Conjugated linoleic acid (CLA) promotes endurance capacity via peroxisome proliferator-activated receptor δ -mediated mechanism in mice

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Abstract

Previously, it was reported that conjugated linoleic acid (CLA) with exercise training potentially improved endurance capacity via the peroxisome proliferator-activated receptor δ (PPAR δ)-mediated mechanism in mice. This study determined the role of exercise and/or CLA in endurance capacity and PPAR δ -associated regulators. Male 129Sv/J mice were fed either control (soybean oil) or CLA (0.5%) containing diets for 4 weeks and were further divided into sedentary or training regimes. CLA supplementation significantly reduced body weight and fat mass independent of exercise during the experimental period. Endurance capacity was significantly improved by CLA supplementation, while no effect of exercise was observed. Similarly, CLA treatment significantly increased expressions of sirtuin1 (SIRT1) and PPAR γ coactivator-1 α (PGC-1 α), up-stream regulators of PPAR δ , in both sedentary and trained animals. With respect to downstream markers of PPAR δ , CLA up-regulated the key biomarkers needed to stimulate mitochondrial biogenesis, nuclear respiratory factor-1 (NRF-1) and mitochondrial transcription factor A (Tfam). Moreover, CLA supplementation significantly induced overall genes associated with muscle fibers, such as type I (slow-twitch) and type II (fast twitch). Taken together, it suggests that CLA improves endurance capacity independent of mild-intensity exercise via PPAR δ -mediated mechanism.

1. Introduction

There are two distinct types of exercise training: strength and endurance. Strength exercise, such as weightlifting and bodybuilding, promotes protein synthesis resulting in induced hypertrophy of muscle. On the other hand, endurance exercise, such as jogging, swimming and cycling, enhances energy expenditure and triggers a remodeling of the skeletal muscle fiber phenotype from type II (glycolytic fast-twitch fibers) to type I (oxidative slow-twitch fibers) [1]. In general, type I fibers in skeletal muscle have a greater density of mitochondria than type II fibers.

Oxidative type I fibers promote mitochondrial biogenesis and fatty acid β -oxidation to promote fat utilization in the body [2]. Thus, endurance exercise is known to help reduce body fat and potentially contribute to reducing obesity.

During the last two decades conjugated linoleic acid (CLA) has gained significant attention as a bioactive compound to prevent obesity. Although most studies of CLA's anti-obesity effects have focused on body fat reduction, accumulated evidence demonstrated that CLA increased lean body mass and enhanced exercise performances [3-5]. In fact, the previous studies in rodents have reported that CLA improved voluntary activity and endurance capacity accompanied with modulation of carnitine palmitoyltransferase 1 (CPT1), uncoupling protein 2 (UCP2) and peroxisome proliferator-activated receptor δ (PPAR δ) in skeletal muscle [6-8]. Recently, it was reported that the specific CLA isomers, *cis-9,trans-11* and *trans-10,cis-12*, induced the up-regulation of key molecular markers involved in mitochondrial biogenesis in C2C12 myotubes [9]. However, effects of CLA on skeletal muscle metabolism as well as its interaction with exercise *in vivo* are not clear. Thus, we investigated the mechanisms of CLA in skeletal muscle metabolism to determine how CLA affects endurance capacity in mice, and further to determine the interaction between CLA and exercise in muscle metabolism.

2. Materials and methods

2.1. Materials

CLA was provided by Natural Lipids Ltd. AS (Hovdebygda, Norway). The purity of CLA was 80.7% (37.8% *cis*-9,*trans*-11, 37.6% *trans*-10,*cis*-12 and 5.3% other CLA isomers and the remainder of the preparation was 13.7% oleic acid, 3.2% stearic acid, 0.4% palmitic acid, 0.2% linoleic acid and 1.8% was unknown). Semi-purified powdered diets (TD07518, 95% basal mix) were obtained from Harlan Laboratories (Madison, WI). Serum triacylglyceride (TG), glucose, and total cholesterol assay kits were purchased from Genzyme Diagnostics (Charlottetown, PE, Canada). Serum non-esterified fatty acids (NEFA) and L-Lactate assay kits were obtained from BioAssay Systems (Hayward, CA). The blood urine nitrogen (BUN) assay kit was purchased from BioQuant (San Diego, CA). DC protein assay kit was from Bio-Rad (Hercules, CA). Rabbit antibodies for phosphorylated AMP-activated protein kinase α (p-AMPK α), AMPK α and superoxide dismutase 2 (SOD2) were obtained from Cell Signaling (Berberly, MA). Rabbit antibodies for PGC-1 α , sirtuin 1 (SIRT1), PPAR δ , nuclear respiratory factor 1 (NRF-1), mitochondrial transcription factor A (Tfam), cytochrome c (Cyt C) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Horseradish peroxidase-conjugated goat anti-rabbit IgG antibody was from Boston Bioproducts Inc. (Ashland, MA). Other chemicals used were purchased from either Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Pittsburg, PA).

