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Effects of Conjugated Linoleic Acid Associated With Endurance Exercise on Muscle Fibres and Peroxisome Proliferator-Activated Receptor γ Coactivator 1 α Isoforms

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Conjugated linoleic acid (CLA) has been reported to improve muscle hypertrophy, steroidogenesis, physical activity, and endurance capacity in mice, although the molecular mechanisms of its actions are not completely understood. The aim of the present study was to identify whether CLA alters the expression of any of the peroxisome proliferator-activated receptor γ coactivator 1α (PGC1α) isoforms, and to evaluate the possible existence of fibre-type-specific hypertrophy in the *gastrocnemius* and *plantaris* muscles. Mice were randomly assigned to one of four groups: placebo sedentary, CLA sedentary, placebo trained, or CLA trained. The CLA groups were gavaged with 35 μl per day of Tonalin® FFA 80 food supplement containing CLA throughout the 6-week experimental period, whereas the placebo groups were gavaged with 35 μl sunflower oil each day. Each administered dose of CLA corresponded to approximately 0.7 g/kg or 0.5%, of the dietary daily intake. Trained groups ran 5 days per week on a Rota-Rod for 6 weeks at increasing speeds and durations. Mice were sacrificed by cervical dislocation and hind limb posterior muscle groups were dissected and used for histological and molecular analyses. Endurance training stimulated mitochondrial biogenesis by PGC1α isoforms (tot, α1, α2, and α3) but CLA supplementation did not stimulate PGC1α isoforms or mitochondrial biogenesis in trained or sedentary mice. In the *plantaris* muscle, CLA supplementation induced a fibre-type-specific hypertrophy of type IIx muscle fibres, which was associated with increased capillary density and was different from the fibre-type-specific hypertrophy induced by endurance exercise (of types I and IIb muscle fibres).

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A new class of food supplements known as "fat supplements" have become popular among elite and recreational athletes because they are marketed with claims to induce weight loss, alter lipid profiles, improve performance, increase fat metabolism, and increase testosterone synthesis and spare glycogen stores during endurance exercise (Jeukendrup and Aldred, 2004; Macaluso et al., 2012b). These supplements include conjugated linoleic acid (CLA), long-chain triacylglycerols, medium-chain triacylglycerols, and fish oil (Macaluso et al., 2013). One of the most studied fat supplements is CLA.

CLA is a group of positional and geometrical isomers of conjugated dienoic octadecadienoate fatty acids produced from linoleic acid by rumen bacteria (Kepler et al., 1966). CLA contains two double bonds separated by a single bond in either a *cis* or *trans* configuration. These double bonds can be located in any position on the carbon chain, and are commonly positioned between carbon molecules 8 and 13 (Dilzer and Park, 2012). There are at least 28 known CLA isomers, but the two most common are cis-9, trans-11 (c9:t11), and trans-10,

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cis-12 (t10:c12) (Dilzer and Park, 2012). CLA has been described to possess a variety of beneficial effects such as anti-cancer, -atherosclerosis, -osteoporotic, -oxidant, and -obesity (Lim et al., 2005; Rahman et al., 2011; Kim et al., 2016).

CLA supplementation has the ability to reduce liver glycogen consumption (Kim et al., 2010, 2012), resulting in decreased lactate production (Kim et al., 2012) and increased fat utilization (Mizunoya et al., 2005; Kim et al., 2010, 2012), and therefore elongating time to exhaustion during running (Kim et al., 2010, 2012) and swimming (Mizunoya et al., 2005) in mice. CLA may regulate skeletal muscle metabolism by a newly identified molecular pathway, improving oxidative phosphorylation and antioxidant capacity in rodent models (Choi et al., 2007; Rahman et al., 2009). Recently, Kim and Park (2015) have reported that CLA treatment significantly upregulated peroxisome proliferator-activated receptor γ coactivator 1α (PGC1α) in a murine skeletal muscle cell line. In contrast, Parra et al. (2012) showed that CLA treatment did not affect PGC1α gene expression in sedentary mice supplemented with standard-fat (over 37 days) and high-fat diet (over 65 days).

PGC1α is a transcriptional coactivator that controls the expression of genes involved in the regulation of fatty acid oxidation, glucose metabolism, and antioxidants (St-Pierre et al., 2003; Rodgers et al., 2005; Gerhart-Hines et al., 2007; Garcia-Carrizo et al., 2016). It is considered the "master regulator of mitochondria" (Ventura-Clapier et al., 2008), as it regulates mitochondrial transcription factors (Scarpulla, 2002) such as the nuclear respiratory factor (NRF) family. It has been reported that PGC1α may induce many of the changes associated with endurance training, including mitochondrial biogenesis, fibre-type switching, stimulation of fatty acid oxidation, angiogenesis, and resistance to muscle atrophy (Ruas et al., 2012). Recent studies have found that the PGC1α gene contains more than one promoter and transcribes multiple isoforms (Zhang et al., 2009). Ruas et al. (2012) suggested that PGC1 α4 is preferentially induced during resistance exercise in mice. Similarly, Lundberg et al. (2014) and Ydfors et al. (2013) have also shown that endurance exercise affects PGC1 α4 expression in human skeletal muscle. Recently, we observed that endurance exercise increases the expression of PGC1 α1, α2, and α3 isoforms in murine *soleus* muscle (Barone et al., 2016a).

