vitamin A, has many remarkable effects on lipid and energy metabolism [7-9]. Treatment with all-trans retinoic acid (ATRA) reduces body weight and adiposity independently of changes in food intake and improves insulin sensitivity and glucose tolerance in lean and obese mice [7]. ATRA-induced body fat loss associates with WAT browning [10, 11] and increased capabilities for fatty acid oxidation (FAO), oxidative metabolism and thermogenesis in skeletal muscle [12-14], besides activation of BAT [15] and of FAO in the liver [16]. Along with effects on lipid and energy metabolism, ATRA modulates adipose tissue/adipocyte-born signalling proteins (adipokines) involved in the control of energy balance and insulin sensitivity such as leptin, resistin and retinol binding protein [17-20]. Direct action of ATRA stimulating oxidative metabolism in cultured white adipocytes [11, 21] and hepatic cells [22] has been demonstrated, however, cell-autonomous effects of ATRA on skeletal myocytes and the possible impact of ATRA on myokine production have been less studied.

The objective of this work was to investigate ATRA's potential multifaceted effects on differentiated skeletal muscle cells, addressing its impact on lipid and glucose metabolism and myokine production. These aspects have been studied using ATRA-treated differentiated C2C12 myocytes and intact mice as experimental models.

Materials and Methods

C2C12 cell culture, differentiation and treatment

Mouse C2C12 myoblasts (ATCC-LGC Promochem, Barcelona, Spain) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 25 mM glucose supplemented with 10% w/v fetal bovine serum, antibiotics (50 IU penicillin/mL and 50 µg streptomycin/mL) and 3 mM glutamine (referred to as growth media). Cells were maintained at 37°C in a saturated humidity atmosphere containing 5% CO₂. To induce differentiation, C2C12 myoblasts were plated at an initial density of 1.2×10⁵ cells/well in 12-well culture dishes, which allowed reaching 80% confluency after 24 h; then, growth media was replaced by differentiation media, consisting in DMEM supplemented with antibiotics and 2% w/v horse serum. Differentiation media was replaced every two days until complete differentiation (10 days), when cells were fused into differentiated myocytes (myotubes). Differentiated myocytes were exposed during the 24 h before harvesting to a single ATRA dose (Sigma, Madrid, Spain) of 0.1, 1 and 10 µM final concentration dissolved in DMSO (final concentration 0.1%). No cytotoxicity of ATRA over that of DMSO was detected by colorimetric quantification of lactate dehydrogenase release into medium, measured using the LDH Cytotoxicity Assay Kit following the manufacturer's instructions (Thermo Scientific, Carlsbad, CA). In a second experimental design used, parallel cultures of differentiated myocytes were individually exposed for 24 h to ATRA (10 µM), the retinoid receptors agonists methoprene (isopropyl- (E,E)-(R,S)-11-

methoxy-3, 7,11-trimethyldodeca-2, 4-dienoate, 10 μ M) and TTNPB (p-[(E)-2-(5, 6,7, 8-tetrahydro-5, 5,8, 8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid, 10 μ M) and the selective peroxisome proliferator-activated receptor (PPAR) β/δ agonist GW0742 (0.1 μ M), all from Sigma and dissolved in DMSO. In other experimental designs, differentiated C2C12 myocytes were treated for 30 min or 24 h with ATRA (10 μ M) in the absence and the presence of the AMP-activated protein kinase (AMPK) inhibitor compound C (40 μ M) (6-[4-(2-Piperidin-1-ylethoxy) phenyl]-3-pyridin-4-ylpyrazolo [1, 5-a]pyrimidine; Calbiochem-Merck Millipore, Darmstadt, Germany). Cells receiving compound C were pre-incubated with the agent for 30 min before ATRA administration, and further incubated for the indicated time periods. In all experiments, control cells received the equivalent volume of vehicle (DMSO). Experiments were routinely performed at least twice in duplicates.

Animal study

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) received one daily subcutaneous injection of ATRA at a pharmacological dose of 50 mg/kg body weight during the 4 days before they were sacrificed (six animals per group). Control mice were injected the vehicle (100 μ l olive oil). The animals were kept at 22°C under 12-h light/12-h dark cycles (lights on at 08:00). Body weight and food intake during the treatment period were followed daily on a per-cage basis (three animals per cage). The ATRA-treated animals did not show any external signs of vitamin A toxicity nor liver enlargement at sacrifice (mean relative liver weight, in g per 100 g body weight: controls, 4.35 ± 0.19 ; ATRA, 4.43 ± 0.16), as in previous work using this experimental design [10, 13, 19]. Further, aminotransferase activity in serum, a marker of hepatic damage, was previously shown to be unaffected by the ATRA treatment applied [11, 18]. The animals were euthanized by decapitation at the start of the light cycle. Blood was collected and serum prepared by centrifugation and frozen at -20°C. Tissues including *gastrocnemius* muscle, liver, epididymal, inguinal and retroperitoneal WAT and interscapular BAT were excised in their entirety, weighted, snap-frozen in liquid nitrogen, and stored at -80°C. *Adductor/vastus* muscles were also sampled. Tissue specimens of *gastrocnemius* muscle, liver, BAT, and inguinal and epididymal WAT for histological analysis were sampled and fixed by immersion in 4% paraformaldehyde in 0.1 M sodium phosphate buffer (PB), pH 7.4. The sum of the weight of the individual WAT depots as percentage of body weight was used as adiposity index. All animal experiments were performed following the guidelines for the use and care of laboratory animals of the University of the Balearic Islands and the protocols were submitted to, and approved by, the University institutional review board.

RNA isolation

Total RNA was extracted from C2C12 myocytes and animal *gastrocnemius* muscle biopsies using Tripure Reagent (Roche, Barcelona, Spain) or EaZy Nucleic Acid Isolation Kit E.Z.N.A.™ (Omega Bio-Tek, Vermont, USA). Isolated RNA was quantified using the NanoDrop ND-1000 spectrophotometer (Nadrop Technologies Inc., Wilmington, Delaware, USA) and its integrity confirmed using agarose gel electrophoresis.