2.2. Animals and diet

All work related to animals was done in compliance with the Institutional Animal Care and Use

Committee at the University of Massachusetts, Amherst. Twenty 10-week-old male 129Sv/J mice were obtained from breeding colonies. All mice were placed in individual wire-bottom cages with 12h light and dark cycles in a temperature and humidity-controlled room. All mice underwent a 2-week adaptation period with a control diet. The formulation of diets is shown in Table 1. During the second week of adaptation period, animals were trained on a motorized treadmill (Columbus Instrument, Columbus, OH) for 3 days (described in details in the next section) and were subjected to baseline testing for endurance capacity and voluntary activity. All mice were randomly divided into four groups based on weights and given either control or 0.5% CLA containing diets, and either sedentary or exercise groups. The dose of CLA used for the current study was determined on the basis of previous reports [10,11]. Body weight and food intake were recorded weekly. After a four-week intervention, the mice were fasted for 4 h, exercised for 15 min, and then were sacrificed by CO₂ asphyxiation. For analysis of serum markers, blood was collected using cardiac puncture. Three different parts of adipose tissue (epididymal, retroperitoneal and mesenteric), two designated parts of skeletal muscle (gastrocnemius and quadriceps) and other organs (liver, heart, and spleen) were harvested and weighed.

2.3. Training and endurance capacity test

All mice were subjected to training on the treadmill 3 times (Columbus Instrument, Columbus, OH) during the acclimatization period. Training took 15 min total, with a speed of 10 m/min on a 0° incline with the aid of an electric shock grid. During the treatment period, mice in the exercise groups trained five times a week. The training was for 15 min (10 m/min for the first 10 min, then an increase of 1 m/min every minute for the following 5 min) on a 10° inclination with the

aid of an electric shock grid.

Endurance capacity was evaluated biweekly, including during adaptation period (week 0). The testing protocol used to a speed of 10 m/min at a 10° incline, increased by 1 m/min every minute until reaching 25 m/min, and maintained at the speed of 25 m/min until the mice were determined to be fatigued or exhausted [12,13]. Endurance capacity was determined as a function of running time and distance.

2.4. Voluntary movement measurement (Non-exercise physical activity test)

Voluntary movement was recorded biweekly using LoliTrack Quatro Video Tracking Software Version 1.0 (Loligo Systems, Tjele, Denmark) with a high-resolution infrared camera during the dark cycle (5:00pm-5:00am). Animals were individually placed into cages with free access to diet and water (provided as HydroGel®, Clear H₂O, Portland, ME). The cage (30 × 46 × 40 cm) used for activity monitoring was larger than the typical cage to avoid limited voluntary movements. Activity data between 7:00pm and 3:00am were used, excluding the first 2 h after onset of dark period and last 2 h before light period, to eliminate variable data during adaptation and lack of movement, respectively.

2.5. Serum parameters

Serum was separated from clotted blood by centrifugation at 3,000 g for 20 min at 4°C and used for analyses of glucose, total cholesterol, triglyceride (TG), non-esterified fatty acids (NEFA), blood urea nitrogen (BUN) and L-Lactate using commercial kits.

2.6. Western blot analysis

Each sample from gastrocnemius muscle tissue was homogenized in radioimmune precipitation assay (RIPA) (50 mM Tris-HCl, 10 mM NaCl, 5 mM MgCl₂, and 0.5% NP-40, pH 8.0) buffer (Boston Bioproducts Inc., Ashland, MA), protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO) and a phosphatase inhibitor cocktail (Thermo Scientific, Rockford, IL) and was lysed on ice for 2 h with gentle shaking, then centrifuged at 14,000g for 20 min at 4°C. The protein concentration in tissue lysate was normalized at 50 µg/ml of protein. The normalized samples were mixed with loading buffer, and then separated by a 10-15% (w/v) sodium dodecyl sulfate-polyacrylamide gel and were transferred onto a polyvinylidene fluoride membrane (Millipore, Bedford, MA). After blocking, proteins were probed with primary rabbit antibodies, and were then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody for 1 h at room temperature. For detection, the Enhanced Chemiluminescence solution (Bio-Rad, Hercules, CA) was used and developed under the Image Station 4000MM (Carestream Health, New Heaven, CT) setting. GAPDH was used as a loading control for normalization. The results were quantified using Image J software (U.S. National Institutes of Health).