We hypothesized that CLA supplementation, whether accompanied by endurance training or not, may stimulate mitochondrial biogenesis through activation of the PGC1α isoforms. The aim of the present study was to identify if CLA alters the expression of any of the PGC1α isoforms, and to evaluate the presence of a possible fibre-type-specific hypertrophy in the *gastrocnemius* and *plantaris* muscles.

Materials and Methods

Animals and animal care

Animal experiments were performed at the animal experimental facilities of the Human Physiology Laboratory (University of Palermo, Department of Experimental Biomedicine and Clinical Neuroscience, authorized by the Ministero della Sanità of Rome, Italy). The experimental procedure received prior approval from the Committee on the Ethics of Animal Experiments of the University of Palermo (Italy), and adheres to the Guide for the Care and Use of Laboratory Animals of the National Institute of Health (NIH). Thirty-two young (7 weeks old) male mice (BALB/cAnNHsd) were obtained from Harlan laboratories S.r.l. (Italy). Mice were maintained on a constant 12:12-h light-dark cycle with free access to food and water.

CLA-administration and training protocol

Mice were randomly distributed into 4 groups of 8 mice each: placebo sedentary (PLA-SED); CLA sedentary (CLA-SED); placebo trained (PLA-TR); CLA trained (CLA-TR). The CLA groups (CLA-SED; CLA-TR) were gavaged with 35 $\mu l \bullet d^{-1}$ of Tonalin® FFA 80 (Cognis Nutrition and Health, Germany) food supplement containing CLA throughout the 6-week experimental period, whereas the placebo groups (PLA-SED; PLA-TR) were gavaged with 35 $\mu l \bullet d^{-1}$ of sunflower oil. Each administered dose of CLA corresponded to approximately 0.7 g/kg, or 0.5%, of the dietary daily intake. Tonalin® FFA 80 contained 84% CLA (40% c9:t11, 40% t10:c12, and 4% other CLA isomers), 12% oleic acid, 3% stearic acid, 0.5% palmitic acid, and 0.5% linoleic acid. This supplementation procedure has been previously by our research group (Barone et al., 2013).

Trained groups (PLA-TR; CLA-TR) ran 5 days/week for 6 weeks at progressively increasing durations and intensities on a motorized Rota-Rod (Ugo Basile, Biological Research Apparatus, Italy). The training protocol and procedures have been described previously by our research group (Bellafiore et al., 2007a,b; Chimenti et al., 2007; Di Felice et al., 2007). Sedentary mice (PLA-SED; CLA-SED) did not perform any controlled physical activity. Forty-eight hours after the completion of the final exercise session, all mice were sacrificed by cervical dislocation and hind limb posterior muscle groups (the *gastrocnemius* and *plantaris* muscles) were dissected. Muscles from the right hind limb were frozen in liquid nitrogen and stored at −80°C, whereas the muscles from the left hind limb were fixed (acetone, methanol, and water—2:2:1; for 12 h) and embedded in paraffin (Rappa et al., 2014; Barone et al., 2016b).

Immunohistochemistry

For immunohistochemical analysis, serial cross sections (5 μm) of paraffin-embedded muscles were mounted on a slide and incubated in an "antigen unmasking solution" (10 mM tri-sodium citrate, 0.05% Tween-20) for 10 min at 75°C. Following this, the MACH1 kit (M1u539g, Biocare, CA) was used according to the manufacturer's instructions. The immunohistochemistry protocol and procedures have been described previously by our research group (Macaluso et al., 2012a; Pomara et al., 2016). The primary antibodies used were anti-myosin heavy chain-I (MHC-I; A4951, Hybridoma Bank, IA) and anti-myosin heavy chain-II (MHC-II; A474, Hybridoma Bank). Immunohistochemical images were captured using a Leica DM5000 upright microscope (Leica Microsystems, Heidelberg, Germany). Cross-sectional area (CSA) of types I and II muscle fibres from the *plantaris* and *gastrocnemius* muscles were measured using ImageJ 1.41 software. Analyses were performed using five fields per section, five sections per mouse (40 μm between sections), and eight mice per group. An average of 287 fibres were analyzed for each mouse.

Immunofluorescence and confocal analysis

For immunofluorescence, deparaffinized sections were incubated in the "antigen unmasking solution" (10 mM tri-sodium citrate, 0.05% Tween-20) for 10 min at 75°C and treated with a blocking solution (3% BSA in PBS) for 30 min. Next, the primary antibody (anti-Isolectin GS-IB4, Alexa Fluor 488 conjugate I21411, Life Technologies, Monza, Italy or anti-laminin, rabbit polyclonal AB2034, Millipore, Temecula, CA) diluted 1:50, was applied, and the sections were incubated in a humidified chamber overnight at 4°C. The sections were then incubated for 1 h at 23°C with a conjugated secondary antibody (anti-rabbit IgG–FITC antibody produced in goat, F0382, Sigma–Aldrich, St. Louis, MO). Nuclei were stained with Hoechst Stain Solution (1:1000, Hoechst 33258, Sigma–Aldrich). The slides were treated with PermaFluor Mountant (Thermo Fisher Scientific, Inc., Waltham, MA) and cover slipped. The images were captured using a Leica Confocal Microscope TCS SP8 (Leica Microsystems) and analyses were performed using four fields per section, five sections per mouse, and six mice per group.

