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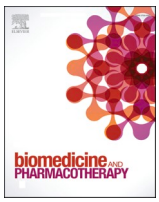

Furan fatty acid extracted from *Hevea brasiliensis* latex increases muscle mass in mice

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ABSTRACT

Skeletal muscle is essential for locomotion and plays a crucial role in energy homeostasis. It is regulated by nutrition, genetic factors, physical activity and hormones. Furan fatty acids (FuFAs) are minor fatty acids present in small quantities in food from plants and animals origin. Recently, we showed that a preventive nutritional supplementation with furan fatty acid in a DIO mouse model reduces metabolic disorders. The present study was designed to determine the influence of FuFA-F2 extracted from *Hevea brasiliensis* latex on skeletal muscle phenotype. In C2C12 myotubes we found that FuFA-F2 whatever the concentration used increased protein content. We revealed that in C2C12 myotubes FuFA-F2 (10 μM) increases protein synthesis as shown by the stimulation of mTOR phosphorylation. Next, to confirm *in vivo* our results C57Bl6 mice were supplemented by oral gavage with vehicle or FuFA-F2 (20 mg/kg) for 3 and a half weeks. We found that mice supplemented with FuFA-F2 had a greater lean mass than the control mice. In line with this observation, we revealed that FuFA-F2 increased muscle mass and promoted more oxidative muscle metabolism in mice as attested by cytochrome c oxidase activity. In conclusion, we demonstrated that FuFA-F2 stimulates muscle anabolism in mice *in vitro* and *in vivo*, mimicking in part physical activity. This study highlights that *in vivo* FuFA-F2 may have health benefits by increasing muscle mass and oxidative metabolism.

1. Introduction

Furan fatty acids (FuFAs) are fatty acids present in small quantities, which have a furan ring [1]. FuFAs are synthesized in plants [2] and microorganisms [3,4]. Through food, they are also found in animals and humans [1]. Fish is a particularly important source of FuFAs, due to its high consumption of algae and marine microorganisms rich in FuFAs [5,6]. In commercial fish oils, up to 1 % of the total fatty acids can be found in the form of FuFAs [7]. Interestingly, these fatty acids are particularly abundant in certain clones of *Hevea brasiliensis* latex used to produce natural rubber [8,9].

FuFAs have various biological effects, which lead to beneficial health effects [10]. In particular, FuFAs are known to have antioxidant properties [11–13] due to the electron-rich furan ring that makes it highly susceptible to oxidation. In the presence of ROS (reactive oxygen species), it will open and form an unstable and highly reactive dioxoene [14]. However, due to the lack of commercially available FuFAs only a few studies have been performed to test their biological function. *In vitro*, in 3T3-L1 adipocyte cells, FuFAs increase the expression of adiponectin, a protein known for its anti-inflammatory and

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insulinosensitive effects [15]. Further, *in vivo*, in a rat model of arthritis, FuFAs have a greater anti-inflammatory activity than eicosapentaenoic acid (EPA) [16]. A least, CMPF (3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid) a degradation product of FuFAs but also a metabolite derived from the catabolism of PUFAs of the ω-3 family [17], may contribute to these health effects. CMPF supplementation prevents hepatic steatosis and insulin resistance induced by a high-fat diet in mice [17,18].

Furthermore, many works indicate that the ω-3 PUFAs contained in fish may contribute to the improvement or prevention of cardiovascular disease (CVD) [19,20]. However, several studies suggest that FuFAs present in fish may also be involved in the beneficial effect of fish consumption on CVD [21]. Thus, several studies conducted in humans show that the consumption of fish oil increases the levels of FuFA [22] and CMPF in the plasma [23]. In addition, *in vitro* studies show that FuFAs inhibit platelet aggregation induced by arachidonic acid [24], reduce the oxidation of Low-Density Lipoproteins (LDL) [12] and inhibit the progression of non-enzymatic lipid peroxidation [25].

Recently, we showed that a preventive nutritional supplementation with furan fatty acid in a DIO mouse model reduces metabolic disorders and increases muscle mass [26]. The skeletal muscle plays a crucial role in energy homeostasis and is a major target of insulin action. In the present study, our aim is to better understand the influence of FuFA-F2 extracted from *Hevea brasiliensis* latex [9] on skeletal muscle phenotype. To this end, we treated a murine myoblast cell line C2C12 with FuFA-F2 and then supplemented healthy C57Bl6 mice with FuFA-F2 by gavage. In this study, we chose to work using a FuFA-F2 (10,13-epoxy-11-methyl-octadecan-10,12-dienoic acid) with a purity of over 96 %. *In vitro*, we tested at different concentration the impact of FuFA-F2 on C2C12 murine myoblasts differentiation. Then, we studied the influence of FuFA-F2 directly on myotubes. We showed that the FuFA-F2 had no effect on the differentiation of C2C12 myoblasts but significantly increased the protein content of myotubes. In addition, we found that oral gavage with FuFA-F2 for 3 weeks and half in C57Bl6 mice increased muscle mass and promoted more oxidative muscle metabolism in mice.

2. Materials and methods

2.1. FuFA-F2 purification

Fresh latex from PB235 *Hevea* clone was collected from a plantation of Visahakit Thai Rubber Co., Ltd., Chanthaburi, Thailand. Lipid extraction was performed by adding freshly harvested latex dropwise into continuously stirred ethyl acetate (Univar, Illinois, USA) with a