2.7. mRNA analysis

Total RNA was isolated from the gastrocnemius muscle tissue (50 mg) in TRIzol reagent (Invitrogen, Calsbad, CA), and was then reversely transcribed to cDNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Forster, CA). cDNA with predesigned TaqMan gene expression assay kits were used for the relative quantitation. The target genes were lipoprotein lipase (LPL, Mm00434764_m1), fatty acid binding protein 4 (Fabp4, Mm00445878_m1), PPAR α (Mm00440903_m1), CPT1 β (Mm00487200_m1) and UCP2 (Mm00627597_m1) for lipid metabolism, and phosphoinositide 3-kinase (PI3K)

(Mm00803160_m1), pyruvate dehydrogenase kinase, isoform 4 (PDK4, Mm01166879_m1), protein kinase B1 (AKT1, Mm01331626_m1) and glucose transporter type 4 (GLUT4, Mm01245502_m1) for glucose metabolism. For analysis of muscle fiber type composition we used four target genes, myosin heavy chain 1 (MHC1, Mm01332493_m1), MHC2 (Mm01332564_m1), MHC3 (Mm01332463_m1) and MHC7 (Mm01332518_m1). GAPDH (Mm99999915_g1) or 18S ribosomal RNA (18S rRNA, Mm03928990_g1) were used for internal standards. All reactions were triplicated with the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster, CA). The program of reaction was 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Data were calculated using $2^{-\Delta\Delta C_t}$.

2.8. Statistical analyses

Data were analyzed by PROC MIXED of the SAS software (Version 9.3, SAS Institute Inc., Cary, NC, USA). For data on body weight, food intake, endurance capacity and voluntary movement, two-way repeated measure analysis of variance (ANOVA) and the slice option in the Least Square (LS) means statement were used to examine the group differences at each time point. For tissue weights, serum parameters, the relative protein and mRNA expression level data, two-way ANOVA along with LS means statement was used. The Tukey-Kramer's method in SAS was used for the multiple comparisons among the experimental groups. Data were shown as the mean $\pm$ S.E. *P*-values less than 0.05 are reported as statistically significant.

3. Results

3.1. Body weights and food intake

CLA-fed mice gained less body weight than the control from week 2, and maintained this trend

throughout the experiment (overall CLA effect, $p=0.0047$). Along with CLA effects, time effects on body weight gain were significant (overall time effect, $p<0.0001$), but no exercise effect was observed. There was no significant 3-way interaction (Exercise x CLA x Time), thus 2-way interactions were further evaluated. Among three possible 2-way interactions, the interaction for CLA and time was observed in body weight gain ($p<0.0001$, Fig. 1A).

There were significant overall CLA and time effects, but not exercise effect, on food intake ($p=0.0187$ and $p<0.0001$, respectively) (Fig. 1B). There was no effect on 3-way interaction (Exercise x CLA x Time), but a significant interaction between CLA and time on food intake ($p=0.0007$) was observed. A significant difference in food intake at week 2 was detected between CLA-fed group and control diet group, without any additional difference in food intake at other times between all treatment groups.

3.2. Tissue weights

Total and individual adipose tissue (epididymal, retroperitoneal, and mesenteric adipose tissue), skeletal muscle (gastrocnemius and quadriceps), liver, heart and spleen weights are shown in Table 2. Overall CLA-fed mice showed significantly less adipose tissue mass, as % of body weight, than control groups. No exercise effect was observed in adipose tissue weights. The gastrocnemius muscle weights (as % of body weight) were significantly increased by CLA supplementation ($p=0.0035$). No differences of exercise effect were observed in the liver, heart and spleen weights, whereas CLA supplementation significantly influenced the liver weight.

3.3. Endurance capacity and voluntary movement (non-exercise physical activity)

To evaluate endurance capacity, we determined the maximum running time and distance on the

motorized treadmill. Animals in the CLA groups significantly improved the maximum running time and distance over mice in the control diet groups (Fig. 2A and 2B, $p=0.0166$ and $p=0.0174$, respectively). No exercise effect was observed in endurance capacity, while significant time effect (improved over experimental period) on running time and distance was observed. There were no significant 3-way interactions (Exercise x CLA x Time) nor any 2-way interactions (Exercise x CLA, Exercise x Time, or CLA x Time) for both maximum running time and distances. For depicting overall trend of maximum running distance change (%), we fitted the Least-squares line with a zero intercept. Although there were no significant differences (as shown in Fig. 2B), all experimental groups except the sedentary control group steadily increased the maximum running distance over time (Fig. 2C).

We determined the effects of CLA on voluntary movement during the experimental period. No differences in voluntary movement by either CLA or exercise were observed, although time effect was significant ($p=0.0113$) (Fig. 3). No significant interactions were observed.

3.4. Serum parameters

The concentrations of serum metabolic parameters are shown in Table 3. The CLA supplemented animals had significantly lower levels of serum TG than mice in the control diet groups ($p=0.0431$). No difference of overall CLA and exercise effects were observed for glucose, total cholesterol, NEFA, BUN and L-Lactate in mice.