Immunoblotting

Frozen skeletal muscles (approximately 250 mg each, inclusive of both the *gastrocnemius* and the *plantaris* muscles) were homogenized by hand (using mortar and pestle) in an ice-bath in lysis buffer. The protocol and procedures for immunoblotting and sample preparation have been described previously by our research group (Pomara et al., 2016). Primary antibodies used were anti-MnSOD (diluted 1:1000, rabbit polyclonal antibody ADI-SOD-110, Enzo Life Sciences Inc., Farmingdale, NY), anti-phospho-AMPKα (diluted, 1:1000, rabbit polyclonal antibody 07-681SP, Millipore), anti-AMPKα1 (diluted 1:1000, rabbit polyclonal antibody 07-350SP, Millipore), anti-PGC1α (diluted 1:1000, mouse monoclonal antibody ST1202, Calbiochem Inc., San Diego, CA), anti-TFAM (diluted 1:1000, rabbit polyclonal antibody DR1071, Calbiochem Inc.), anti-Hsp60 (diluted 1:3000, mouse monoclonal antibody ab13532, Abcam, UK), and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; diluted 1:3000, rabbit polyclonal antibody ADI905784, Enzo Life Sciences). The detection of immunopositive bands was performed using ECL Western Blotting Detection Reagent (Amersham Biosciences, Valley Stream, NY) according to the manufacturer's instructions. The immunoblotting bands were analysed using ImageJ software version 1.41 (NIH; http://rsb.info.nih.gov/ij). GAPDH immunoblotting band intensity was examined as the loading control. Each experiment was performed at least five times.

Total RNA and DNA isolation

Total RNA and DNA (genomic and mtDNA) were extracted using Tri-reagent (Sigma–Aldrich) according to the manufacturer's instructions (Campanella et al., 2015) from the frozen muscles of 8 mice for each group. Sample RNA and DNA concentrations were quantified by spectrophotometric analysis using the NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, IL).

Quantitative real-time PCR (qRT-PCR)

Reverse transcription was performed using 300 ng of the extracted RNA and the ImProm-II Reverse Transcriptase Kit (Promega, Madison, WI) according to the manufacturer's instructions. Complementary deoxyribonucleic acid (cDNA, 20 ng) was amplified by qRT-PCR, which was performed using GoTaq qPCR Master Mix (A6001, Promega) and a Rotor-gene™ 6000 Real-Time PCR Machine (Qiagen GmbH, Hilden, Germany) using primers indicated in Table 1. Changes in transcription level were calculated using the $2^{-\Delta\Delta CT}$ method and normalized to transcription levels of GAPDH (Di Felice et al., 2015). PCR fragments purified using the Nucleospin PCR and Gel Clean-up Kit (Macherey-Nagel GmbH & Co. KG, Germany) were sequenced by Eurofins Sequencing Service (Germany). Sequences were then analyzed with the BLASTn Web Tool on the NIH website (http://www.ncbi.nlm.nih.gov/BLAST/). All PCR-amplified fragments corresponded to the desired target.

Relative mitochondrial copy number method

qRT-PCR analysis was performed using GoTaq qPCR Master Mix (A6001, Promega) using three optimized PCR primer pairs targeting two mitochondrial (*mt*-12S and *mt*-Cyt_b) and one nuclear (*Becn1*) gene (Table 1). The protocol, procedures, and mathematical formulae for calculating mitochondrial copy number have been described previously by our research group (Barone et al., 2016a). The amplification primers were validated for matching amplification efficiency and the absence of any amplification of mitochondrial pseudogenes by sequencing of PCR products and comparing amplicon sequences with BLASTn database.

Statistical analysis

Normality of the data and homogeneity of the variance were verified. Two-way ANOVA for single measurements followed by Bonferroni post-hoc test was used for data analysis. All statistical analyses were performed using the program GraphPad Prism™ 4.0 (GraphPad Software Inc., San Diego, CA). All data are presented as the mean ± SD, and the level of statistical significance was set at $P < 0.05$.

Results

Effects of endurance training and CLA supplementation on the CSAs of posterior hind limb muscle fibres

To evaluate the hypertrophic effects of endurance exercise and CLA supplementation on the hind limb posterior muscle group (*gastrocnemius* and *plantaris* muscles), CSA was evaluated in the different muscle fibre types. Morphometric analysis showed that endurance training increased the CSA of type IIb fibres in the *plantaris* and *gastrocnemius* muscles and of type I fibres exclusively in the *gastrocnemius* ($P < 0.0001$). CLA supplementation induced a main effect on the CSA of type IIa and x fibres in the *plantaris* muscle ($P < 0.05$), although the significant difference for IIx muscle fibres was only detected between the PLA-TR and CLA-TR groups ($P < 0.01$). Analysis on type I fibres of the *plantaris* muscle was not performed because of their relative paucity (three to four fibres for each muscle). CLA supplementation did not induce any effect in the *gastrocnemius* muscle. Morphometric analysis of the *plantaris* and *gastrocnemius* muscles are shown in Figure 1.