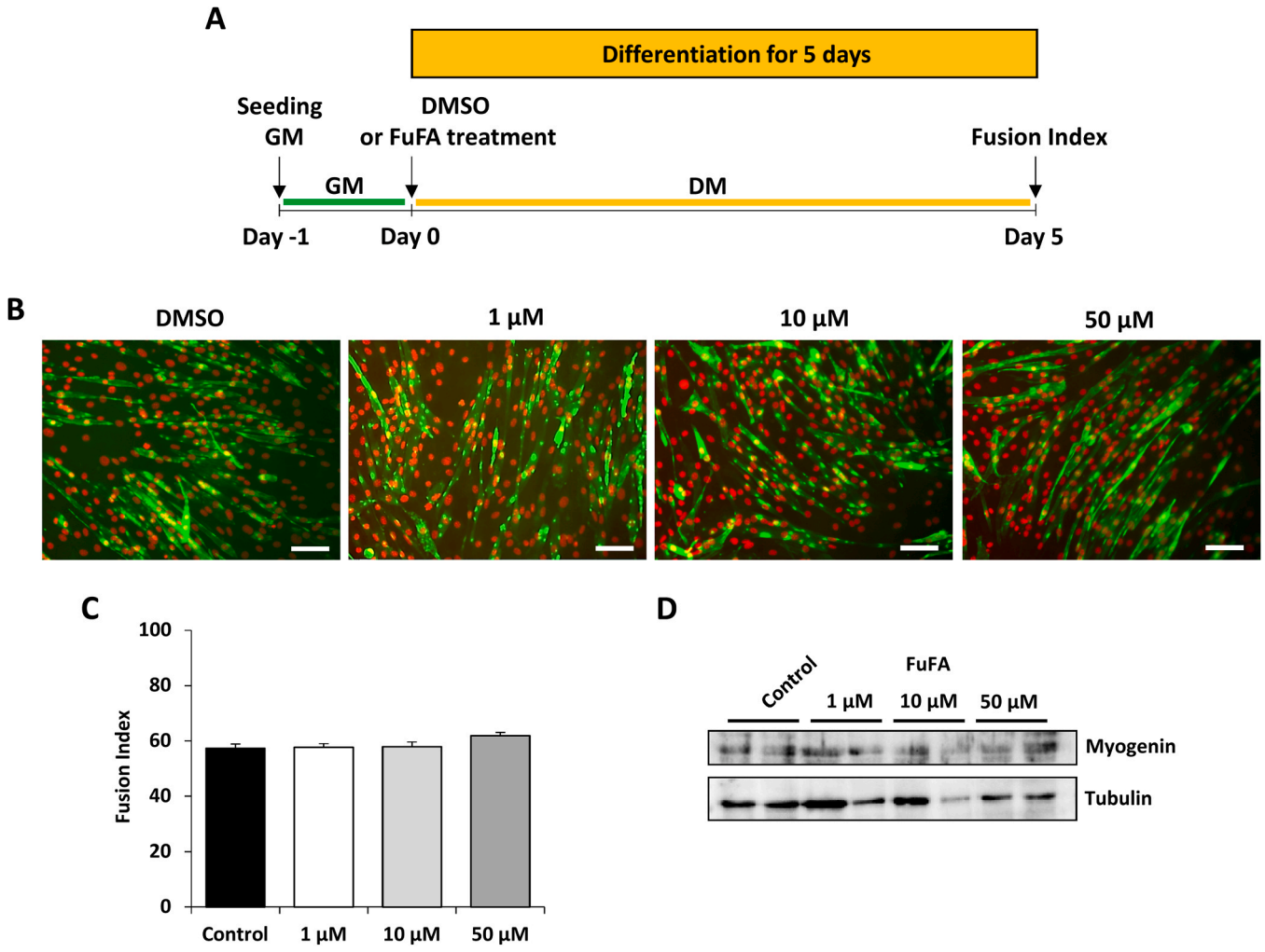

Fig. 1. FuFA-F2 supplementation had no influence on C2C12 myoblast differentiation. (A) Scheme of the experiment. GM: growth medium; DM: differentiation medium. (B) Cytoimmunofluorescence studies using antibodies raised against Troponin T were performed on C2C12 myoblasts after 5 days in differentiation medium. Nuclei are stained in red and troponin T (a myotube differentiation marker) in green. FuFA-F2 was added at the induction of the differentiation at the indicated concentration ($n = 6$ for each group). Scale bar, 100 µm. (C) The extent of differentiation was assessed by the fusion index (percentage of nuclei incorporated into myotubes relative to the total number of nuclei). (D) Myogenin protein levels were analyzed by Western blot ($n = 6$ for each group). Typical blots are shown. Proteins were quantified with Image Lab™ Touch Software. Results are expressed as means ± SEM.

ratio latex:solvent of 1:5 (v/v). Coagulum made mainly of insoluble polyisoprene was removed by filtration and the extract was let decanted in separating funnel overnight. The upper fraction of lipid-containing ethyl acetate was collected and evaporated. Total lipid extract was further purified using silica gel column chromatography. Lipid extract (1.8 g of lipid solution in hexane) was loaded onto a silica gel column (column 4.0 cm diameter × 16 cm length) containing 42 g of silica gel 60 (0.040–0.063 mm, Merck, Germany). Mobile phase was made of 760 mL of hexane:diethyl ether (90:1, v/v) at the flow rate of 1.8 mL/min and the collection volume was 19 mL/fraction. The separation quality was checked by spotting an aliquot of each fraction on a thin layer chromatography plate following the method described for neutral lipid by Liengprayoon et al. [27]. The selected fractions containing only Trifuranoylglycerols (TGF) were saponified and the purity of the produced free FuFA-F2 was analyzed by GC-FID on methyl ester derivatives (Supplemental Fig. 1). The identity of FuFA was confirmed by ^{1}H and ^{13}C NMR (Supplemental Fig. 2) as described previously [27]. The purity of FuFA-F2 used in this study was more than 96 % (Supplemental Fig. 1). FuFA-F2 was dissolved in DMSO before used in C2C12.

2.2. Animals

Male C57Bl/6 J wild-type mice were obtained from Janvier Labs at 12 weeks of age and were randomly assigned to one of the two groups based on body weight. Mice were housed 2 per cage and maintained on a 12-hour light/dark cycle (lights on at 7:30 pm). Food and water were provided ad libitum. Enrichment (nesting cotton squares) was provided in each cage. Mice were treated once daily by oral gavage with vehicle (50 % PEG-400, 0.5 % Tween 80 %, and 49.5 % distilled water) or FuFA-F2 (20 mg/kg) as described Fig. 6A. All animal experiments were performed according to European directives (86/609/CEE and 2010/63/CEE) and approved by the regional animal experimentation ethics committe: Région Languedoc-Roussillon (No36). Our institutional guidelines for the care and use of laboratory animals were observed. Our animal facility is approved by the Departmental Veterinary Services (No. E34–172–10, 2019/03/04) and the Ministry of Research (DUO No. 7053, 2020/02/26). According to the European Directive 210–63-EU, mice were observed daily for the general health status and mortality scoring. The animals were euthanized in the fed state.

2.3. Body composition

Mice whole-body composition (fat and lean masses) was measured every week using an EchoMRI-700 whole-body composition analyzer, according to the manufacturer's instructions.

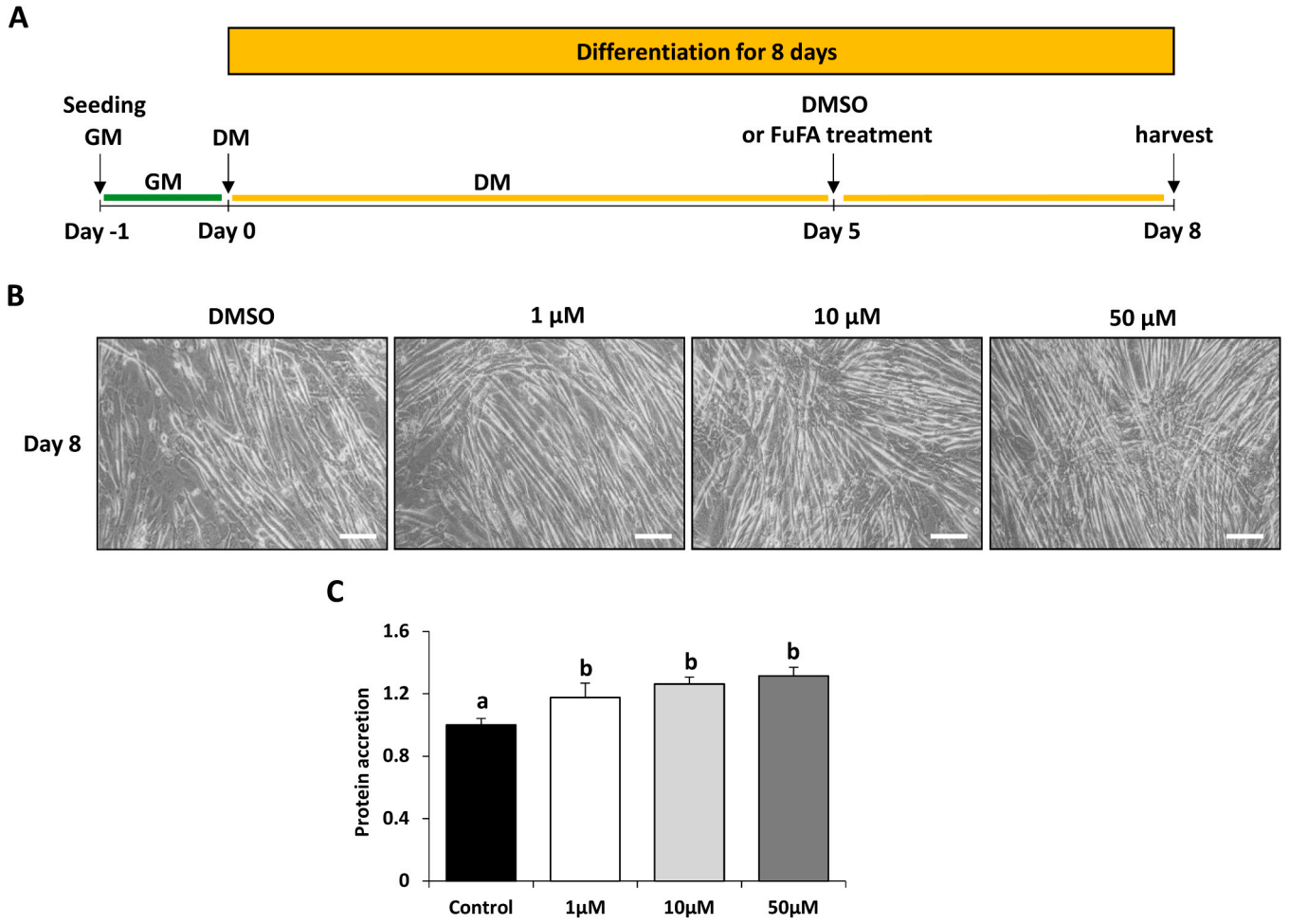

Fig. 2. FuFA-F2 supplementation increases protein content in C2C12 myotubes. (A) Scheme of the experiment. GM: growth medium; DM: differentiation medium. (B) Representative pictures of myotubes using a bright field microscope after 8 days in differentiation medium. FuFA-F2 was added on the 5th day of differentiation at the indicated concentration ($n = 6$ for each group). Scale bar, 100 µm. (C) Relative protein content (n = 6 for each group). Statistical Significance: $p < 0.05$ vs Control, the effects of the different dose of FuFA-F2 were tested by one way ANOVA followed by Tukey's post hoc test. The means with different letters were significantly different. Results are expressed as means ± SEM.