3.5. Effects on biomarkers in skeletal muscle energy metabolism

To determine the effects of CLA and/or exercise on skeletal muscle metabolism, we investigated

the AMPK-related molecular biomarkers in the regulatory pathway [6,9]. There were no significant overall effects of CLA or exercise on total AMPK α in all treatments (Fig. 4B), while phosphorylated AMPK α (the active form of AMPK α) was significantly up-regulated in the exercise trained mice ($p=0.0342$ for overall exercise effect, Fig. 4C). As a consequence, the ratio of phosphorylated AMPK α to AMPK α was significantly increased in the exercise trained animals ($p=0.0197$ for overall exercise effect, Fig. 4D). SIRT1, a NAD-dependent deacetylase cross-talking to AMPK, was significantly influenced by CLA supplementation ($p=0.0009$ for overall CLA effect, Fig. 4E). However, there was no significant effect of exercise in SIRT1 expression. Similarly, the expression of PGC-1 α showed significant increase by CLA treatment ($p=0.0127$ for overall CLA effect), but not by exercise (Fig. 4F).

3.6. Effects on biomarkers in mitochondrial biogenesis signaling cascades

On the basis of the evidence that CLA up-regulated SIRT1 and PGC-1 α in the CLA-fed mice in Fig. 4, we further studied the effects of CLA and/or exercise on mitochondrial biogenesis signaling cascades in the gastrocnemius muscle. It is known that endurance exercise training stimulates mitochondrial biogenesis, resulting in improved oxidative capacity and reduced muscle fatigue [14]. In fact, PGC-1 α is a master regulator in mitochondrial biogenesis, which orchestrates several nuclear encoded proteins. Among them, we primarily investigated three major molecular biomarkers, PPAR δ , NRF-1 and Tfam. In addition, the expressions of mitochondrial proteins, cytochrome c and SOD2, were evaluated for changes of mitochondrial function by nutritional and physical treatments. There were significant overall CLA effects on PPAR δ expression compared to controls ($p=0.0051$ for overall CLA effect, Fig. 5B), while no exercise effect was observed for PPAR δ . The CLA-fed mice significantly increased NRF-1

expression, a marker that regulates nuclear-encoding mitochondrial proteins, in comparison to controls ($p=0.0016$ for overall CLA effect). The expression levels of Tfam, a major regulator for mitochondrial DNA replication and transcription, in the CLA supplemented groups were greater than the controls ($p=0.0195$ for overall CLA effect). There were no exercise effects in either NRF-1 or Tfam (Fig. 5C and 5D).

We further determined expression levels of mitochondrial proteins; cytochrome c which is located in the inner membrane of mitochondria, and SOD2 which protects mitochondria from reactive oxygen species. No significant overall effects of CLA or exercise were observed on cytochrome c and SOD2 (Fig. 5E and 5F).

3.7. Effects on muscle fiber type composition

The effects of CLA and/or exercise on changes in the muscle fiber types were determined by the composition of the four different genes in myosin heavy chain (MHC) of the gastrocnemius muscle (Fig. 6). These myosin heavy chain genes include MHC7 (slow-twitch fiber type I), MHC3 (embryonic fiber), MHC2 (slow/fast-twitch fiber type IIa) and MHC1 (fast-twitch fiber type IIx/d). Significant differences in MHC7 ($p<0.0001$), MHC3 ($p=0.0019$) and MHC1 ($p=0.0041$) were observed in the CLA-fed animals. On the other hand, the exercise trained mice showed significantly increased gene expression levels of MHC2 ($p=0.0205$ for overall exercise effect).

3.8. Effects on lipid and glucose metabolism in skeletal muscle

To determine the effects of CLA and/or exercise on lipid metabolism in skeletal muscle the selected genes were measured from the gastrocnemius muscle (Fig. 7). CLA supplementation

significantly increased the mRNA expression of LPL (the master regulator of fatty acid uptake, $p=0.0019$), PPAR α (the key regulator of lipid metabolism, $p=0.00154$) and CPT 1 β (the key enzyme of fatty acid β -oxidation, $p=0.039$), while no effects of CLA were observed on Fabp4 and UCP2 in muscle. No overall exercise effect was observed in these genes.

The relative mRNA expression levels of PI3K, PDK4, AKT1 and GLUT4, markers for glucose and insulin metabolism, were also determined in the gastrocnemius muscle (Fig. 8). Among these targets, mRNA levels of GLUT4 (the insulin-regulated glucose transporter) were significantly up-regulated by CLA supplementation ($p=0.0447$), but not by exercise training.