Real-time quantitative PCR (RT-qPCR)

Real-time polymerase chain reaction was used to measure the mRNA expression levels of the following genes: muscle-type carnitine palmitoyltransferase 1 (*Cpt1b*), acyl CoA oxidase (*Acox1*), uncoupling protein 3 (*Ucp3*), pyruvate dehydrogenase kinase 4 (*Pdk4*), lipoprotein lipase (*Lpl*), fatty acid translocase (*Cd36*), hormone sensitive lipase (*Lipe*), adipose triglyceride lipase (*Pnpla2*), peroxisome proliferator activated receptor d (*Ppard*), peroxisome proliferator activated receptor γ coactivator 1 α (*Ppargc1a*), glucose transporter 4 (*Slc2a4*), hexokinase (*Hk2*), insulin receptor (*Insr*), interleukin 6 (*Il6*), fibroblast growth factor 21 (*Fgf21*), fibronectin type III domain-containing 5 (*Fndc5*), and cytochrome P450 26A1 (*Cyp26a1*). For retrotranscription to cDNA, 0.25 μ g of total RNA (in a final volume of 5 μ l) was denatured at 65°C for 10 min and then incubated with MuLV reverse transcriptase (Applied Biosystem, Madrid, Spain) at 20°C for 15 min, followed by 30 min at 42°C, and finally 5 min at 95°C, in an Applied Biosystems 2720 Thermal Cycler (Applied Biosystem, Madrid, Spain). Each PCR was performed from diluted (1/5) cDNA template, forward and reverse primers (1 μ M each), and Power SYBR Green PCR Master Mix (Applied Biosystems, CA, USA). Primers were obtained from Sigma (Madrid, Spain) and are detailed in Table 1. Real time PCR was performed using the Applied Biosystems StepOnePlus™ Real-Time PCR Systems (Applied Biosystems) with the following profile: 10 min at 95°C, followed by a total of 40 two-temperature cycles (15 s at 95°C and 1

min at 60°C). In order to verify the purity of the products, a melting curve was produced after each run according to the manufacturer's instructions. The threshold cycle (Ct) was calculated by the instrument's software (StepOne Software v2.0) and the relative expression of each mRNA was calculated using Pfaffl's method [23], except when we compared the relative expression of *Fcnd5* mRNA between tissues that we used the $2^{-\Delta\Delta C_t}$ method [24]. Beta-actin (*Actb*) and 18S rRNA were used as reference genes.

Table 1. Primers used in the PCR reactions

gene	primer sequence		Accession number
18S rRNA	5'-CGCGGTTCTATTTTGTGGT-3'	5'-AGTCGGCATCGTTTATGGTC-3'	
Acox1	5'-TGGTGAAGAAGATGAGGGAGT-3'	5'-AGCAAGGTGGGCAGGAAC-3'	NM_025729
Actb	5'-TACAGCTTCACCCACAGCT-3'	5'-TCTCCAGGAGGAAGAGGAT-3'	NM_007393.3
Cd36	5'-GCTCAAGATGGTCCATTG-3'	5'-GTCCTGGCTGTGTTGGA-3'	NM_007643
Cpt1b	5'-AAGGGTAGATGGGCAGAGG-3'	5'-GCAGGAGATAAGGGTGAAGA-3'	NM_009948
Fgf21	5'-ACAGATGACGACAGGACAC-3'	5'-AGGCTTTGACACCCAGGATT-3'	NM_020013
Fcnd5	5'-ATGAAGGAGATGGGAGGAA-3'	5'-GCGGCAGAAGAGAGCTATAACA-3'	NM_027402
Hk2	5'-CAGCTAGACAGAGCATCC-3'	5'-CGCATCTTCCATGTAGCA-3'	NM_013820
Il6	5'-TGGGAAATCGTGGAAATGAG-3'	5'-GAAGGACTCTGGCTTTGTCTT-3'	NM_031168
Insr	5'-GTCGGCGTTTCATCAGAG-3'	5'-CTCCTGGGATTTCATGCTGT-3'	NM_010568
Lipe	5'-TCACGCTACATAAAGGCTGCT-3'	5'-CCACCCTAAAGAGGGAAT-3'	NM_010719.5
Lpl	5'-CTGATGACGCTGATTTTGT-3'	5'-TATGCTTTGCTGGGGTTTC-3'	NM_008509
Pdk4	5'-TCCTTCACACCTTCACCACA-3'	5'-AAAGAGGCGGTCAAGTATCC-3'	NM_013743
Pnpla2	5'-TGTGGCTCATTCCTCTAC-3'	5'-AGCCCTGTTTGACATCTCT-3'	NM_025802
Ppargc1a	5'-AGGAGGGTCATCGTTTGTGG-3'	5'-GGAGGCAGAAGAGCCGTC-3'	NM_008904
Pppard	5'-GCCTCGGGCTTCCACTAC-3'	5'-TCCGTCCAAGCGGATAG-3'	NM_011145
Slc2a4	5'-GGCATGCGTTTCCAGTATGT-3'	5'-GCCCTCAGTCATTCTCATC-3'	NM_009204
Ucp3	5'-GGAGGAGAGAGGAAATACAGAGG-3'	5'-CCAAAGGCAGAGCAAAGTGA-3'	NM_009464