2.4. Protein synthesis measurement in mice

Protein synthesis was measured by determining puromycin incorporation into proteins as previously described [28]. In this aim, mice were intraperitoneally injected with 10 mM puromycin (100 μL of puromycin solution/25 g body weight, Sigma Aldrich). Twenty-five minutes after puromycin injection, animals were euthanized. Puromycin incorporation in proteins in gastrocnemius muscle was assessed by immunoblotting on 4–20 % acrylamide gels and using an anti-puromycin primary antibody (anti-puromycin antibody (1/3000), clone 12D10 from EMD Millipore). The optical density of the entire sample lane was assessed and normalized with Stain Free total protein staining [29].

2.5. Histological study

After euthanasia by cervical dislocation, tibialis anterior muscles were collected, freshly frozen in Tissue-Tek, and then stored at −80 °C. For immunostaining, the sections (10 μm thickness) were permeabilized 30 min in PBS, 20 % horse serum, 0.1 % triton at RT, and incubated for 1 h at 37 C with the anti-laminin (1/200, rabbit polyclonal, Sigma L9393) in a solution of PBS, 1 % BSA. Sections were washed in PBS 1X for 10 min 3 times and incubated with the secondary antibody (Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488) for 1 h at 37 C. Sections were washed in PBS 1 × 2 times for 10 min, incubated 30 s with DAPI, and washed once in PBS for 10 min and mounted with Aqueous Mounting Medium PermaFluor™. For the morphometric analysis, muscle sections were scanned using a NanoZoomer (Hamamatsu Photonics) with a 20X objective. Image J free software was used to analyze and quantify the pictures for each entire area.

2.6. Cell culture and treatments

Mouse myoblasts of the C2C12 cell line (ATCC) were seeded at a plating density of 7000 cells/cm2 in 6-well plates. They were grown in DMEM high glucose (4.5 g/l) supplemented with gentamycin (50 μg/mL), amphoterecin (50 μg/mL), and fetal calf serum (10 %). Terminal differentiation was induced at cell confluence by lowering the medium serum concentration (0.5 %). Protein content is determined by measuring the total amount of protein in each culture dish using Bio-Rad Protein Assay. The amount is expressed relative to the control, which has a value of 1.

When indicated, FuFA-F2 was added to the culture medium at the indicated concentration. For the differentiation studies, FuFA-F2 was added at the confluence at the same time as the change in media condition. Three doses of FuFA-F2 were studied at a final concentration of 1, 10, or 50 μM. Human insulin was used at a concentration of 100 nM.

2.7. Cytoimmunofluorescence

Cytoimmunofluorescence myoblast differentiation was assessed by observation of morphological changes and accumulation of muscle-specific markers. After methanol fixation and three washes with PBS-gelatin (0.2 %), cells were labeled with an antibody raised against Troponin T (T6277, Sigma, diluted at 1:50). Nuclei were stained with Hoechst 33258 (1 μg/mL). To assess the extent of differentiation, the fusion index (percentage of nuclei incorporated into myotubes relative to the total number of nuclei) was calculated 120 h after adding the differentiation medium. Nuclei were counted on 10 images/dish using Image J software.

2.8. Western blotting

Protein levels were assessed by Western blotting. Total proteins were measured using the Bio-Rad protein assay according to the manufacturer's instructions. Total proteins were lysed in RIPA buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 % NP40, pH 7.5 + complete cocktail protease inhibitors + Pierce phosphatase inhibitors tablet). 40 μg of cell extracts were loaded and blotted onto a nitrocellulose membrane. The membranes were washed, blocked with 5 % nonfat milk and incubated in primary antibody overnight at 4 °C. The membranes were then washed in TBS-Tween (10 mM Tris, 140 mM NaCl, and 0.2 % Tween-20), incubated with the appropriate secondary antibody coupled to horseradish peroxidase, and washed again in TBS-Tween. Signals were revealed using a Clarity™ Western ECL Substrate Kit (Bio-Rad), and proteins were visualized by enhanced chemiluminescence using the ChemiDoc Touch Imaging System (Bio-Rad) and quantified with Image Lab. Touch Software (version 5.2.1).

2.9. Antibodies

Primary antibodies used include: anti-AMPK (1/500, Cell Signaling #2603); anti-Phospho-AMPK (Thr172) (1/500, Cell Signaling #2535); anti-AKT (1/500, Cell Signaling #9272); anti-Phospho-AKT (Ser473) (1/500, Cell Signaling #9271); anti-ULK1 (1/500, Cell Signaling #8054); anti-Phospho-ULK1 (Ser757) (1/500, Cell Signaling #6888); anti-mTOR (1/500, Cell Signaling #2972); anti-Phospho-mTOR (Ser2448) (1/500, Cell Signaling #2971); anti-myogenin (1/500, Santa Cruz Biotechnology Sc-398002); anti-LC3A (1/1000, Cell Signaling #4599); anti-4E-BP1 (1/500, Cell Signaling #9644); anti-Phospho-4E-BP1 (Thr37/46) (1/500, Cell Signaling #2855); Anti-α-Tubulin (1/6000, Cell Signaling #3873); Anti-Myosin (Fast) (1/1000, Sigma M4276); Anti-Myosin (Slow) (1/1000, Sigma M8421); Anti-PPARδ (1/1000, [30]); Anti-SIRT1 (1/1000, Cell Signaling #8469); Anti-PGC-1α (1/1000, Merck 516557).

2.10. Gene Expression Studies

Total RNAs were extracted from quadriceps muscle using Trizol®, and cDNAs were generated using the PrimeScript-1st strand cDNA Synthesis Kit (Takara Bio). Real-time PCR was performed using SYBR® Premix Ex Taq-II (Takara Bio) and Applied Biosystems Step-One Plus (Thermo Fisher Scientific). Gene expression was normalized to the expression of the housekeeping gene Rps9. The primers for the determination of MyHC were previously described [31].

The PCR overall efficiency (E) was calculated from the slopes of the standard curves according to the equation $E = [10^{(-1/slope)}] - 1$, and this value was higher than 95 % for all assays. The relative abundance of each sample was then normalized according to the equation: relative quantity $= 2^{-\Delta Ct}$. All of the experiments were performed according to the minimum information for publication of quantitative real-time PCR experiment (MIQE) guidelines [32,33].

2.11. Mitochondrial Enzymatic Activities

Mitochondrial respiratory complex activities were determined as previously described [31]. Citrate synthase (CS) activity was measured according to Srere [34] by following the color formation of 5-thio-2-nitrobenzoic acid at 412 nm. The enzymatic activity of complex IV (cytochrome c oxidase) was measured according to Wharton and Tzagoloff [35], a spectrophotometer following the oxidation of reduced cytochrome c at 550 nm, pH 7.4, and 37 °C. Cytochrome c was reduced with sodium dithionite.

2.12. Statistical analyses

All results are presented as means ± SEM, or as percentages. In culture cells, the effects of the different dose of FuFA-F2 were tested by one way ANOVA followed by Tukey's post hoc test. Statistical analysis data was performed using GraphPad Prism 8.0 (GraphPad Software, Inc., San Diego, CA). The means with different letters were significantly

different. In mice, statistical significances of the differences compared to Controls were evaluated with Student's t-test. The limit of statistical significance was set at $p < 0.05$.

3. Results

3.1. FuFA-F2 supplementation had no effect on C2C12 myoblast differentiation

For *in vitro* studies, the C2C12 cell line is classically used because it recapitulates the different phases observed *in vivo*: proliferation, withdrawal from the cell cycle, and fusion into myotubes. After a proliferation phase during 24 h, the C2C12 myoblasts reach confluence and their terminal differentiation is induced by lowering the serum level in the culture medium. C2C12 cells were grown for 5 days in a differentiation medium, with or without FuFA-F2 (1, 10, and 50 μM) (Fig. 1A). In humans, the levels of FuFAs in plasma are around 1 μM [22,36,37]. We found that FuFA-F2 had no significant effects on myoblast fusion index (Fig. 1B-C). Consistent with this observation, FuFA-F2 had no effect on the expression of myogenin, a key myogenic factor for differentiation (Fig. 1C).