4. Discussion

In this study, we investigated effects of CLA on metabolic alteration in skeletal muscle along with regular exercise. Overall, CLA supplementation led to enhanced endurance capacity and positively altered molecular markers in skeletal muscle metabolism, whereas exercise effects were limited in this study. These results are consistent with the previous studies that demonstrate increased maximum swimming or running capacity by CLA treatment in mice [6,15-18].

Earlier studies reported that 0.5% CLA supplemented mice increased voluntary movement (non-exercise physical activity) [7,8,19,20]. However, we did not observe an overall CLA effect on voluntary movement, consistent with a previous publication with exercise [6]. This inconsistency may originate in the different experimental designs (regular exercise vs. no exercise), as regular exercise may mask the effects of CLA on voluntary movement. However, further study is needed for better understanding the relationship between exercise and voluntary movement.

It is known that exercise activates various signaling cascades in skeletal muscle energy

metabolism. AMPK plays a central role in the adaptive response to exercise, since AMPK senses increase in the ratios of AMP/ATP and in turn leads to the activation of energy production via PGC-1 α [21]. In addition to AMPK, SIRT1 contributes to the deacetylation of PGC-1 α to activate it [22]. Our current results demonstrated that the exercise-trained animals had significantly upregulated phosphorylation of AMPK in comparison to the sedentary animals, while no CLA effect was observed. Meanwhile, there was significant effect of CLA on SIRT1 regardless of exercise. **Previously we reported that CLA treatment activated AMPK and PGC-1 α independent of SIRT1 in C2C12 myotubes and female mice [8,9], whereas CLA supplementation upregulated all AMPK, SIRT1 and PGC-1 α in wild type male animals [19]. This discrepancy might be due to the different experimental methods such as in *in vitro* vs. *in vivo*, males and females, or duration of CLA supplementation in animal models. Thus, we need to further investigate effects of CLA on AMPK and SIRT1 as upstream regulators of PGC-1 α .**

PGC-1 α is a master regulator in mitochondrial biogenesis, which is a complex process that increases mitochondrial volume and alters mitochondrial composition in response to physical and nutritional stimuli [23]. Activated PGC-1 α triggers the modulation of nuclear and mitochondrial regulators such as PPAR δ , NRF-1 and -2, and Tfam [24]. The significant role of PGC-1 α in muscle fiber type composition in skeletal muscle is supported by the observation in mice that ectopically overexpressed PGC-1 α showed a remarkable conversion of muscle fiber type II to type I [25]. In the current study, CLA supplementation facilitated PGC-1 α , PPAR δ and PGC-1 α -NRF-1-Tfam signaling cascades, implying that CLA may have potentiated mitochondrial biogenesis. This observation is consistent with the previous study where CLA isomers induced mitochondrial biogenesis via modulating the same signaling pathway [8,9]. Although we have not determined total mitochondrial DNA copy numbers and shown cross-

sectional analysis in the current study, our group previously reported that CLA indeed significantly increased total mitochondrial DNA copy numbers in murine C2C12 cells and supplementation with a cognate of CLA, conjugated nonadecadienoic acid (CNA), prevented muscle dystrophy in a rodent model (increased muscle size determined by muscle cross section area analysis) [5,9].

Along with PGC-1 α , one of the critical modulators in mitochondrial biogenesis and muscle fiber type composition is PPAR δ [26-28]. In the present study, feeding CLA led to an increase of PPAR δ expression and the induction of overall MHC genes. This is also consistent with previous reports on CLA and increased skeletal muscle PPAR δ expression [6,8,19]. These imply that CLA supplementation could affect the overall physiology of skeletal muscle based on the PPAR δ -interacted metabolic events. In this study, CLA treatment significantly induced overall MHC gene expressions. Furthermore, CLA supplementation significantly increased the relative gastrocnemius muscle weight (as % body weight) (Table 2). There is supporting evidence that muscle mass is attributed to the number of muscle fibers, especially muscle fiber type II [29]. We here showed CLA treatment significantly increased gene expression levels associated with fiber type II (MHC1) rather than regular exercise. Moreover, CLA-fed animals highly expressed muscle fiber type I gene (MHC 7) in the present study, which implies an increased capacity to burn fat for fuel, oxidative capacity and insulin sensitivity. However, the current observation of MHC changes is rather limited and indirect evidence to support CLA's effect on muscle fiber types. Thus, additional studies determining the effects of CLA on muscle fiber type transformation would be needed to support the current findings.

Our data demonstrated that CLA significantly increased three key genes associated with lipid metabolism in skeletal muscle, LPL, PPAR α and CPT1 β , which is consistent with previous

studies in mice [6,7,10,15,30,31]. Along with previous reports that CLA reduces respiratory quotient values in mice [7,31], this implies that CLA supplementation may have improved energy homeostasis to use fatty acids as an energy source over glucose. Although PPAR δ is also known to regulate genes in glucose and insulin metabolism, overall our results suggest no effects of CLA on muscle glucose metabolism, which is consistent with results on serum glucose levels found in these animals [8,31].