CLA supplementation induced the increase of isolectin levels in *plantaris* muscle

To assess the effects of endurance exercise and CLA supplementation on capillary density, immunofluorescence staining using a specific antibody against isolectin GS-IB4 was performed on the hind limb *gastrocnemius* and *plantaris* muscles (Fig. 2A). Isolectin immunoreactivity levels were analyzed by confocal microscopy using the Leica Application Suite Advanced Fluorescences software. Two-way ANOVA analysis

TABLE 1. Forward and reverse primers used for qRT-PCR

Primer	Forward	Reverse
PGC1 tot	5′-TGATGTGAATGACTTGGATACAGACA-3′	5′-GCTCATTGTTGTACTGGTTGGATATG-3′
PGC1 a1	5′-GGACATGTGCAGCCAAGACTCT-3′	5′-CACTTCAATCCACCCAGAAAGCT-3′
PGC1 a2	5′-CCACCAGAATGAGTGACATGGA-3′	5′-GTTCAGCAAGATCTGGGCAAA-3′
PGC1 a3	5′-AAGTGAGTAACCGGAGGCATTC-3′	5′-TTCAGGAAGATCTGGGCAAAGA-3′
PGC1 a4	5′-TCACACCAAACCCACAGAAA-3′	5′-CAGTGTGTGTATGAGGGTTGG-3′
HSP60	5′-ACGATCTATTGCCAAGGAGG-3′	5′-TCAGGGGTTGTCACAGGTTT-3′
GADPH	5′-CAAGGACACTGAGCAAGAGA-3’	5’-GCCCCTCCTGTTATTATGGG-3’
BECN1	5′-CGAGTGCCTTCATCCAAAAC-3′	5′-GTCCTGGCACCTCTCTAATG-3′
mt-Cytb	5′-TAGCAATCGTTCACCTCCTC-3′	5′-TGTAGTTGTCTGGGTCTCCT-3′
mt_12s	5′-GATAAACCCCGCTCTACCTC-3′	5′-CATTGGCTACACCTTGACCT-3′

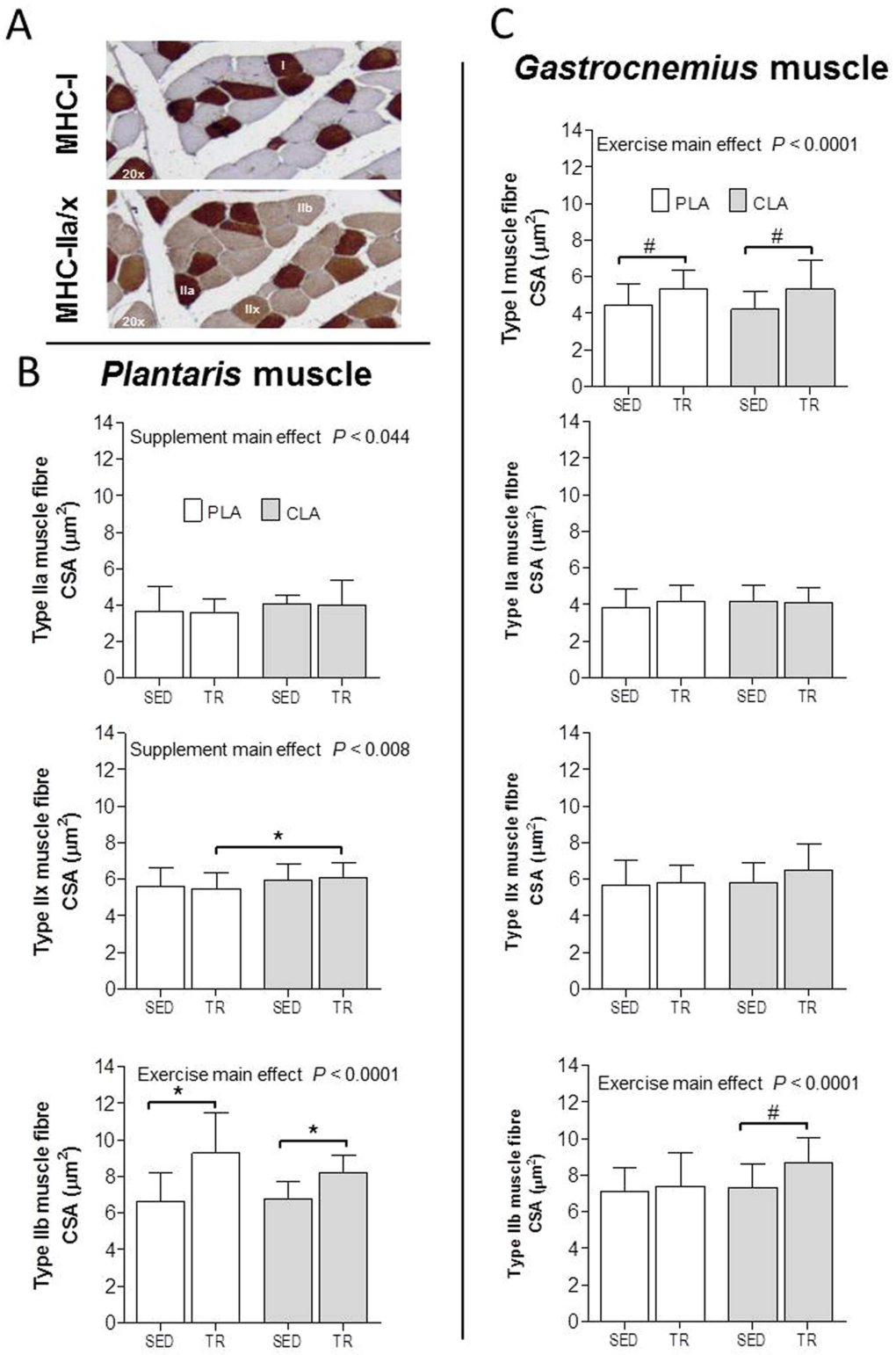

Fig. 1. Histological evaluation of hind limb posterior muscle group (*gastrocnemius* and *plantaris* muscles). (A) Explanatory immunohistochemistry for MHC-I and MHC-IIa/x in serial cross-sections of *gastrocnemius* muscle. Type IIb fibres are negative to antibodies anti-MHC-I and anti-MHC-IIa/x. Skeletal muscle fibres cross sectional area (CSA) of *plantaris* (B) and *gastrocnemius* (C) muscles of trained (TR) and sedentary (SED) mice supplemented with conjugated linoleic acid (CLA) or placebo (PLA). Data are presented as the mean ± SD. #Significantly different ($P < 0.05$), *($P < 0.01$).