FAO measurements in C2C12 myocytes

Oxidation of uniformly (U) ^{14}C -labeled palmitate to CO_2 and acid-soluble products (ASPs) in cultured cells was measured as previously described [21, 22], with minor modifications. Briefly, C2C12 myoblasts were differentiated as described above. Differentiated myocytes were treated for 22.5 h with 10 μM ATRA or vehicle (DMSO), after which the medium was removed and cells were further incubated at 37°C for 1.5 h in fresh medium containing 0.2 mM L-carnitine (Sigma, St. Louis, MO) and 200 μM [^{14}C (U)] palmitate (0.1 $\mu\text{Ci}/\text{mL}$, from Perkin Elmer, Boston, MA), in the continued presence of ATRA or vehicle. Prior the 1.5 h incubation, each well was individually covered with a piece of Whatman filter paper and the entire plate was sealed with parafilm. Following the 1.5 h incubation, the Whatman filter paper was soaked with 0.1 mL of methylbenzylamine:methanol (1:1) to trap the CO_2 produced, and 0.2 mL of HCl 6 M was injected into the wells to release the CO_2 present in the liquid phase to the gaseous phase. After 1 h of CO_2 capturing at room temperature, the pieces of Whatman filter paper were removed and transferred to scintillation vials for radioactivity counting. Total ASPs, which include ketone bodies, acyl-carnitines, Krebs cycle intermediates and acetyl-CoA [25], were separated by precipitation of the intact [^{14}C (U)] palmitate in 1 mL of the culture medium by adding 0.5 mL cold 1.5 M HClO_4 . After centrifugation (15 min, 1800 $\times g$), isolated ASPs present in the supernatant was measured using Hi-safe 3 scintillation cocktail (Perkin Elmer, Shelton, CT, USA) using a Beckman Coulter LS 6500 multi-purpose liquid scintillation counter (Beckman Coulter, Brea, CA, USA). Protein concentration in cell lysates of parallel culture wells was measured using the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA) as an indication of cell number.

2-deoxyglucose uptake in C2C12 myocytes

Cellular uptake of 2-deoxy-D-glucose (2-DOG) was evaluated in differentiated C2C12 myocytes pre-exposed for 24 h to ATRA or vehicle (DMSO) as described previously [26]. In brief, cells were first washed with Krebs-Ringer phosphate buffer (10 mM- KH_2PO_4 , pH 7.4, containing 136 mM-NaCl, 4.7 mM-KCl, 1.25 mM- CaCl_2 , 1.25 mM- MgSO_4 and 0.05 % bovine serum albumin) and then incubated for 15 min at 37°C in Krebs-Ringer phosphate buffer without insulin or with 100 nM insulin. The transport assay was then initiated by the addition of [^3H]-2-DOG (0.1 $\mu\text{Ci}/\text{mL}$ in 1 μM unlabeled 2-DOG) and developed for 10 min with or without 100 nM insulin at 37°C. Cells were then washed three times with ice-cold PBS and lysed with 0.1 M NaOH. Radioactivity in cell lysates was counted as described above. Protein concentration in the same cell lysates was measured using the BCA protein assay kit.

Quantification of myokines

Irisin concentration in the cells-conditioned culture media and mouse sera was measured using ELISA kit EK-067-52 from Phoenix Pharmaceuticals Inc. (Burlingame, CA, USA), considered the best validated ELISA kit for irisin to date [6], following the manufacturers' instructions. IL-6 and FGF21 concentration in the same samples were measured through ELISA kits from Thermo Fisher Scientific (Former Pierce, EM2IL6; Waltham, MA, USA), and R&D Systems Inc. (MF2100; Minneapolis, MN, USA) respectively.

Immunoblotting analyses

The phosphorylation status of AMPK and acetyl-CoA carboxylase (ACC) in cell lysates containing a protease inhibitor cocktail (Roche Diagnostic, Barcelona, Spain) was assessed by immunoblotting using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA), as previously described [27]. Polyclonal antibodies against p-ACC (Ser79) and ACC (#3661 and #3662, respectively) and monoclonal antibodies against p-AMPK α (Thr172) and AMPK α (#2535 and #2793, respectively) were used as primary antibodies (all from Cell Signaling Technology, Inc., Danvers, MA, USA). Corresponding infrared -dyed secondary antibodies (LI-COR Biosciences) were used.

Lipid measurements in C2C12 myocytes and mouse sera

Aliquots of cell lysates obtained in PBS were used to measure intracellular triacylglycerol content by means of an enzymatic colorimetric kit (Trygliceride INT20, Sigma, St. Louis, MO, USA). Commercial enzymatic colorimetric kits were used for the determination of serum nonesterified fatty acid (NEFA) (Wako Chemicals GmbH, Neuss, Germany) and 3-hydroxybutyrate (BEN Srl, Milan, Italy), according to the supplier's instructions.

Histology and immunohistochemistry

After washing in PB overnight, fixed tissue samples were dehydrated in a graded series of ethanol and embedded in paraffin blocks for light microscopy and immunohistochemistry. Sections (5 μ m) were immunostained for FNDC5 by means of the avidin-biotin technique. Briefly, sections preincubated with 5% H₂O₂ in water to block endogenous peroxidase and then with normal goat serum (Vector Laboratories, Burlingame, CA, USA; diluted 1:50 in PBS) to block unspecific sites were incubated overnight at 4°C with primary polyclonal anti-FNDC5-C-terminal antibody raised in rabbits (ab181884; Abcam, Cambridge, UK; diluted 1:200 in PBS), then with biotinylated anti-rabbit IgG secondary antibody raised in goat (Vector Laboratories; diluted 1:200 in PBS), and finally, with ABC complex (Vectastain ABC kit; Vector Laboratories). Peroxidase activity was revealed using 0.075% 3, 3'-diaminobenzidine hydrochloride as chromogen (Sigma, St. Louis, MO) in Tris buffer 0.05 M, pH 7.6. Appropriate positive controls were used to check antibody specificity. Sections were counterstained with hematoxylin and mounted in Eukitt (Kindler, Freiburg, Germany). Sections were observed with Zeiss Axioskop 2 microscope equipped with AxioCam Icc3 digital camera (Carl Zeiss, S.A., Barcelona, Spain). For the quantification of FNDC5 positive muscle fibers, ~210 fibers in discrete fascicles (occupying a total area of ~0.37 mm²) were examined per animal using a quantitative morphometric method, at 10 $\times$ magnification, with the assistance of Axio Vision software.

Statistical analysis

Data are expressed as means $\pm$ SEM. Statistical significance was assessed by Student's *t*-test or one-way ANOVA followed by least-significance difference (LSD) *post-hoc* comparison or two-way ANOVA. Results were considered statistically significant when *P*<0.05. In the animal experiment, Pearson correlation analyses between selected parameters were performed with pooled ATRA-treated and vehicle-treated animals. Statistical analyses were performed with SPSS 19.0 for windows (Chicago, IL, USA).