Previously it was reported that the *trans*-10,*cis*-12 CLA isomer is responsible for its anti-obesity effect and improved endurance capacity in mice [6,32]. However, both isomers increased total mitochondrial DNA copy number and PGC-1 α -NRF-1-Tfam signaling cascade, while the *trans*-10,*cis*-12 isomer potentiated AMPK activation and the *cis*-9,*trans*-11 isomer increased cytochrome c expression in C2C12 myotubes [9]. Thus, it is likely that both isomers contributed to overall effects in muscle metabolism observed in the current study.

The potential health concerns related to CLA supplementation have been reviewed recently, including glucose intolerance, fatty liver, oxidative stress and milk fat depression [11,33-35]. Overall, the effects of CLA on glucose homeostasis have been inconsistent in both animal and human studies [4,11,36,37]. In the current study, only some markers of glucose metabolism from the gastrocnemius muscle were measured, which was rather limited and no direct measurement to determine the effects of CLA on glucose homeostasis was made. However, previously we reported that the same dose of CLA up to 8 weeks had no effects on glucose intolerance, including the same strain of mice used in the current study [7,8,19]. It was suggested that increased fatty acid β -oxidation from the skeletal muscle, one of down-stream targets of activated AMPK and/or SIRT via PGC-1 α , may have contributed to altered glucose intolerance [5]. This is further supported by several long-term (longer than 6 months) human studies, which

reported no adverse effects of CLA on glucose and/or insulin levels [4,11,36,37]. In addition, CLA supplementation reduced or caused no effects on serum levels of NEFA and TG [6,7,19], however, elevated levels of these are often associated with diabetes and/or metabolic syndrome [38,39].

In contrast, there are consistent reports of CLA being linked to enlarged liver, including in the species used in the current study [3,5-8,11,19,40]. Liver tissue was not analyzed in the current study, however, it was reported that enlarged liver associated with CLA as mainly non-alcoholic fatty liver is suggested to be linked to CLA's significant influence on lipid metabolism, such as reduced fat accumulation in adipose tissue along with increased hepatic lipogenesis [11,36,37]. Rodents may be more sensitive to CLA affecting fatty liver than other species, including humans [3,11]. Although it was suggested that the effects of CLA on fatty liver is reversible in rodents [41], it would be important to determine if the fatty liver caused by CLA is the consequence or cause of altered lipid metabolism and/or glucose homeostasis.

Other potential health concerns associated with CLA consumption are oxidative stress and milk fat depression. CLA consistently increased oxidative markers such as isoprostanes, which may be related with its metabolism rather than representing oxidative stress, as no other oxidative stress markers were increased with CLA supplementation [3,11]. Lastly, it was also suggested that milk fat depression is species specific, of minimal concern for humans [3,11].

In the current study, we observed minimal effects of exercise itself. Endurance exercise is known to induce mitochondrial biogenesis and muscle fiber type transformation via the modulation of intracellular biomarkers, however, the magnitude of exercise intensity and duration influences these physiological and phenotypic alterations in skeletal muscle [42]. Consistently, Tadashi *et al.* [43] reported that PGC-1 α expression levels were significantly

correlated with the intensity of running endurance in mice. Since the current study used a relatively low-intensity exercise protocol, it is possible that the exercise protocol used had minimal physiological and biochemical effects. Further investigation would be needed to determine the effect of CLA and high-intensity exercise, including measurement of VO₂ max value.

In conclusion, we demonstrated that CLA supplementation promotes endurance capacity and increases relative skeletal muscle weight (as % of body weight), by stimulating mitochondrial biogenesis and inducing muscle fiber gene expression, resulting in increased lipid metabolism in mice. PPAR δ may play a significant role in CLA's effect on these metabolic events. Although the precise mechanism of how CLA regulates PPAR δ is not clear, it is suggested that CLA exerts its action via the activation of PGC-1 α through SIRT1. Our results suggest that CLA, independent of mild-intensity exercise, may provide an effective method to prevent obesity by the modulation of skeletal muscle metabolism.

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518

Table 1. Composition of experimental diet.