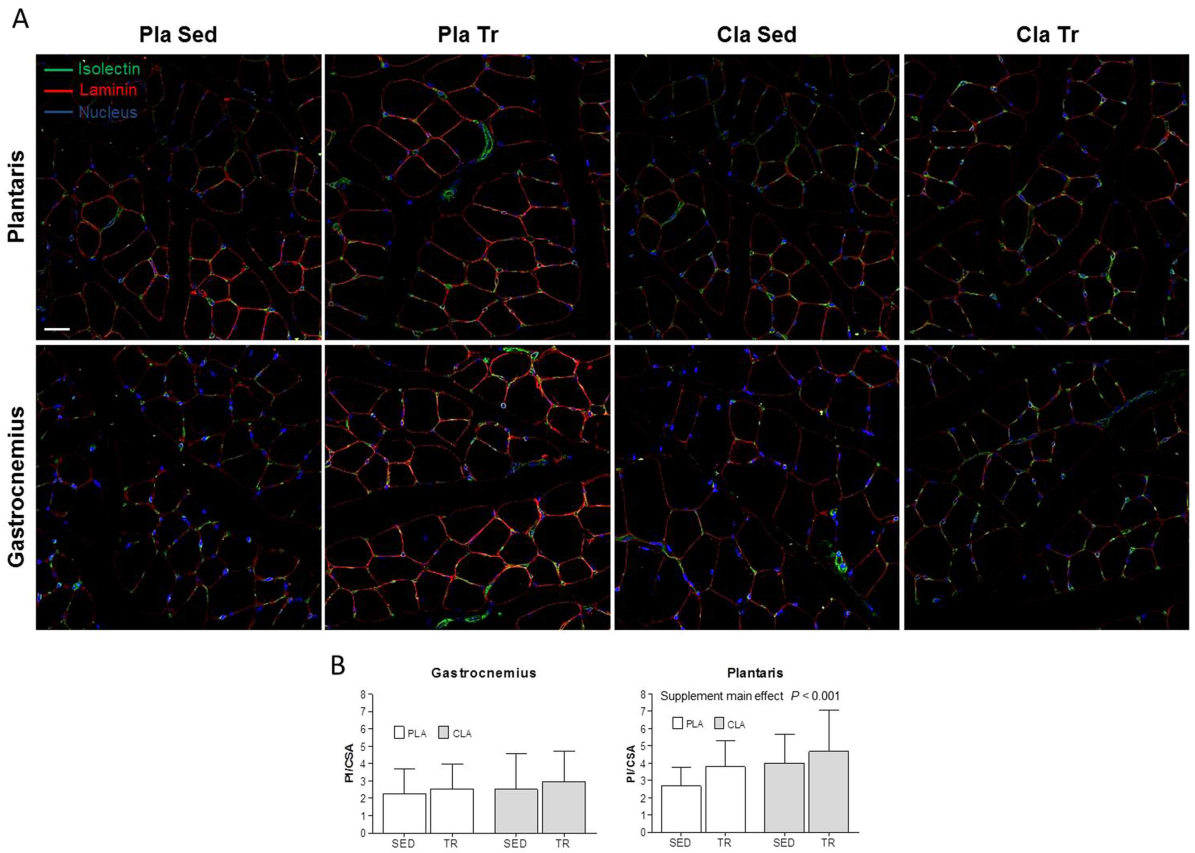

Fig. 2. Confocal microscopy analysis for isolectin GS-IB4 in *plantaris* and *gastrocnemius* muscles. A, immunofluorescence for isolectin GS-IB4 (green) and laminin (red) of cross-sections of *plantaris* and *gastrocnemius* muscles of trained (TR) and sedentary (SED) mice supplemented with conjugated linoleic acid (CLA) or placebo (PLA). Bar 25 μm. B, the staining intensity for isolectin GS-IB4 (bars) was expressed as the mean pixel intensity (PI) normalized to the CSA (cross-sectional area) using the software Leica Application Suite Advanced Fluorescences software. Open bar, groups of the study. Data are presented as the mean ± SD.

did not show any significant difference between the groups whereas isolectin immunoreactivity was significantly higher in response to CLA supplementation ($P < 0.001$) in the *plantaris* muscle (Fig. 2B).

Endurance training induced the increase of p-AMPKα and PGC1α protein levels

MnSOD, p-AMPKα, AMPKα1, PGC1α, TFAM, and Hsp60 protein analyses were performed on the hind limb posterior muscle group (*gastrocnemius* and *plantaris* muscles) using western blot analysis (Fig. 3). p-AMPKα and PGC1α protein levels were significantly higher in both trained groups (PLA-TR and CLA-TR) compared to the sedentary groups (PLA-SED and CLA-SED; $P < 0.01$). As shown in Figure 3, p-AMPKα and PGC1α protein levels significantly increased as a consequence of endurance training ($P < 0.0001$) and not as an effect of CLA supplementation. Mn SOD, AMPKα1, TFAM, and Hsp60 protein levels were not affected by training and/or supplementation.