3.2. FuFA-F2 supplementation increased protein content in C2C12 myotubes

We next employed C2C12 myotubes that had been differentiated from myoblasts during 5 days in order to test the influence of FuFA-F2 at this stage (Fig. 2A). We observed that whatever the dose, 3 days of FuFA-F2 supplementation significantly increase total protein content in C2C12 myotubes (1 μM, +18 %, $p < 0.05$; 10 μM, +26 %, $p < 0.05$; 50 μM, +31 %, $p < 0.05$) (Fig. 2B-C).

3.3. FuFA-F2 supplementation decreases autophagy in C2C12 myotubes

We have shown that FuFA-F2 increased protein content in myotubes. Protein homeostasis is determined by the dynamic balance between protein synthesis and degradation. Protein synthesis was evaluated by measuring the phosphorylation of AKT (Ser473), mammalian target of rapamycin (mTOR) (Ser2448) and Eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) (Thr37/46), which are major regulators of translation and protein synthesis. Unfortunately, basal level of AKT and 4E-BP1 phosphorylation was undetectable with C2C12 myotubes in our experiments (Fig. 3A). In addition, we found no difference in mTOR phosphorylation with or without FuFA-F2 (Fig. 3A-B).

AMP activated protein kinase (AMPK) is a crucial cellular energy sensor protein and is activated when intracellular ATP level decreases to regulate autophagy initiation [38]. Activation level of AMPK as assessed by phosphorylation at Thr172 was significantly decreased by FuFA-F2 for the lower dose (1 μM, −27 %, $p < 0.05$) but did not reach significativity for the higher doses (Fig. 3C-D). We further evaluate the autophagy-related signaling by measuring the MTORC1 (mammalian target of rapamycin complex 1) mediated phosphorylation by ULK1 (Unc-51 like autophagy activating kinase 1) at Ser757 which disrupts the interaction between ULK1 and AMPK. We found that FuFA-F2 had no impact on ULK1 (Fig. 3C-D). Therefore, we assessed the autophagic flux by measuring the conversion of LC3-I (Microtubule-associated proteins 1 A/1B light chain 3B) to LC3-II, through lipidation. We found that the ratio of LC3-II/LC3-I was decreased by FuFA-F2 whatever the dose (1 μM, −62 %; 10 μM, −72 %; 50 μM, −67 %, $p < 0.05$) (Fig. 3C-D) suggesting that FuFA-F2 inhibited the autophagy process in C2C12 myotubes.

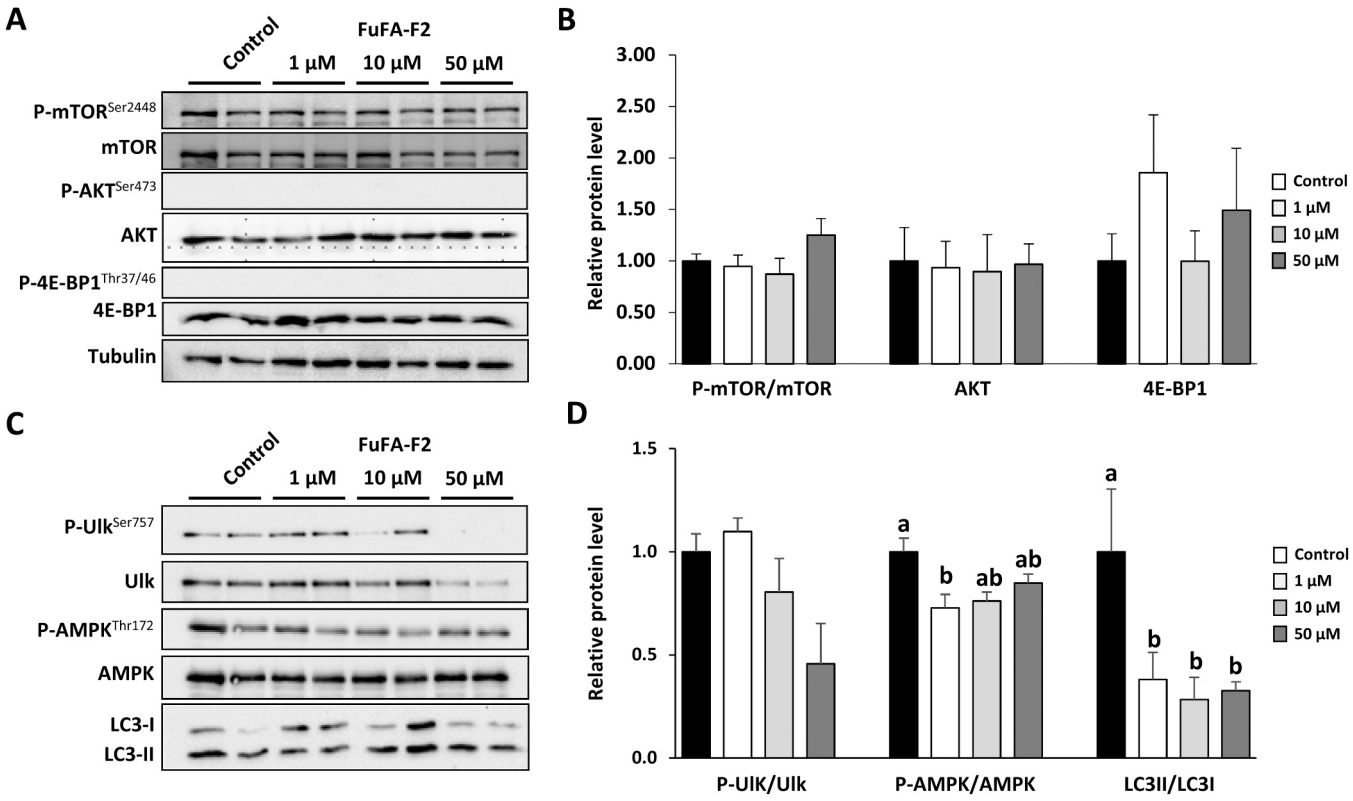

Fig. 3. FuA-F2 supplementation decreases autophagy in C2C12 myotubes. (A) Western-blot analysis of the indicated proteins ($n = 6$ for each group). Typical blots are shown. (B) Quantification of relative protein expression in C2C12 myotubes. (C) Western-blot analysis of the indicated proteins ($n = 6$ for each group). Typical blots are shown. (D) Quantification of relative protein expression in C2C12 myotubes. Proteins were quantified with Image Lab™ Touch Software. Statistical Significance: $p < 0.05$ vs Control, the effects of the different dose of FuFA-F2 were tested by one way ANOVA followed by Tukey's post hoc test. The means with different letters were significantly different. Results are expressed as means ± SEM.

3.4. FuFA-F2 supplementation increase mTOR phosphorylation in C2C12 myotubes

Next, we treated C2C12 myotubes as before using a 10 μM FuFA-F2 concentration and then cultured them for 2 h in low-glucose medium (1 g/l) before adding again the different treatments as indicated for 30 min (Fig. 4A). In this condition, we showed that insulin, used as positive control, induced a strong increase in AKT phosphorylation at Ser473 (x38, $p < 0.05$) (Fig. 4B-C). Compared to low glucose medium, the normal high-glucose medium with insulin and FuFA-F2 induced a similar increase of AKT phosphorylation (x31, $p < 0.05$) (Fig. 4B-C). In addition, treatment with FuFA-F2 did not alter insulin-induced phosphorylation of AKT (Fig. 4B-C). This indicates that FuFA-F2 treatment does not appear to regulate AKT phosphorylation. Furthermore, in FuFA-F2 treated myotubes we observed a significant increase in mTOR phosphorylation at Ser2448 (10 μM, +59 %, $p < 0.05$) while no increase was detected in the presence of insulin alone (Fig. 4B-C). This data set suggests that FuFA-F2 stimulates mTOR phosphorylation and protein synthesis.