Results

ATRA activates FAO in differentiated C2C12 myocytes

ATRA treatment *in vivo* increases lipid oxidation and mitochondrial uncoupling capacity in skeletal muscle of mice [12-14]. To determine if effects on muscle metabolism reflect direct effects of ATRA on myocytes or are secondary to systemic effects of treatment, differentiated C2C12 myocytes were used as a cell-autonomous model system. The cells were exposed to a wide range of ATRA concentrations, from 0.1 to 10 μ M, and the mRNA levels of key genes in fatty acid handling and oxidation were analyzed (Fig. 1A). Exposure to ATRA resulted in a 1.4-fold increase in the mRNA levels of *Cpt1b*, which was already evident at the lowest dose tested and not increased at higher dosages, and in dose-dependent increases in the mRNA levels of *Ucp3* and *Pdk4* (up to ~3-fold increase for both genes at 10 μ M ATRA). Exposure to ATRA also induced the gene expression of *Cd36* (by 1.8-fold, already at the lowest dose tested) and *Pnpla2* (encoding adipose triglyceride lipase) (up to 3-fold). Gene expression of *Lpl* was decreased by ~2.5-fold by ATRA treatment, whereas gene expression of *Acox1*,

Ppard, *Ppargc1a*, and *Lipe* (encoding hormone sensitive lipase) was unaffected.

The functional consequences of the aforementioned changes in gene expression were analysed by evaluating the impact of ATRA exposure on the oxidation rate of exogenously supplemented radiolabelled fatty acids. Compared with vehicle-treated cells, C2C12 myocytes pre-exposed to 10 μ M ATRA displayed a significant 44% increase in palmitate oxidation to CO₂ together with a significant 17% decrease in palmitate oxidation to ASPs (Fig. 1B), indicating a more efficient mitochondrial FAO in the ATRA-treated cells. We also studied the impact of ATRA interaction with lipid metabolism on the accumulation of intramyocellular lipids. After 24 h treatment with 0.1 to 10 μ M ATRA, the intracellular triacylglycerol content of C2C12 myocytes was decreased by ~20% compared with vehicle-treated control cells (Fig. 1C). Changes in palmitate oxidation and triacylglycerol content took place in the absence of changes in the total amount of protein recovered per culture well (Fig. 1D).

ATRA does not affect 2-deoxyglucose uptake in differentiated C2C12 myocytes

The interaction of ATRA with glucose utilization in C2C12 myocytes was assessed by analyzing effects on the expression of selected related genes and on the actual cellular uptake of radiolabeled 2-DOG, a non-metabolizable glucose analogue widely used in measurements of cellular glucose uptake. Exposure to ATRA (up to 10 μ M, for 24 h) resulted in a 3-fold upregulation of *Slc2a4* mRNA levels (encoding GLUT4) without affecting gene expression of *Insr* and *Hk2*, which were used as indicators of the cellular capabilities for insulin sensing and glycolysis, respectively (Fig. 2A). 2-DOG uptake under basal or insulin-stimulated conditions were unaffected by exposure to ATRA. As expected, insulin exposure enhanced 2-DOG uptake (Fig. 2B).

ATRA induces irisin production in C2C12 myocytes

Reported effects of the myokine irisin favoring oxidative metabolism in skeletal muscle cells [28, 29] and WAT browning [3] are reminiscent of effects triggered by ATRA treatment. This, together with the fact that the promoter of the irisin precursor gene *Fndc5* contains a RXR heterodimer binding site in the mouse [30], prompted us to study the impact of ATRA

Fig. 1. ATRA treatment increases fatty acid oxidation in C2C12 myocytes. Cells on day 9 of differentiation were treated for 24 h with vehicle (DMSO) or ATRA at the indicated concentrations. The expression of the indicated lipid metabolism-related genes (normalized to the expression of 18S rRNA) (A), palmitate oxidation to CO₂ and acid soluble products (ASP) (B), cellular triacylglycerol content (C) and protein content (D) are shown. Separate cultures were used for each type of analysis. Data on gene expression and palmitate oxidation are expressed relative to the mean value in the vehicle-treated cells, which was set to 100%. Triacylglycerol content is expressed relative to protein content, and protein content is expressed as absolute values per well. Data are the mean $\pm$ SEM of at least two independent cultures made in triplicate (n=6). Statistical significance was assessed by Student's t-test (*, P<0.05, ATRA-treated vs vehicle-treated cells) or one-way ANOVA followed by LSD post hoc comparisons (P<0.05 for values not sharing a common letter).

Fig. 2. Effect of ATRA treatment on glucose uptake in C2C12 myocytes. Cells on day 9 of differentiation were treated for 24 h with vehicle (DMSO) or ATRA at the indicated concentrations. The expression of the indicated glucose metabolism-related genes (normalized to the expression of 18S rRNA) (A) and 2-deoxyglucose (2-DOG) uptake under basal and insulin stimulated conditions (B) are shown. Separate cultures were used for the two types of analysis. Data on gene expression and 2-DOG are expressed relative to the mean value in the vehicle-treated cells under basal conditions, which was set to 100%, and are the mean $\pm$ SEM of at least two independent cultures made in triplicate (n=6). Statistical significance was assessed by one-way ANOVA followed by LSD post hoc comparisons ($P < 0.05$ for values not sharing a common letter) or two-way ANOVA (*, $P < 0.05$, effect of insulin stimulation).

Fig. 3. Effect of ATRA treatment on irisin, FGF21 and IL-6 gene expression and secreted levels in C2C12 myocytes. Cells on day 9 of differentiation were treated for 24 h with vehicle (DMSO) or ATRA at the indicated concentrations. Gene expression (normalized to the expression of 18S rRNA) (A) and myokine levels in the cell's conditioned culture media (B) are shown. Irisin levels were determined by ELISA and are expressed as absolute values; other data are expressed relative to the mean value in vehicle-treated cells, which was set to 100%. Data are the means $\pm$ SEM of at least two independent cultures made in triplicate (n=6). Statistical significance was assessed by one-way ANOVA followed by LSD post hoc comparisons: values not sharing a common letter were considered statistically different ($P < 0.05$).

treatment on the production of irisin in skeletal muscle cells. Exposure of differentiated C2C12 myocytes to ATRA led to a dose-dependent upregulation of *Fndc5*/irisin expression, which was evidenced both at the mRNA level (Fig. 3A) and at the level of the irisin protein accumulated in the cells'-conditioned culture medium (Fig. 3B).