CLA supplementation did not induce variation in PGC1α isoform gene levels

Quantitative RT-PCR analysis showed a significant increase in total PGC1 α1, α2, and α3 isoforms ($P < 0.05$; Fig. 4A) in response to endurance training in the hind limb posterior muscle group (*gastrocnemius* and *plantaris* muscles). CLA supplementation did not induce any significant changes on PGC1α isoform gene expression levels. Moreover, PGC1 isoform α4 gene expression was not detected in any muscle samples.

Endurance training induced the increase of mitochondrial content in *gastrocnemius* and *plantaris* muscles

As shown in Figure 4B, mtDNA copy number in hind limb posterior muscle groups (*gastrocnemius* and *plantaris* muscles) significantly increased in response to endurance training ($P < 0.01$), but not in response to CLA supplementation.

Discussion

The results obtained in our study suggested that endurance training may stimulate mitochondrial biogenesis in mice and activate three PGC1 isoforms (α1, α2, and α3) whereas CLA supplementation, whether associated or not with endurance exercise, did not stimulate further mitochondrial biogenesis. The protein and gene expression levels of PGC1α were not directly affected by CLA supplementation. Interestingly, our endurance training protocol induced hypertrophy of types I and IIb muscle fibres in *plantaris* and *gastrocnemius* muscles, in

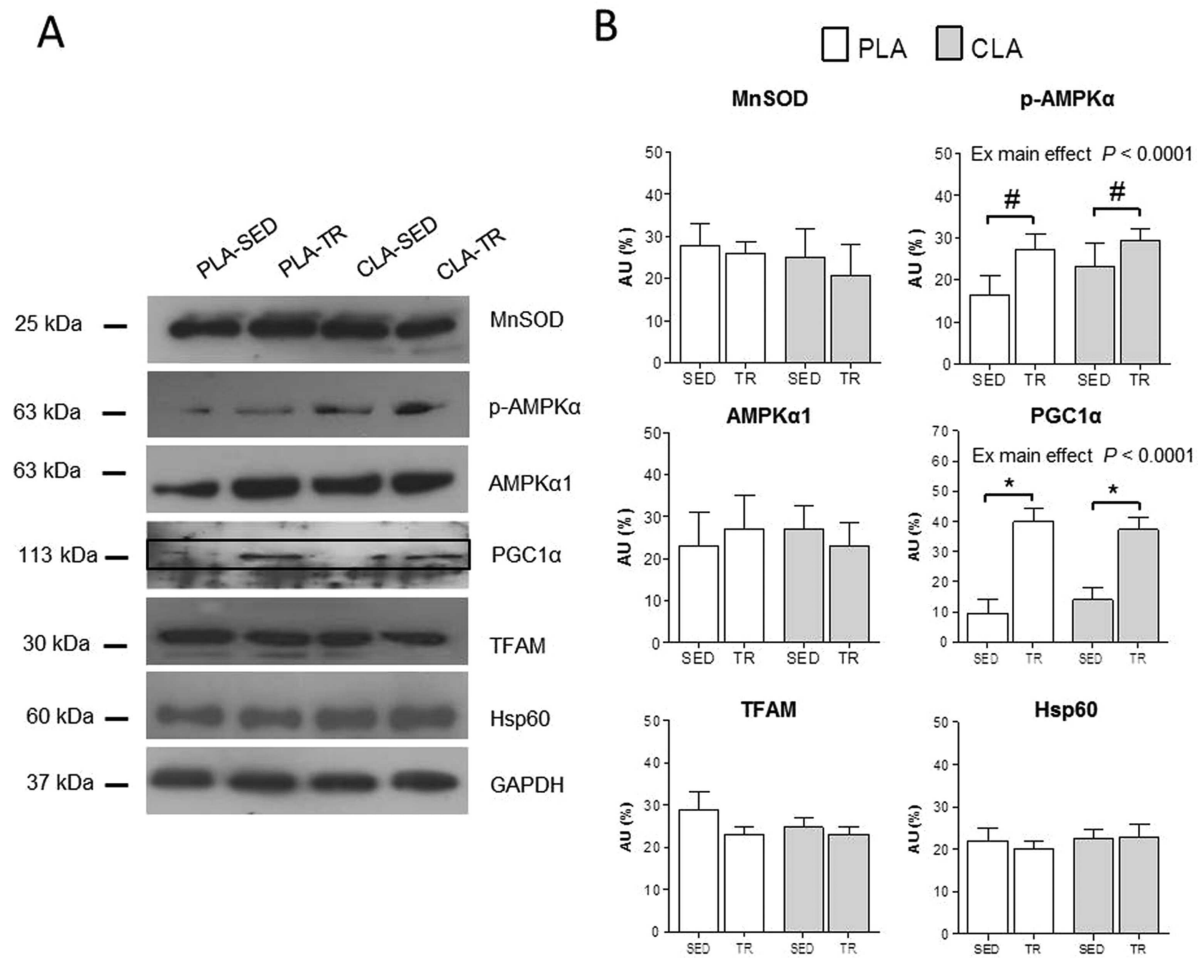

Fig. 3. Protein levels of MnSOD, p-AMPKα, AMPKα1, PGC1α, TFAM, and Hsp60 of hind limb posterior muscle group (*gastrocnemius* and *plantaris* muscles). Representative western blots (A) and relative expression levels (B) of MnSOD, p-AMPKα, AMPKα1, PGC1α, TFAM, and Hsp60 in hind limb posterior muscle group of trained (TR) and sedentary (SED) mice supplemented with conjugated linoleic acid (CLA) or placebo (PLA). Data are presented as the mean ± SD. *Significantly different ($P < 0.001$) #($P < 0.01$).

sharp contrast to CLA supplementation, which only induced a specific hypertrophy of type IIx muscle fibres in the *plantaris* muscle.