To determine whether mTOR phosphorylation is induced only by 30 min of incubation in the presence of FuFA-F2 or whether it is an effect partly related to the 3-day treatment with FuFA-F2, we repeated the experiment without pretreatment (Fig. 4D). In this condition we still observed that only insulin is able to induce a strong increase in AKT phosphorylation (x10, $p < 0.05$) (Fig. 4E-F). However, in contrast to the previous experiment we did not observe a rapid effect of FuFA-F2 on mTOR phosphorylation (Fig. 5E-F). This observation suggests that the activation of mTOR by FuFA-F2 was related to the 3-day treatment.

3.5. Influence of FuFA-F2 supplementation on metabolic and contractile characteristics of C2C12 myotubes

We further explored if this increase in myotubes anabolism is associated with a change in the metabolic and contractile characteristics of muscle fibers. To address this issue, we tested the influence of FuFA-F2 on mitochondrial activity in C2C12 myotubes. We found that 3 days of FuFA-F2 treatment of C2C12 myotubes had no effect on citrate synthase activity (Fig. 5A), commonly used as a quantitative marker of intact mitochondria. In addition, we found no significant influence on Complex IV activity (Fig. 5B), a key enzymatic activity of the mitochondrial respiratory chain. Next, we studied the expression of slow and fast myosin heavy chain (MyHC) by western-blot. Skeletal muscle contains myofibers differing in contractile function, mitochondrial content, and consequently metabolic properties. Slow-twitch fibers are characterized by type I myosin heavy chain (MyHC) expression and a high mitochondrial density leading to a prominent oxidative metabolism. Fast-

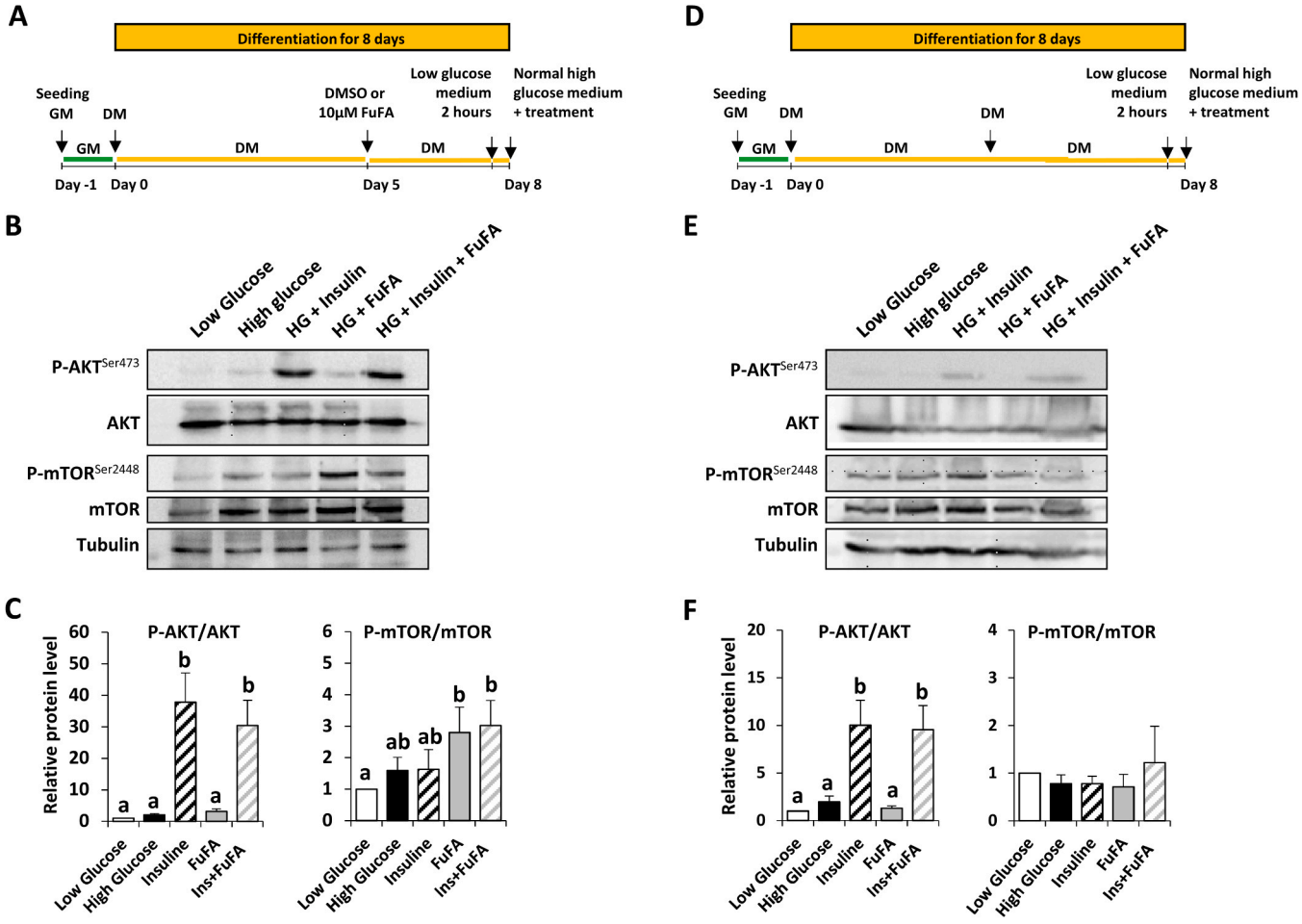

Fig. 4. FuFA-F2 supplementation increase mTOR phosphorylation in an Akt independent manner. (A) Scheme of the experiment. GM: growth medium; DM: differentiation medium. (B) Western-blot analysis of the indicated proteins ($n = 6$ for each group). Typical blots are shown. (C) Quantification of relative protein expression in C2C12 myotubes. (D) Scheme of the experiment. (E) Western-blot analysis of the indicated proteins ($n = 6$ for each group). Typical blots are shown. (F) Quantification of relative protein expression in C2C12 myotubes. Statistical Significance: $p < 0.05$ vs Control, the effects of the different dose of FuFA-F2 were tested by one way ANOVA followed by Tukey's post hoc test. The means with different letters were significantly different. Results are expressed as means ± SEM. Low glucose: DMEM glucose 1 g/l; High Glucose: DMEM glucose 4.5 g/l; HG + Insulin: DMEM glucose 4.5 g/l + Insulin 100 nM; HG + FuFA: DMEM glucose 4.5 g/l + FuFA-F2 10 μM; HG + Insulin + FuFA: DMEM glucose 4.5 g/l + Insulin 100 nM + FuFA-F2 10 μM.