We also studied ATRA effects on myocyte production of FGF21 and IL-6, two signaling proteins that, similar to irisin, are secreted by the skeletal muscle (though not exclusively) and exert endocrine effects including WAT browning [31, 32]. *Fgf21* mRNA levels were equal in C2C12 myocytes exposed to vehicle or 10 μ M ATRA but higher in the cells exposed to 10 μ M ATRA than in those exposed to lower ATRA doses (Fig. 3A). FGF21 protein levels in the culture media were significantly increased in the 10 μ M ATRA-exposed cells relative to control (vehicle-exposed) cells (Fig. 3B). For IL-6, the results revealed a marked and dose-dependent down-regulation of gene expression in C2C12 myocytes by ATRA (Fig. 3A) that was accompanied by similarly decreased levels of IL-6 protein accumulated in the culture medium (Fig. 3B). Overall these data indicate that ATRA regulates myokine production in C2C12 myocytes.

ATRA modulation of lipid metabolism-related genes and Fndc5 in C2C12 myocytes is reproduced by PPAR β / δ and RXR agonists

ATRA interacts with nuclear receptors: it is the specific ligand for the retinoic acid receptors (RARs) and it can also bind to and activate PPAR β / δ (but not the other PPAR subtypes) [7]. Further, ATRA can isomerize in the cells to 9-*cis* retinoic acid, which activates

Fig. 4. RXR and PPAR β/δ activation mimic the effects of ATRA on fatty acid oxidation-related genes and the irisin gene in C2C12 myocytes. Cells on day 9 of differentiation were treated for 24 h with vehicle (DMSO), ATRA, the pan-RAR agonist TTNPB, the pan-RXR agonist methoprene, or the selective PPAR β/δ agonist GW0742 at the indicated concentrations. Gene expression of the indicated genes was measured and normalized to the expression of Actb. Data are expressed relative to the mean value in the vehicle-treated cells, which was set to 100%, and are the mean $\pm$ SEM of three independent cultures made in duplicate (n=6). Statistical significance was assessed by one-way ANOVA and LSD post hoc comparisons: values not sharing a common letter were considered statistically different (P<0.05).

certain nuclear receptor heterodimers containing the retinoid X receptor (RXR), the so-called permissive heterodimers that respond to ligands of either moiety, among them the PPAR:RXR heterodimers [7]. We addressed the possible involvement of RARs, RXRs and PPAR β/δ in the induction of lipid metabolism-related genes and *Fndc5* in C2C12 myocytes by employing selective agonists of these nuclear receptors (Fig. 4). We first measured *Cyp26a1* expression, since this gene, which encodes a retinoic acid hydroxylase involved in the clearance of bioactive retinoids, is a well-established RAR target [33]; only ATRA and the pan-RAR agonist TTNPB had a significant effect on *Cyp26a1* expression upregulation, as expected (Fig. 4). Gene expression of *Cpt1b*, *Ucp3*, *Cd36* and *Fndc5* was insensitive to the pan-RAR agonist TTNPB but strongly induced by the pan-RXR agonist methoprene and the selective PPAR β/δ agonist GW0742. These results suggest that the ATRA-dependent induction of *Cpt1b*, *Ucp3*, *Cd36* and *Fndc5* in C2C12 myocytes is independent of RAR activation and could be mediated by PPAR β/δ :RXR activation.

Involvement of AMPK activation in ATRA effects on lipid metabolism and Fndc5 in C2C12 myocytes

Besides regulating gene expression by direct binding to nuclear receptors, ATRA can impact gene expression and cell function by modulating key regulatory cellular protein kinases [7]. In particular, ATRA was shown to affect cytoskeletal rearrangement and glucose uptake in skeletal muscle cells through AMPK-dependent pathways [34]. Here, we examined the possible involvement of AMPK activation in ATRA effects on lipid metabolism and *Fndc5* induction in C2C12 myocytes. First, we confirmed that ATRA impacts the AMPK-ACC axis in these cells. As shown in Fig. 5A, the phosphorylation of the catalytic subunit (α) of AMPK at the active Thr¹⁷² site, which is essential for enzyme activity, was increased in the cells exposed to ATRA (10 μ M, for 30 min), and so was the phosphorylation of the AMPK downstream target ACC at Ser⁷⁹. Administration of compound C (40 μ M), a cell-permeable inhibitor of AMPK, significantly inhibited p-AMPK and tended to inhibit p-ACC induced by ATRA in C2C12 myocytes, as expected (Fig. 5A). To examine the dependence of ATRA induction of lipid metabolism-related genes and *Fndc5* on AMPK activation, C2C12 myocytes were exposed to ATRA (10 μ M) for 24 h in the absence or the presence of compound C (40 μ M). Co-administration of compound C suppressed the stimulatory effect of ATRA on *Ucp3*, *Cd36* and *Fndc5*, but not *Cpt1b*, gene expression (Fig. 5B). Taken together, the results suggest that activation of AMPK is involved in the stimulation of FAO and irisin production promoted by ATRA in skeletal muscle cells.

Fig. 5. Involvement of AMPK in ATRA effects on fatty acid oxidation-related genes and the irisin gene in C2C12 myocytes. Cells on day 9 of differentiation were treated with 10 μ M ATRA for 30 min (A) or 24 h (B), in the absence and the presence of the AMPK inhibitor compound C (C.C., 40 μ M). Cells receiving C.C. were pre-incubated with the agent for 30 min before ATRA administration. Control cells received the vehicle (DMSO). To assess short-term ATRA effects on AMPK activation, the phosphorylation state of AMPK (at Thr¹⁷²) and of the AMPK target ACC (at Ser⁷⁹) in cell lysates was analyzed by immunoblotting; changes in the phospho-protein to total protein ratio are shown in (A), together with representative immunoblots

(two cultures per experimental condition). The long-term (24 h) incubation experiment was designed to assess effects on selected gene expression (B), which was measured and normalized to the expression of Actb. All data are expressed relative to the mean value in vehicle-treated cells, which was set to 100%, and are the mean $\pm$ SEM of two independent cultures made in duplicate (n=4). Statistical significance was assessed by Student's t-test (*, P<0.05, ATRA-treated vs vehicle-treated cells) or one-way ANOVA followed by LSD post hoc comparisons (P<0.05 for values not sharing a common letter).