The current literature indicates that CLA supplementation affects skeletal muscle metabolism (Kim et al., 2016). Studies conducted in animals and in humans showed controversial results on the effect of CLA supplementation on lean body mass due to different factors, such as subject characteristics and form of supplementation, dosage, duration period, study design, treatment type, etc. A comprehensive and updated review has been recently published to provide a systematic assessment of skeletal muscle metabolic adaptation in response to CLA supplementation in humans and in rodents (Kim et al., 2016). Our research group has previously shown that 6 weeks of CLA supplementation associated with progressive endurance training, increased lean body mass, and muscle hypertrophy (Di Felice et al., 2007; Barone et al., 2013). In this study, our endurance training protocol increased the CSA of types I and IIb muscle fibres in the *gastrocnemius* and type IIx fibres in the *plantaris* muscle. It is known that endurance exercise, as it mainly stimulates slow oxidative fibres (i.e., type I fibres), induces muscle hypertrophy of this fibre (Schiaffino and Reggiani, 2011). Type IIb fibres, fast-glycolytic fibres, are more susceptible than other fibre types to hypertrophic and atrophic stimuli induced by training or muscle wasting, respectively (Schiaffino and Reggiani, 2011). Moreover, we have shown that the hypertrophy present in trained mice supplemented with CLA was fibre-type and muscle-specific. In fact, the CSA of type IIx muscle fibres of the *plantaris* muscle was significantly higher in trained mice supplemented with CLA than in trained mice supplemented with placebo, but this difference was not detected in the *gastrocnemius* muscle. The difference in muscle-specific hypertrophy effects may be due to the different biomechanical functions of these two muscles during quadruped plantigrade locomotion, as suggested by Schiaffino and Reggiani (2011). After CLA supplementation, hypertrophy of the *plantaris* muscle was accompanied by an increase of capillary density, possibly due to the higher oxygen demand. However, we did not observe the same effects in the *gastrocnemius* muscle because it is rich in type IIb muscle fibres, which are less involved in the types of physical activity induced by this exercise protocol. To the best of our knowledge, there is no data regarding CLA supplementation and capillary density in skeletal muscles.

It has been recently reported that CLA (c9:t11 and/or t10:c12 isomers), in a murine skeletal muscle cell line, engaged the