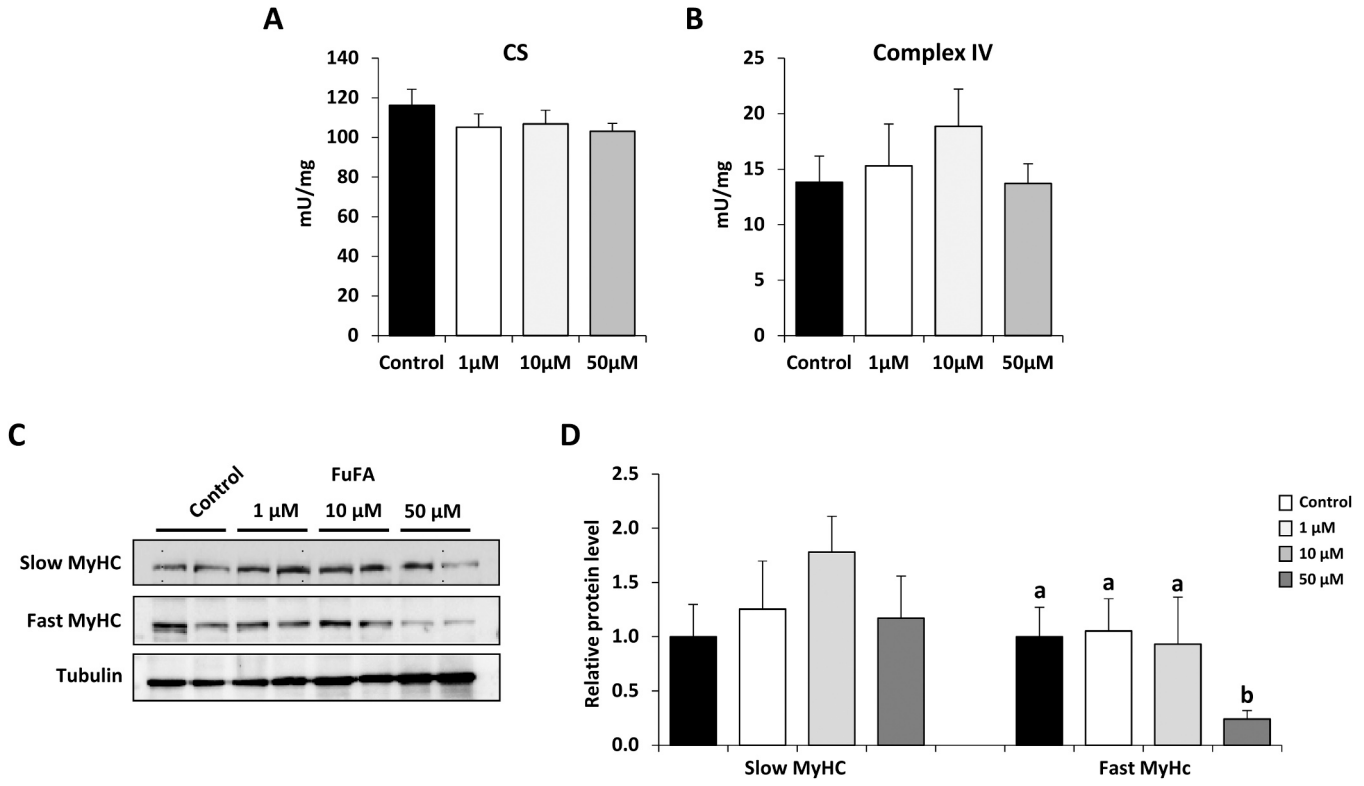

Fig. 5. Influence of FuFA-F2 supplementation on metabolic and contractile characteristics of C2C12 myotubes. (A) Citrate synthase activity of C2C12 myotubes. Enzymatic activity was measured 3 days after FuFA-F2 treatment at the indicated concentration (n = 6 for each group). (B) Complex IV activity of C2C12 myotubes. Enzymatic activity was measured 3 days after FuFA-F2 treatment at the indicated concentration (n = 6 for each group). (C) Slow and Fast MyHC protein levels were analyzed by Western blot ($n = 6$ for each group). Typical blots are shown. (D) Quantification of relative MyHC expression in C2C12 myotubes. Proteins were quantified with Image Lab™ Touch Software. Statistical Significance: $p < 0.05$ vs Control, the effects of the different dose of FuFA-F2 were tested by one way ANOVA followed by Tukey's post hoc test. The means with different letters were significantly different. Results are expressed as means ± SEM.

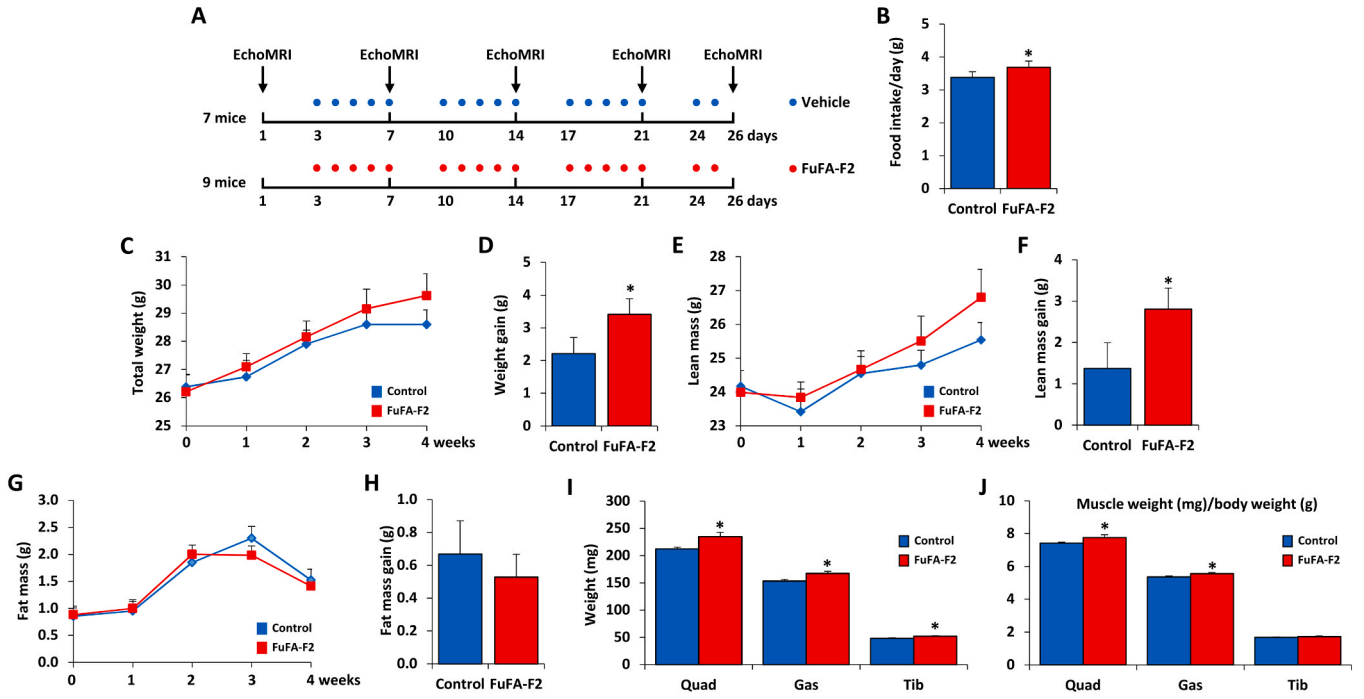

Fig. 6. FuFA-F2 supplementation in C57Bl6 mice increases muscle mass. (A) Design of the study. (B) Food intake. (C) Evolution of body weight. (D) weight gain at the end of the experiment. (E) Evolution of lean mass. (F) Lean mass gain at the end of the experiment. (G) Evolution of fat mass. (H) Fat mass gain at the end of the experiment. (I) Quadriceps, gastrocnemius, and tibialis muscles weight. (J) Quadriceps, gastrocnemius, and tibialis muscles weight were normalized to body weight. Statistical Significance: *$p < 0.05$ vs Control, Student's *t*-test. Results are expressed as means ± SEM. n = 7 for control and n = 9 for FuFA-F2.

twitch fibers express type II MyHCs (3 subtypes: IIa, IIx, and IIb) and are characterized by a lower mitochondrial density and a higher glycolytic metabolism. The oxidative capacity of type IIa and IIx fibers is intermediate between that recorded in type I and IIb fibers [39]. We found that slow MyHC was no affected by FuFA-F2 (Fig. 5C-D). Surprisingly, a decrease of fast MyHC was observed for the highest dose of FuFA-F2 (−76 %, $p < 0.05$) (Fig. 5C-D).

3.6. FuFA-F2 supplementation in C57Bl6 mice increase muscle mass

To investigate *in vivo* the potential impact of FuFA-F2 on muscle characteristics, C57Bl6 mice were supplemented by oral gavage with vehicle or FuFA-F2 (20 mg/kg) for 3 and a half weeks (Fig. 6A). We observed that mice receiving FuFA-F2 ate more (3.38 g/day for control vs 3.69 g/day for FuFA-F2, $p < 0.05$) and had a significantly higher body weight (weight gain: 2.21 g/day for control vs 3.41 g/day for FuFA-F2, $p < 0.05$) than animals receiving the vehicle alone (Fig. 6B-D). In addition, analysis of the body composition using an EchoMRI revealed that the mice supplemented with FuFA-F2 had a greater lean mass than the control mice (weight gain: 1.37 g for control vs 2.81 for FuFA, $p < 0.05$) (Fig. 6E-F). However, fat mass was similar between the two groups (Fig. 6G-H). In agreement with these observations, after euthanizing mice in the fed state, we found that the weight of the quadriceps, gastrocnemius and tibialis muscles of the hindlimbs, were significantly increased in mice receiving FuFA-F2 (Quadriceps: 212 mg for control vs 235 for FuFA-F2; Gastrocnemius: 153 mg for control vs 167 for FuFA-F2; Tibialis: 48 mg for control vs 52 for FuFA-F2, $p < 0.05$) (Fig. 6I). This influence of FuFA-F2 remains true even when muscle weight is normalized by the body weight of the mice (Fig. 6J). These data demonstrated that 3 weeks of FuFA-F2 supplementation was sufficient to increase muscle in mice.