Fig. 6. Effect of ATRA treatment on irisin, FGF21, and IL-6 circulating levels and gene expression in mice. Twelve-week-old NMRI male mice were subcutaneously treated with ATRA (50 mg/kg body weight per day) or vehicle (olive oil) during 4 days. Panels show serum irisin, FGF21, and IL-6 levels (A); Fndc5, Fgf21 and Il6 gene expression in gastrocnemius skeletal muscle (B); Fndc5 expression in liver and adipose tissues (C); and relative Fndc5 expression among tissues in vehicle-treated mice (D). Gene expression was normalized to that of 18S rRNA (B) or Actb (C, D). Gene expression data are expressed relative to the mean value in vehicle-treated mice (B, C) or to the mean value in iWAT (D), which were set to 100%. Data are the means $\pm$ SEM of at least 6 animals per group. Statistical significance was assessed by Student's t-test (*, P<0.05, ATRA-treated vs vehicle-treated mice) or one-way ANOVA followed by LSD post hoc comparisons (P<0.05 for values not sharing a common letter). BAT, brown adipose tissue; iWAT, inguinal white adipose tissue (WAT); rpWAT, retroperitoneal WAT; eWAT, epididymal WAT.

Data are the means $\pm$ SEM of at least 6 animals per group. Statistical significance was assessed by Student's t-test (*, P<0.05, ATRA-treated vs vehicle-treated mice) or one-way ANOVA followed by LSD post hoc comparisons (P<0.05 for values not sharing a common letter). BAT, brown adipose tissue; iWAT, inguinal white adipose tissue (WAT); rpWAT, retroperitoneal WAT; eWAT, epididymal WAT.

ATRA impacts Fndc5/irisin in mice in vivo

Results obtained in the C2C12 muscle cell model prompted us to study the impact of ATRA treatment on *Fndc5*/irisin *in vivo*. For this, we repeated a short-term protocol in mice that has been previously used in our laboratory to study ATRA effects on lipid and energy metabolism and adipokine production [10, 11, 13, 19]. ATRA treatment triggered as expected a significant ~10% decrease in body weight ($P=0.000$, Student's *t* test) that was independent of changes in food intake and associated with trends to decreased adiposity index (3.1 ± 0.2 vs 4.2 ± 0.5 , $P=0.097$, Student's *t* test), increased relative *gastrocnemius* muscle weight (0.53 ± 0.01 vs 0.42 ± 0.05 g per 100 g body weight, $P=0.092$, Student's *t* test) and increased serum levels of ketone bodies (0.942 ± 0.094 vs 0.560 ± 0.143 mM 3-hydroxybutyrate, $P=0.050$, Student's *t* test) in the ATRA-treated animals. These results confirmed that the ATRA treatment applied favored as expected lipid mobilization from WAT stores and fatty acid catabolism in tissues.

Serum levels of irisin were 5-fold higher in the ATRA-treated mice compared to the vehicle-treated mice, whereas serum levels of FGF21 and IL-6 remained unchanged (Fig. 6A). Despite the marked increase in circulating irisin levels, *Fndc5* mRNA levels in the *gastrocnemius* muscle were unaffected by ATRA treatment (Fig. 6B). Lack of induction of muscle *Fndc5* mRNA levels was confirmed in an independent experiment in other skeletal muscles, namely *adductus/vastus* (data not shown). Skeletal muscle *Fgf21* mRNA and *Il6* mRNA levels were not significantly affected by ATRA treatment, although for *Fgf21* a trend towards increased expression levels in the ATRA-treated mice was apparent (Fig. 6B).

Upregulated serum irisin levels in the face of unchanged skeletal muscle *Fndc5* mRNA levels in ATRA-treated mice prompted us to analyze *Fndc5* gene expression in additional tissues that have been recently demonstrated to be a source of irisin, namely adipose tissues [5, 35] and the liver [36], and to examine FNDC5 protein by immunohistochemistry in all of them. As shown in Fig. 6C, ATRA treatment led to significant increases of *Fndc5* mRNA levels in liver, BAT and epididymal WAT. Comparatively, in vehicle-treated mice *Fndc5* expression was 16-fold higher in skeletal muscle than in the liver and ~35-fold higher in the liver than in the adipose tissues; among the latter, *Fndc5* was maximally expressed in BAT, followed by subcutaneous (inguinal) WAT, and to a lesser extent in the visceral WAT depots, particularly the epididymal depot (Fig. 6D). These results are in concordance with a higher production of irisin in subcutaneous than in visceral fat, already described [35], and are first to unveil substantially higher basal expression levels of the *Fndc5* gene in the liver compared to adipose tissues in mice. In good concordance with the mRNA results, FNDC5 could be detected by immunostaining in skeletal muscle and to lower levels in liver and BAT in vehicle-treated mice (Fig. 7 A, C, E). In skeletal muscle, FNDC5 staining was observed mainly at the fiber sarcolemma but also in the sarcoplasm as dots (Fig. 7A), and not in all fibers, in agreement with a previous report [37]. In liver and BAT, FNDC5 staining was observed in hepatocytes and adipocytes, especially in cells surrounding blood vessels (Fig. 7C and E). WAT was negative for FNDC5: only one animal out of six showed some positivity for FNDC5 in the inguinal depot, associated with multilocular adipocytes (containing multiple intracellular lipid droplets) (Fig. 7G and data not shown). ATRA treatment had opposite effects on FNDC5 immunostaining in non-adipose and adipose tissues. The treatment led to decreased FNDC5 immunostaining of skeletal muscle fiber sarcolemma (Fig. 7B), decreased number of FNDC5 positive muscle fibers (70.7 ± 1.6 % vs 80.3 ± 1.6 %, $P=0.004$, Student's *t* test), and decreased FNDC5 positivity in hepatocytes surrounding blood vessels (Fig. 7D). Conversely, ATRA treatment led to increased FNDC5 immunostaining in BAT (Fig. 7F) and inguinal WAT (Fig. 7H), mainly at the cell membrane. Activation of BAT and induction of multilocularity in inguinal WAT (a sign of WAT browning) in ATRA-treated mice was apparent from the corresponding tissue micrographs, in keeping with previous reports [10, 11, 15].