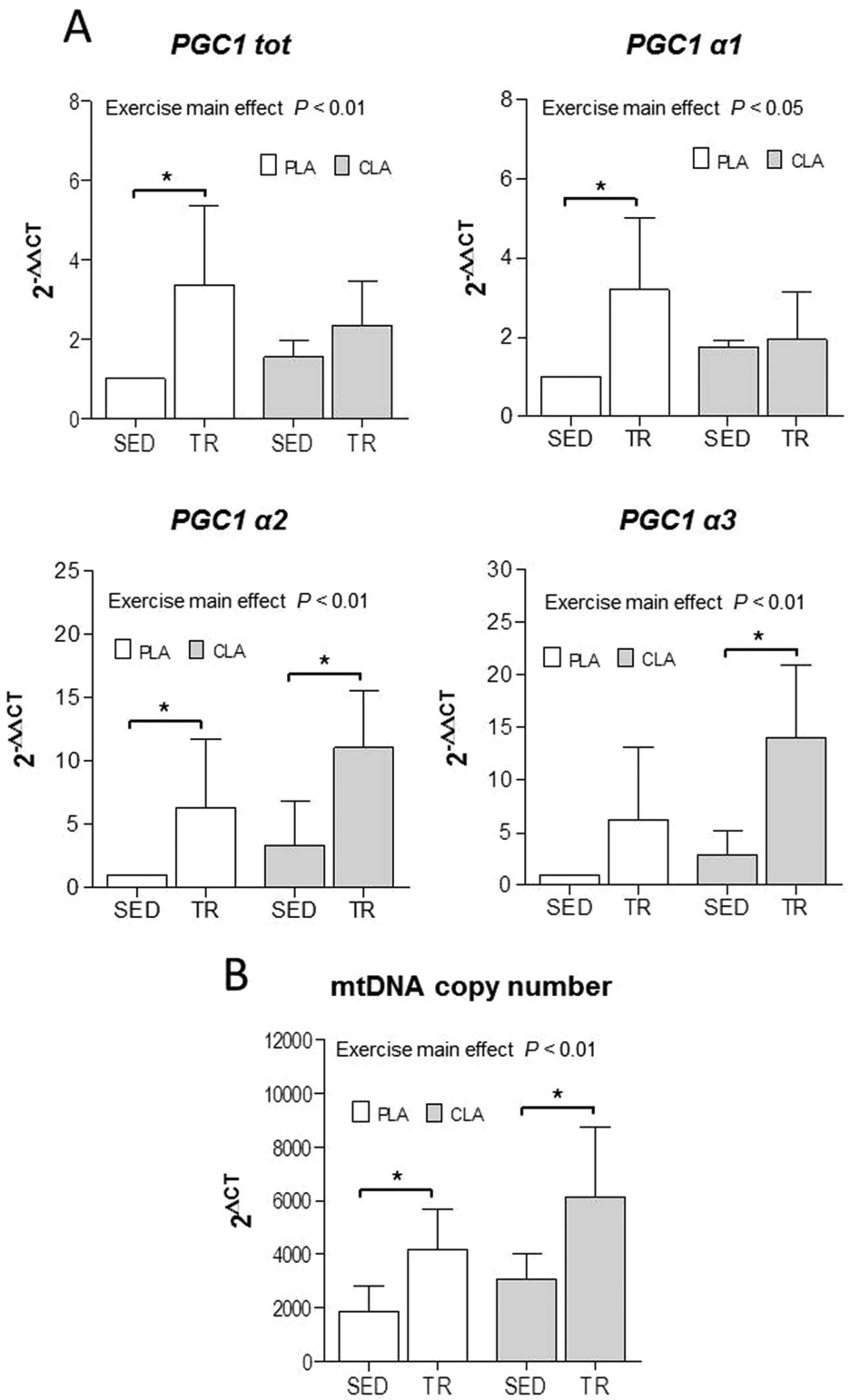

Fig. 4. qRT-PCR analysis of PGC1α isoforms and mitochondrial DNA copy number. (A) PGC1α isoforms (PGC1α total (αtot), isoform 1 [α1], 2 [α2], 3 [α3]) gene expression normalized for the reference genes and (B) copy number of mitochondrial genes in hind limb posterior muscle group of trained (TR) and sedentary (SED) mice supplemented with conjugated linoleic acid (CLA) or placebo (PLA). Data are presented as the mean ± SD. *Significantly different ($P < 0.01$).

mitochondrial biogenesis process through modulation of the PGC1α-TFAM cascade. On the other hand, Parra et al. (2012) reported that a commercial CLA mixture did not affected PGC1α gene expression in the *gastrocnemius* muscle of mice fed with standard or high-fat diets. Moreover, CLA treatment did not affect PGC1α activity despite increasing the mitochondrial content in human skeletal muscle cells (Vaughan et al., 2012). To the best of our knowledge, no study has been conducted to investigate the effect of CLA supplementation on mitochondrial biogenesis in the skeletal muscle of a trained model.

As expected, exercise training increased the protein expressions of PGC1α, p-AMPKα, and the gene expression levels of PGC1 α1, α2, and α3 isoforms relative to sedentary controls. These results confirmed our previous finding: that a progressive endurance exercise protocol of 6 weeks was able to induce mitochondrial biogenesis, likely via activation of the transcription factor PGC1α; even though we did not observe an increase in the expression of TFAM, MnSOD, or AMPKα1, which are key factors of the mitochondrial biogenesis pathway (Powers et al., 2011; Barone et al., 2016a). CLA supplementation failed to induce changes in the levels of these factors in both sedentary and trained mice.

Our previous data demonstrated that elevated PGC1α levels were paralleled by increased levels of Hsp60 both in vitro and in vivo. In particular, Hsp60 protein expression was fibre-specific (type I, IIa) and increased after 45 days of endurance training in the *soleus* muscle (Barone et al., 2016a). This protein is classically described as a mitochondrial protein involved in assisting the correct folding of other mitochondrial proteins (Kon, 1985). The chaperonin may be induced by different types of stress, as well as physical exercise (Morton et al., 2009; Cappello et al., 2014). In the present study, Hsp60 protein levels were not different between the experimental groups. These results are in disagreement with our previous data, probably because different muscle types were investigated. We herein carried out a study on the hind limb posterior muscle group (*gastrocnemius* and *plantaris* muscles), which is rich in type IIb muscle fibres expressing low levels of Hsp60 (Barone et al., 2016a).

Our analysis of muscle mitochondrial content showed that only endurance exercise and not CLA supplementation induced an increase in mtDNA. We reported that short term CLA supplementation (45 days) in trained mice had the ability to increase endurance exercise capacity by improving the distance run prior to exhaustion onset (Barone et al., 2013). Therefore, this previously observed improvement in endurance exercise capacity was not due to mitochondrial biogenesis. The levels of the PGC1α isoforms transcribed by the activation of the proximal or the alternative promoter, or as a result of an alternative splicing of exon 1, were also investigated to further study the relationship between CLA supplementation and endurance exercise capacity. The results obtained indicated that CLA supplementation, either alone or associated with endurance training, did not induce any changes in the expression of the PGC1α protein or its isoforms (α1, α2, and α3). Primary aerobic adaptations induced by endurance training, other than increased mitochondrial number and size (Holloszy and Coyle, 1984), also include increased skeletal muscle blood flow (Krustrup et al., 2004), increased mitochondrial enzyme activity for oxidized fatty acids (Holloszy and Coyle, 1984), delayed onset of anaerobic metabolism (Hurley et al., 1984), and reduced accumulations of catabolic products (Hurley et al., 1984). Therefore, it is not possible to exclude the possibility of other adaptations induced by CLA supplementation on skeletal muscle.

In conclusion, the data obtained showed that short-term CLA supplementation, whether associated with endurance exercise or not, did not stimulate mitochondrial biogenesis by PGC1α isoform activation, but did induce hypertrophy in type IIx fibres in the *plantaris* muscle after endurance exercise. We therefore believe that further studies using a different time course of CLA supplementation are required to better understand the effects of this food supplement on skeletal muscle.

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