3.7. Effect of FuFA-F2 supplementation in C57Bl6 mice on muscle phenotype

To determine if this increase in muscle mass was accompanied by changes in the contractile and metabolic characteristics of the muscle fibers, we measured the expression of myosin heavy chains and mitochondrial activity in the quadriceps. Our results did not indicate any significant difference in the expression of the different myosin heavy chains between mice treated or not with FuFA. However, we observed a trend towards an increase in MyHCIIa expression at the expense of MyHCIIb with FuFA supplementation (Fig. 7A). Citrate synthase (CS), a marker of mitochondrial activity, was not modified (Fig. 7B). We found a significant increase of the complex IV activity of mitochondrial respiratory chain complexes in FuFA group (96mU/mg for control vs 116mU/mg for FuFA-F2, $p < 0.05$) (Fig. 7C). These set of data indicates that FuFA-F2 supplementation promotes more oxidative muscle metabolism in mice.

To better understand the influence of FuFA-F2 on muscle growth, we examined the cross-sectional area of the tibialis muscle (supplemental Fig. 3). Morphometric analysis of tibialis indicated that FuFA-F2 supplementation had no impact on total fiber number and mean cross-sectional area (CSA) (Fig. 7D-E). Overall analysis of the distribution of the number of fibers in each surface range also revealed no significant difference (Fig. 7F). However, when considering all fibers over 500 µm2, we found that FuFA-F2 induced a significant increase of these fibers (+9 %, $p < 0.05$) (Fig. 7G). These data indicated that FuFA-F2 supplementation resulted in an overall increase in all fiber categories, with the exception of the smallest fibers.

Next, for measuring the relative rate of protein synthesis, we determined puromycin incorporation into proteins as previously described [28]. In this aim, mice were intraperitoneal injected with puromycin and twenty-five minutes after, animals were euthanized. However, our results indicated that there was no significant difference between control mice and those supplemented with FuFA-F2 (Fig. 8A-B).

In addition, to investigate whether FuFA-F2 increases muscle mass in

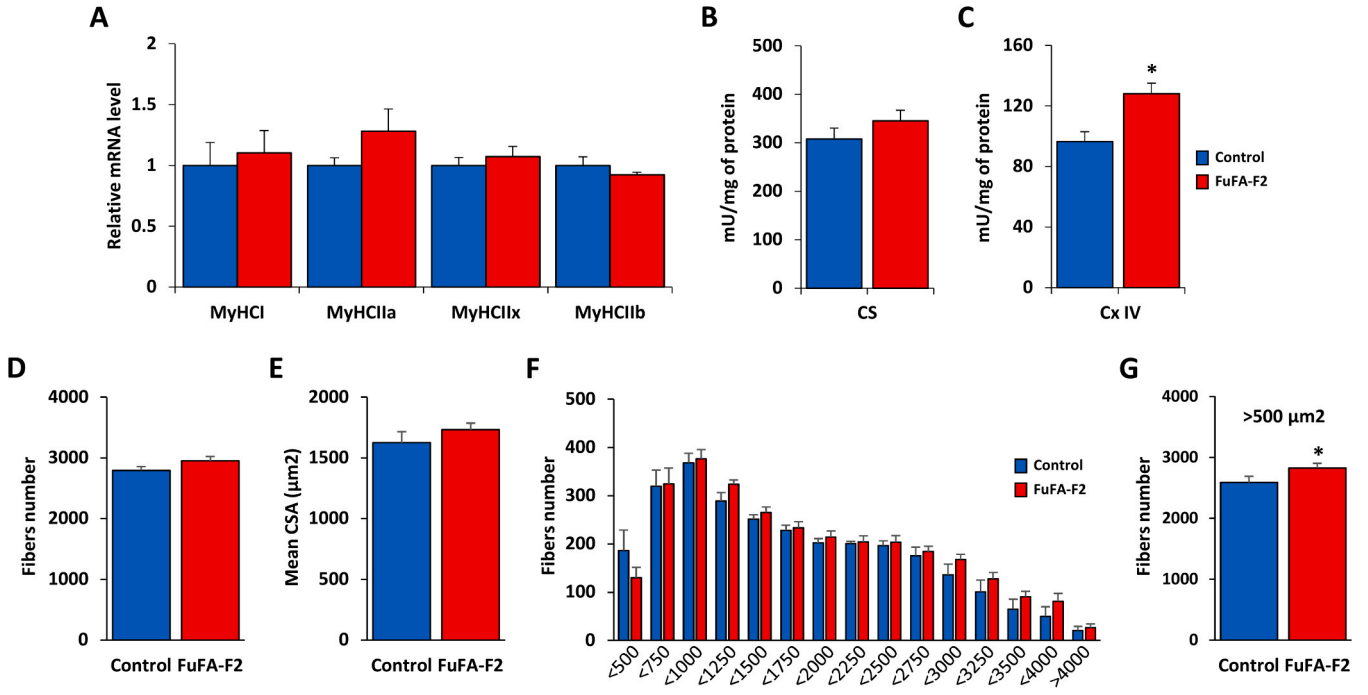

Fig. 7. FuFA-F2 supplementation in C57Bl6 mice increases mitochondrial complex IV activity in quadriceps muscle. (A) Relative expression of myosin heavy chains. (B) Citrate synthase activity. (C) Mitochondrial complex IV activity. (D) Total fibers number. (E) Mean fibers area (μm^2). (F) Fiber size distribution in tibialis muscles. (G) Total number of fibers over 500µm2. Statistical Significance: *$p < 0.05$ vs Control, Student's *t*-test. Results are expressed as means ± SEM. n = 7 for control and n = 9 for FuFA-F2.