Discussion

Vitamin A is essentially involved in the control of many biological processes including lipid and energy metabolism in homeostatic tissues, mainly in its carboxylic form ATRA

and both through direct and indirect (humoral signal-mediated) mechanisms [7, 9]. In this work, we show that ATRA activates AMPK and FAO in differentiated myocytes in culture. We also demonstrate, for the first time to our knowledge, that ATRA induces the production of irisin, a secreted signal that holds therapeutic potential in obesity and type 2 diabetes [6]. The implications of these findings are discussed below.

ATRA induces FAO in C2C12 myocytes – Previous results from animal studies suggested that, besides effects on adipose tissues and the liver, activation of lipid oxidation in skeletal muscle contributes to beneficial effects of ATRA treatment on adiposity and metabolic health in mice [12–14]. However, evidence of cell-autonomous effects of ATRA in skeletal muscle cells was lacking. This evidence is provided here, supported by gene expression results revealing the induction of important genes in fatty acid uptake, intracellular mobilization and oxidation (*Cd36*, *Cpt1b*, *Ucp3*, *Pdk4*, *Pnpla2*) and results of functional analyses indicating activation of the AMPK-ACC axis, increased complete oxidation of exogenously added palmitate and decreased intracellular triacylglycerol content following exposure of differentiated C2C12 myocytes to ATRA. ATRA effects on skeletal muscle cells shown in this work are in accordance with previous studies in animals [12–14] and such effects are of interest in the control of obesity and type 2 diabetes mellitus, as both excess muscle triacylglycerol content and defects in complete muscle FAO have been linked to obesity and insulin resistance [38, 39].

Our nuclear receptor agonists study points to PPAR β/δ :RXR as a likely mediator of the transcriptional effects of ATRA on lipid metabolism-related genes (in particular *Cpt1b*, *Ucp3* and *Cd36*) in skeletal muscle cells. This is in concordance with previous observations from ATRA treatment studies *in vivo* in mice [13, 14] and with the key role attributed to PPAR β/δ in the transcriptional control of oxidative metabolism/FAO in muscle cells [40]. Additionally, results in this work provide first evidence that activation of AMPK is likely to be involved in ATRA effects on lipid metabolism in skeletal muscle cells. AMPK activates FAO through its effects on the ACC-CPT1 axis, which lead to enhanced fatty acid entry into mitochondria for oxidation, and induces the expression of FAO-related genes by modulating the activity of specific transcriptional regulators [41]. In particular, AMPK stimulates basal and ligand-dependent PPAR β/δ transcriptional activity through direct AMPK-PPAR β/δ

Fig. 7. Immunostaining of FNDC5/irisin in tissues of vehicle- and ATRA-treated mice. Representative micrographs of FNDC5/irisin immunostained sections of: gastrocnemius muscle (A, B), liver (C, D), BAT (E, F), and inguinal WAT (G, H) from vehicle-treated (A, C, E, G) and ATRA-treated (B, D, F, H) mice. Polyclonal anti-FNDC5-C-terminal antibody raised in rabbits (ab181884; Abcam) was used as primary antibody. Treatment was 50 mg ATRA/kg body weight per day during the 4 days prior euthanization. Magnification 100 $\times$ (A–B) and 40 $\times$ (C–H); v = blood vessel.

protein-protein interaction and/or by phosphorylating the PPAR β/δ co-modulator PGC1 α [42, 43]. We confirmed that ATRA activates AMPK in C2C12 myocytes and demonstrate that the ATRA-dependent induction of *Cd36* and *Ucp3* – whose protein products favor fatty acid uptake and oxidation by muscle mitochondria [44, 45] – is suppressed when the cells are co-treated with the AMPK inhibitor compound C. Lack of suppression by compound C of ATRA induction of *Cpt1b* suggests that, for this gene, direct effects of the retinoid on transactivating transcription factors (such as PPAR β/δ) constitute a main mechanism of activation independent of AMPK. Even if caution is required in the interpretation of these results, since compound C can exert AMPK-independent effects [46] and steps from ATRA to AMPK activation remain to be defined, altogether the involvement of AMPK activation in ATRA effects on FAO in muscle cells is strongly suggested.

ATRA does not impair glucose metabolism in differentiated C2C12 myocytes – Increased fatty acid oxidation often leads to decreased glucose utilization in cells [47]. It was therefore of interest to examine the interaction of ATRA with glucose metabolism in our skeletal muscle cell model. In good concordance with previous observations in cardiac myocytes [48], ATRA exposure increased the mRNA levels of the insulin-regulated glucose transporter GLUT4 in C2C12 skeletal myocytes. However, GLUT4 mRNA levels only indirectly reflect glucose uptake capacity, as this is primarily regulated by transport of GLUT4 to the plasma membrane, and in fact in our hands basal and insulin-stimulated 2-DOG uptake in C2C12 myocytes were unaffected by ATRA treatment. The latter result is in contrast with upregulation of glucose uptake by ATRA previously reported in the same cell model by Lee et al. (2008) [34]. Differences may relate to differences in the duration of the ATRA treatment, much longer in our case (24 h vs 1 h in the study of Lee et al.), and are suggestive of time-course effects of ATRA on glucose and lipid metabolism in skeletal muscle cells, beginning with the activation of glucose metabolism. In any case, to be highlighted is that ATRA increases FAO in skeletal myocytes apparently without provoking adverse effects on cellular glucose uptake and metabolism.