<i>Ingredient</i>	<i>g/kg</i>
Casein, 'vitamin-free' tested	169.1
L-Cystine	2.2
Sucrose	100
Cornstarch	288.5
Maltodextrin	132
Cellulose	50
Soybean oil	195
CLA or Soybean oil	5
Mineral mix, AIN-93M-MX (TD 94049) ^a	42.8
Vitamin mix, AIN-93-VX (TD 94047) ^b	12.4
Choline bitartrate	3
<i>tert</i> -Butylhydroquinone	0.04
Total	1,000

^aMineral mix, g/kg; Calcium carbonate, 357.0; Potassium phosphate, monobasic, 196.0; Potassium citrate, monohydrate, 70.78; Sodium chloride, 74.0; Potassium sulfate, 46.6; Magnesium oxide, 24.3; Ferric citrate, 6.06; Zinc carbonate, 1.65; Manganous carbonate, 0.63; Cupric carbonate, 0.31; Potassium iodate, 0.01; Sodium selenite, 0.01025; Ammonium paramolybdate, 0.00795; Sodium meta-silicate, 1.45; Chromium potassium sulfate, 0.275; Lithium chloride, 0.0174; Boric acid, 0.0815; Sodium fluoride, 0.0635; Nickel carbontate, hydroxide, tetrahydrate, 0.0318; Ammonium vanadate, 0.0066; and Sucrose, 220.716.

^bVitamin mix, g/kg; Nicotinic acid, 3.0; Calcium pantothenate, 1.6; Pyridoxine HCl, 0.7; Thiamin HCl, 0.6; Riboflavin, 0.6; Folic acid, 0.2; D-Biotin, 0.02; Vitamin B₁₂ (0.1% in mannitol), 2.5; DL-Alpha tocopheryl acetate (500 IU/g), 15.0; Vitamin A palmitate (500,000 IU/g), 0.8; Vitamin D₃ (cholecalciferol, 500,000 IU/g), 0.2; Vitamin K (phyloquinone), 0.075; and Sucrose, 974.705.

531 **Table 2. Organ weights**

	Treatment				Effects (<i>P</i> -value)		
	Sedentary		Exercise		Exercise (E)	CLA (D)	E x D
	Control	CLA	Control	CLA			
<i>% Body Weight</i>							
Adipose tissue							
Epididymal fat	4.75 ± 0.92	2.70 ± 0.34	5.39 ± 0.85	3.16 ± 0.60	n.s.	0.0089	n.s.
Retroperitoneal fat	1.14 ± 0.35	0.35 ± 0.04	1.12 ± 0.14	0.44 ± 0.09	n.s.	0.0004	n.s.
Mesenteric fat	3.35 ± 0.61	2.54 ± 0.20	3.86 ± 0.25	2.53 ± 0.12	n.s.	0.0073	n.s.
Total	9.25 ± 1.78	5.59 ± 0.60	10.4 ± 1.03	6.13 ± 0.77	n.s.	0.0032	n.s.
Skeletal muscle tissue							
Gastrocnemius	0.96 ± 0.06	1.13 ± 0.04	0.99 ± 0.08	1.23 ± 0.06	n.s.	0.0035	n.s.
Quardricep	6.53 ± 0.16	6.68 ± 0.43	6.27 ± 0.31	6.72 ± 0.37	n.s.	n.s.	n.s.
Organ							
Liver	3.45 ± 0.10	5.41 ± 0.14	3.57 ± 0.11	5.25 ± 0.16	n.s.	< 0.0001	n.s.
Heart	0.51 ± 0.12	0.59 ± 0.02	0.49 ± 0.07	0.57 ± 0.04	n.s.	n.s.	n.s.
Spleen	0.27 ± 0.05	0.38 ± 0.05	0.28 ± 0.06	0.29 ± 0.05	n.s.	n.s.	n.s.

532 Values represent means ± S.E. (n = 5). *P*-values in table are from two-way ANOVA. CLA, conjugated linoleic acid; n.s., not significant.

533 **Table 3. Serum parameters**

	Treatment				Effects (<i>P</i> -value)		
	Sedentary		Exercise		Exercise (E)	CLA (D)	E x D
	Control	CLA	Control	CLA			
<i>mmol/L</i>							
Glucose	8.31 ± 0.97	9.07 ± 0.50	8.08 ± 1.10	9.72 ± 1.14	n.s.	n.s.	n.s.
Triglyceride	0.42 ± 0.04	0.38 ± 0.05	0.47 ± 0.05	0.33 ± 0.02	n.s.	0.0431	n.s.
Total cholesterol	11.3 ± 1.02	9.75 ± 0.20	11.9 ± 1.22	10.5 ± 0.50	n.s.	n.s.	n.s.
NEFA	2.20 ± 0.18	2.15 ± 0.08	2.20 ± 0.23	2.08 ± 0.16	n.s.	n.s.	n.s.
BUN	8.74 ± 0.83	8.07 ± 0.19	8.93 ± 0.19	8.52 ± 0.22	n.s.	n.s.	n.s.
L-Lactate	5.44 ± 0.61	5.53 ± 0.50	5.38 ± 0.60	5.32 ± 0.52	n.s.	n.s.	n.s.