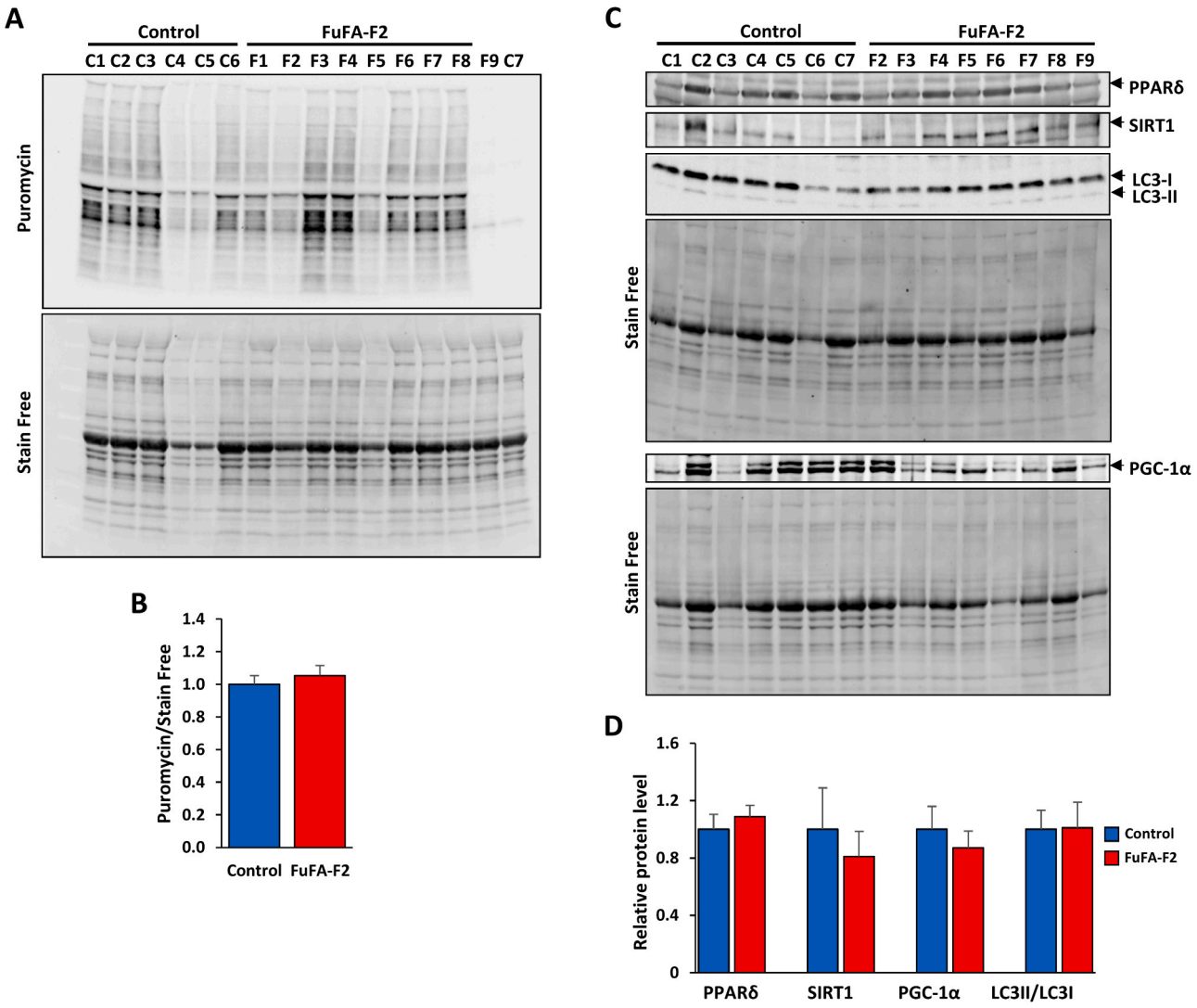

Fig. 8. Influence of FuFA-F2 supplementation on puromycin incorporation and on expression of PPARδ, SIRT1, PGC-1α and LC3. (A) Western-blot analysis of puromycin incorporation in gastrocnemius muscle of C57BL6 mice. (B) Quantification of relative puromicyn incorporation. (C) Western-blot analysis of the indicated proteins in gastrocnemius muscle of C57BL6 mice. (D) Quantification of relative protein expression. Proteins were quantified with Image Lab™ Touch Software. Results are expressed as means ± SEM. n = 7 for control and n = 9 for FuFA-F2. Protein were normalized with Stain Free total protein staining.

mice and regulates oxidative metabolism, skeletal muscle expression of PPARδ (a master regulator of fatty acid catabolism in muscle), SIRT1 (a NAD+ sensitive deacetylase that increases β-oxidation and inhibits muscle atrophy) and PGC-1α (a master regulator of mitochondrial activity), was measured by western-blot. Next, we also determined the expression of LC3-II/LC3-I ratio, whose value dropped in the presence of FuFA-F2 in C2C12 myotubes. We observed that the expressions of PPARδ, SIRT1 and PGC-1 α were not changed by FuFA-F2 supplementation (Fig. 8C-D). Furthermore, in contrast to our previous findings on C2C12 mytotubes, the LC3II/LC3I ratio was not affected in the presence of FuFA-F2 (Fig. 8C-D).

4. Discussion

Recently, we showed that a preventive nutritional supplementation with furan fatty acid in a DIO mouse model reduces metabolic disorders and increase muscle mass [26]. In the present study, our aim is to determine the influence of FuFA-F2 extracted from *Hevea brasiliensis* latex [9] on skeletal muscle phenotype. To address this potential effect of FuFA-F2 we used mouse C2C12 skeletal muscle cell lines and 3 different doses of FuFA-F2 (1, 10 and 50 μM) tested. The lowest dose of 1 μM is comparable to the concentration found in human plasma [22,36,37].

We showed that 3 days of FuFA-F2 treatment significantly increase total protein content in C2C12 myotubes whatever the dose (Fig. 1). To understand how FuFA-F2 induces an increase in total protein content and muscle anabolism we investigated protein homeostasis in C2C12 myotubes. After 5 days of differentiation, myotubes were treated for 3 days with 10 μM FuFA-F2. Then, we cultured C2C12 myotubes for 2 h in low-glucose medium before adding the different treatments for additional 30 min. Under this condition, we observed that 3 days of FuFA-F2 treatment induced a strong increase in mTOR phosphorylation (Fig. 3). This observation indicates that FuFA-F2 stimulates protein synthesis. However, we did not observe in these experiments any influence of FuFA-F2 on AKT phosphorylation. Although the classical pathway of mTOR regulation involves AKT, other mechanisms of activation of this pathway have already been shown [40]. We next investigated protein degradation. Autophagy is a major intracellular degradation process that is essential for the clearance of altered proteins and organelles. In addition, autophagy has been shown to be critical for skeletal muscle homeostasis [41]. This process involves the formation of double-membraned structures known as autophagosomes. LC3-II is a

standard marker for autophagosomes, so we assessed autophagy by measuring the LC3-II/LC3-I ratio. We found that FuFA-F2 decreased significantly this ratio whatever the dose indicating that this fatty acid inhibited the autophagy process in C2C12 myotubes (Fig. 3). We also found that FuFA-F2 decreased AMPK activation, a regulator of autophagy initiation [38]. However, the observation that the LC3II/LC3I ratio is not altered in the muscles of mice supplemented with FuFA-F2 suggests that there is no impact on autophagy *in vivo*. Our results *in vitro* on C2C12 myotubes and *in vivo* in mice indicate that FuFA-F2 mainly increases muscle mass by stimulating protein synthesis.

To investigate *in vivo* the potential impact of FuFA-F2 on muscle characteristics, C57Bl6 mice were supplemented by oral gavage with vehicle or FuFA-F2 during 3 and a half weeks. We revealed that FuFA-F2 increased muscle mass and promoted a more oxidative metabolism (Figs. 6–7). Furthermore, our morphometric analyses indicate that FuFA-F2 supplementation resulted in an overall increase in all fiber categories except the smallest fibers. Next, to investigate whether FuFA-F2 increases muscle mass in mice and regulates oxidative metabolism, we studied the skeletal muscle expression of PPARδ, SIRT1 and PGC-1α. However, our results indicate that the expression of these different major regulators of muscle metabolism was not modified by FuFA-F2 supplementation (Fig. 8). Thus, the mechanisms of action and metabolic pathways of FuFA-F2 at the muscle and cellular level remain to be explored.

Endurance exercise and resistance exercise induce different adaptive changes to the skeletal muscle. Endurance exercise promotes transformation of glycolytic muscle fibers to oxidative muscle fibers and increases in mitochondrial density and oxidative function. On the other hand, resistance exercise increases muscle mass and strength, and promotes development of more glycolytic muscle fibers. Interestingly, FuFA-F2 both promotes an increase in muscle mass and promotes a more oxidative metabolism. This double effect of FuFA-F2 could allow to fight against muscle wasting but also to promote endurance and energy expenditure, which would be particularly beneficial given our increasingly sedentary lifestyles.

Previously, we showed that a preventive nutritional supplementation with furan fatty acid in a DIO mouse model reduces metabolic disorders. These results highlight that FuFA-F2 supplementation could be particularly beneficial 1) in patients with cancer cachexia, 2) in elderly people suffering from sarcopenia, 3) during prolonged immobilization responsible for muscle wasting, 4) in patients suffering from insulin resistance. Furthermore, FuFA-F2 supplementation could also be used to improve the performance of athletes. Work is currently underway in mice to test these various hypotheses, which will of course have to be confirmed by studies in patients. Last, FuFA-F2 supplementation could also be of interest in farm animals, race horses and for aging pets in order to prevent muscle wasting.

In conclusion, we demonstrated that FuFA-F2 extracted from *Hevea brasiliensis* latex stimulates muscle anabolism both *in vitro* and *in vivo*, and promotes a more oxidative metabolism. Thus, our studies highlight that FuFA-F2 is a new biosourced molecule derived from the latex currently used for natural rubber production, produced in an environmentally sustainable way, with a strong therapeutic potential.

CRediT authorship contribution statement

F.P., E.D., J.C., C.B., L.P., L.V., S.L., T.B., C.B-G., and F.C acquisition of data. E.D., C.C.,S.G., A.S., C.F-C. and FC analysis and interpretation of data. C.F-C. and F.C. conception and design, wrote the paper. All the authors have read and approved the final version of this manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Animals and ethics statement

Our animal facility is approved by the Departmental Veterinary Services (No. E34–172-10, 2019/03/04) and the Ministry of Research (DUO No. 7053, 2020/02/26). According to the European Directive 210–63-EU, mice were observed daily for the general health status and mortality scoring. The animals were euthanized in the fed state.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2023.115330.

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