ATRA stimulates irisin production – Novel results in this work show that ATRA treatment induces *Fndc5*/irisin expression in differentiated C2C12 myocytes, independently of *Ppargc1a* induction (although increases in PGC1 α activity cannot be discarded) and through mechanisms that likely involve PPAR β/δ , RXR and AMPK activation. Previous studies showed that *Fndc5* gene expression is induced by ATRA treatment during neural differentiation of embryonic carcinoma cells (P19) and mouse embryonic stem cells [30, 49]. ATRA effects on *Fndc5* gene transcription could be direct or indirect and mechanistic details remain to be established. Analysis of the mouse *Fndc5* gene promoter regions predicted a VDR:RXR binding site but not RAR:RXR or PPAR:RXR binding sites [30]. Interestingly, treatment with irisin increases mitochondrial oxidative metabolism, mitochondrial uncoupling and fatty acid oxidation in skeletal muscle cells [28, 29]. Thus, irisin induction may contribute, in a paracrine/autocrine manner, to the effects of ATRA treatment on C2C12 myocyte metabolism observed in the present work.

Our *in vivo* results are in keeping with an up-regulatory effect of ATRA on irisin, since serum irisin levels were markedly increased in ATRA-treated mice. Although this increase was independent of changes in the mRNA levels of the irisin gene *Fndc5* in the skeletal muscles examined, relative muscle mass tended to be increased after ATRA treatment, and we did find increased *Fndc5* gene transcription in liver and adipose tissues of ATRA-treated mice. Furthermore, our results strongly suggest that ATRA treatment promotes FNDC5/irisin release from skeletal muscle and the liver and its accumulation in serum, BAT and inguinal WAT, as it would be expected for a secreted protein targeting adipose tissues such as irisin – which induces WAT browning and also thermogenesis-related gene expression in BAT [3] – and as part of reported ATRA effects in the whole animal – which include BAT activation [15] and WAT browning [10, 11]. Supporting this interpretation, the antibody used in our study also recognizes the secreted FNDC5/irisin protein, as indicated by positive staining of fixed plasma proteins inside blood vessels (data not shown). In fact, antibodies (as ours) against the endogenous portion of FNDC5 protein have been previously shown to recognize secreted

irisin, which has led to the proposal that the full-length FNDC5/irisin may be secreted [50]. Further supporting our interpretation, immunostaining has been successfully used to visualize irisin bound on the cell surface of recombinant irisin-treated 3T3-L1 adipocytes, which has suggested the existence of a yet-to-be-identified irisin receptor on the adipocyte cell membrane [51].

Taken together, our results suggest that the FNDC5/irisin detected in inguinal WAT and BAT of ATRA-treated mice could be partly of endocrine origin and partly of paracrine/autocrine origin. Remarkably, upregulation of endogenous *Fndc5*/irisin expression in primary white adipocytes promotes browning of the cells [52] and induction of browning in the 3T3-L1 white adipocyte cell model (through the knockdown of the retinoblastoma protein [53]) increases secreted irisin levels [5]. Thus, the possibility that ATRA treatment drives a positive feed-back loop involving WAT browning and WAT irisin production as a paracrine/autocrine signal merits further investigation.

Functional similarities of FGF21 and IL-6 with irisin [31, 32] and reported transactivation of the *Fgf21* gene by liganded-RAR in hepatic (HepG2) cells [54] led us to hypothesize that ATRA treatment could induce FGF21 and/or IL-6 production. However, circulating levels of both signals were unaffected in ATRA-treated mice, ruling out FGF21 and IL-6 as endocrine factors mediating metabolic effects of exogenous ATRA treatment. Our results might be compatible with ATRA treatment inducing skeletal muscle FGF21 as an autocrine/paracrine signal, yet skeletal muscle is not considered a main target of FGF21 due to lack of the FGF-receptor cofactor β -klotho [31]. Moreover, ATRA treatment did not induce but suppressed IL-6 expression in C2C12 myocytes and had no effect on *Il-6* gene expression in skeletal muscle of mice. Downregulation of IL-6 by ATRA was previously reported in non-muscular cell models and related to the ability of ligand-bound RARs to antagonize the activity of CCAAT-enhancer-binding protein β and nuclear factor κ B, two transcription factors that normally transactivate the *Il-6* gene [55, 56]. The fact that ATRA effects on *Il-6* and *Fndc5* mRNA levels were observed in C2C12 myotubes but not in skeletal muscles of ATRA-treated mice suggests that, *in vivo*, systemic or muscle tissue-related factors can offset some of the cell-autonomous effects of ATRA on muscle cells.

Conclusion

In summary, results in this work reveal direct effects of ATRA in the stimulation of FAO and irisin production in skeletal muscle cells, and a substantial rise of serum and adipose tissue FNDC5/irisin protein levels in ATRA-treated mice, linked to FNDC5/irisin protein depletion in skeletal muscle and the liver and the induction of *Fndc5* expression in hepatic and adipose tissues. Stimulation of muscle FAO and of FNDC5/irisin may contribute to the leaning effects and metabolic benefits observed in mice upon ATRA treatment, by favoring the mobilization and consumption of stored lipids and WAT browning.

Abbreviations

ACC (acetyl-CoA carboxylase); Acox1 (acyl CoA oxidase); Actb (beta-actin); AMPK (AMP-activated protein kinase); ASP (acid-soluble product); ATRA (all-trans retinoic acid); BAT (brown adipose tissue); Cd36 (fatty acid translocase); Cpt1b (muscle-type carnitine palmitoyltransferase 1); Cyp26a1 (cytochrome P450 26A1); 2-DOG (2-deoxy-D-glucose); FAO (fatty acid oxidation); Fgf21/FGF21 (fibroblast growth factor 21); *Fndc5* (fibronectin type III domain containing five); Hk2 (hexokinase 2); Il6/IL6 (interleukin 6); Insr (insulin receptor); Lipe (hormone sensitive lipase); Pdk4 (pyruvate dehydrogenase kinase 4); Pnpla2 (adipose triglyceride lipase); Ppard (peroxisome proliferator activator receptor delta); Ppargc1a (peroxisome proliferator activated receptor γ coactivator 1 α); RAR (retinoic acid receptor); RXR (retinoid X receptor); Slc2a4 (solute carrier family 2 member 4); TTNPB (p-[(E)-2-(5, 6, 7, 8-tetrahydro-5, 5,8, 8-tetramethyl-2-naphthalenyl)-1-propenyl]benzoic acid); Ucp3 (uncoupling protein 3); WAT (white adipose tissue).

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Disclosure Statement

No conflict of interests exists.

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