534 Values represent means ± S.E. (n = 5). *P*-values in table are from two-way ANOVA. CLA, conjugated linoleic acid; n.s., not significant; NEFA,
535 non-esterified fatty acid; BUN, blood urea nitrogen

Fig. 1. Effects of conjugated linoleic acid (CLA) on body weight **gain** (A) and food intake (B) in sedentary and exercise-trained mice. 10-week-old mice were treated with either control or 0.5% CLA diets for 4 weeks. Values represent means $\pm$ S.E. (n = 5). Means with different letters at the same time points are significantly different ($P < 0.05$). P -values in table are from two-way repeated measures ANOVA. n.s.: not significant.

Fig. 2. Effects of conjugated linoleic acid (CLA) on maximum running time (A) and distance (B) in sedentary and exercise-trained mice. Mice were supplemented with either control or 0.5% CLA diet for 4 weeks. Endurance capacity was tested every 2 weeks. (C) Trend for maximum running distance changes (%) in each treatment group. **Maximum running distance change (%) was calculated by (running distance at week 2 or 4 – running distance at week 0)/(running distance at week 0) x 100.** Values represent means $\pm$ S.E. (n = 5). P -values in table are from two-way repeated measures ANOVA. n.s.: not significant.

Fig. 3. Effects of conjugated linoleic acid (CLA) on voluntary movement (non-exercise physical activity) in sedentary and exercise-trained mice. Mice were fed either control or 0.5% CLA diets for 4 weeks. Voluntary movement was monitored **between 7 pm and 3 am** during the dark cycle biweekly. Values represent means $\pm$ S.E. (n = 5). P -values in tables are from two-way repeated measures ANOVA. n.s.: not significant.

Fig. 4. Effects of conjugated linoleic acid (CLA) on protein expression of AMP-activated protein kinase (AMPK), sirtuin 1 (SIRT1), and peroxisome proliferator-activated receptor γ coactivator 1

α (PGC-1 α). Samples were prepared from the gastrocnemius muscle. Pictures show representative results (A). The graph represents the fold of the sedentary control group of total AMPK α (B), phosphorylated AMPK α at Threonine 172 (C), a ratio of phosphorylated AMPK α to total AMPK expression (D), SIRT1 (E) and PGC-1 α (F). Each protein expression level was normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Values represent means $\pm$ S.E. (n = 5). *P*-values in tables are from two-way ANOVA. n.s.: not significant.

Fig. 5. Effects of conjugated linoleic acid (CLA) on mitochondrial biogenesis-related molecular markers from the gastrocnemius muscle. Pictures show representative results (A). The relative expression levels of peroxisome proliferator-activated receptor δ (PPAR δ , B), nuclear respiratory factor 1 (NRF-1, C), mitochondrial transcription factor A (Tfam, D), cytochrome c (E), and superoxide dismutase 2 (SOD2, F) were determined by immunoblotting. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control. Values represent means $\pm$ S.E. (n = 5). *P*-values in tables are from two-way ANOVA. n.s.: not significant.

Fig. 6. Effects of conjugated linoleic acid (CLA) on skeletal muscle fiber type composition. Samples were obtained from the gastrocnemius muscle. The relative mRNA expression levels of myosin heavy chain 1 (MHC1), MHC2, MHC3, and MHC7 were quantified using real-time PCR. The values in the graph are presented in terms of $2^{-\Delta\Delta Ct}$. 18S ribosomal RNA was used for an internal standard. Data are means $\pm$ S.E. (n = 5). *P*-values in tables are from two-way ANOVA. n.s.: not significant.

Fig. 7. Effects of conjugated linoleic acid (CLA) on mRNA expressions of selected genes

representative of lipid metabolism in the gastrocnemius muscle. LPL, lipoprotein lipase; Fabp4, fatty acid binding protein 4, PPAR α , peroxisome proliferator-activated receptor α ; CPT1 β , carnitine palmitoyltransferase 1 β ; UCP2, uncoupling protein 2. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal standard. Values represent means $\pm$ S.E. (n = 5). *P*-values in tables are from two-way ANOVA. n.s.: not significant.

Fig. 8. Effects of conjugated linoleic acid (CLA) on mRNA expressions of selected genes representative of glucose and insulin metabolism in the gastrocnemius muscle. PI3K, phosphoinositide 3-kinase; PDK4, pyruvate dehydrogenase kinase, isoform 4; AKT1, protein kinase B1; GLUT4, glucose transporter type 4. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal standard. Values represent means $\pm$ S.E. (n = 5). *P*-values in tables are from two-way ANOVA. n.s.: not significant.

Figure 1

597 **Figure 2**

598 **A. Maximum running time (min)**

B. Maximum running time (min)

C. Maximum running distance change (%)

599 **Figure 3**

600

601 **Figure 4**

Figure 6

607 **Figure 7**

608

Figure 8