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L-Carnitine enhances exercise endurance capacity by promoting muscle oxidative metabolism in mice

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ABSTRACT

L-Carnitine (LC), the bioactive form of carnitine, has been shown to play a key role in muscle fuel metabolism during exercise, resulting in increased fatty acid oxidation and energy expenditure. However, whether LC contributes to improved endurance exercise performance remains controversial. This study was designed to investigate the effects of LC administration on endurance capacity and energy metabolism in mice during treadmill exercise. Male C57BL/6 mice were divided into two groups (sedentary and exercise) and received daily oral administration of LC (150 mg/kg) or vehicle with a high-fat diet for 3 weeks. During the experimental period, all animals were trained three times a week on a motorized treadmill, and the total running time until exhaustion was used as the index of endurance capacity. LC administration induced a significant increase in maximum running time with a reduction of body fat compared with the control group when mice were subjected to programmed exercise. The serum levels of triglyceride, non-esterified fatty acid, and urea nitrogen were significantly lower in the LC group than the corresponding levels in the control group, while serum ketone body levels were higher in the LC group. Muscle glycogen content of LC administered-mice was higher than that of control mice, concomitant with reduced triglyceride content. Importantly, muscle mRNA and protein expressions revealed enhanced fatty acid uptake and oxidative metabolism and increased mitochondrial biogenesis by LC administration. These results suggest that LC administration promotes fat oxidation and mitochondrial biogenesis while sparing stored glycogen in skeletal muscle during prolonged exercise, resulting in enhanced endurance capacity.

1. Introduction

It is well established that enhancing fatty acid oxidation is an important metabolic factor to improve endurance capacity in humans and rodents [1,2]. Fatty acid oxidation in muscle mitochondria followed by aerobic reactions of the TCA cycle is adequate to generate a large proportion of the ATP required for muscular contraction during prolonged exercise [3]; thus, enhanced fatty acid oxidation during endurance exercise reduces carbohydrate consumption as an energy source and suppresses lactate production, resulting in improved endurance capacity. In this respect, much research has been performed to improve endurance capacity by nutritional regimens that promote fatty acid oxidation [4–6].

L-Carnitine (LC) is essential for the transport of long-chain fatty acids from the cytosol into the mitochondrial matrix, where long-chain fatty acids are oxidized, resulting in the production of ATP. Recently, it has been suggested that LC plays a key role in muscle fuel metabolism during exercise, resulting in increased fatty acid oxidation and energy expenditure [7]. In addition, a mouse model with systemic carnitine deficiency exhibits a mitochondrial abnormality in muscle and the heart [8], and LC directly improves the fatigue characteristics of muscles enriched in type I fibers in vitro [9]. These results suggest that LC could contribute to improve endurance exercise performance, but it remains controversial. Indeed, there is some evidence showing that LC supplementation improves endurance capacity with increased oxygen uptake or fatty acid oxidation [10–12], however others did not observe the positive influences of LC supplementation on endurance capacity and energy metabolism during exercise [13–15]. Most recently,

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Pandareesh and Anand [11] reported that the supplementation of LC with fat increased the exhaustive swimming time and reduced glycogen consumption in the liver and muscle in rats, but they did not observe changes in skeletal muscle oxidative metabolism. Thus, we here investigated the effects of LC on endurance capacity and related physiological metabolism using the treadmill system, which has the distinct advantage over other forms of exercise, including swimming, in that the total amount of external work done by the experimental animal can be calculated easily [16]. This study may provide a better understanding of LC in controlling energy metabolism and endurance capacity.

2. Materials and methods

2.1. Animals and diet

The care and treatment of experimental animals conformed to a protocol approved by the Institutional Animal Care and Use Committee of Korea University (Seoul, Korea). Male C57BL/6 mice (10-week-old) (Orient Bio Inc., Seongnam-Si, Korea) were housed in standard cages under controlled conditions of temperature (22 ± 0.5 °C), humidity (50%), and lighting (light from 0900 to 2100 h). During a 1-week adaptation period, all mice were fed a control diet and were subjected to running exercise three times (velocity of 10 m/min on a 0° inclination for 15 min with shock grid OFF, followed by 10 min with shock grid ON) to acclimate to the treadmill. At the end of the adaptation period, all animals were subjected to an endurance test for the measurement of their baseline values on the running time to exhaustion. To minimize individual variations on endurance capacity baseline, we selected 30 mice with the closest value to the mean baseline from the original 40 mice [5]. Finally, selected mice were divided into two groups (sedentary and exercise) and fed a high-fat diet (HFD, 45% fat from calories). LC (99.8%, provided by Immunotech, Cheonan, Korea) was dissolved in distilled water and administered orally to mice (150 mg/kg body weight) once daily for 3 weeks with vehicle. At the end of study, the mice were fasted for 4 h, ran for 25 min according to the endurance protocol, and were then immediately euthanized by an overdose of avertin (2,2,2-tribromoethanol). Blood was collected by cardiac puncture, and skeletal muscle was collected for further analysis.

2.2. Exercise training and endurance protocol

During the experimental period, all animals were trained three times a week on a motorized treadmill (Mirae-STCorp, Daejeon, Korea). Training was performed for a total 15 min (10 min at 10 m/min, then an increase of 1 m/min every minute for 5 min) on a 10° inclination with shock grid ON. Mice were encouraged to run with the use of an electric grid placed at the end of the treadmill (0.97 mA, 1 Hz).

Endurance capacity was determined 1 and 3 weeks after baseline measurement by placing animals on an individual treadmill at room temperature. The exercise regimen was started with shock grid ON and 10° inclination at 10 m/min for 10 min before the speed was increased by 1 m/min up to 25 m/min (15 min with increase speed) and then held at 25 m/min until exhaustion. Based on previous studies that measured treadmill endurance capacity [17–19], the mice were defined as exhausted if they sustained the shock grid three times for more than 2 s or remained on the shock grid for five consecutive seconds. At the moment of exhaustion, the mouse was removed from the treadmill. The total running time until exhaustion was recorded and used as the index of endurance capacity.

2.3. Biochemical parameters

Following euthanasia, serum was separated by centrifugation at 3000 g for 20 min at 4 °C. Serum samples were used for determination of triglyceride (TG), glucose, non-esterified fatty acids (NEFA), ketone body, and urea nitrogen using commercial kits as specified by the manufacturer. For the TG measurement in muscle, tissue saponification in ethanolic KOH and neutralization with $MgCl_2$ were performed as previously described [20]. Glycerol content was determined by enzymatic colorimetric methods using a commercially available kit. The glycogen content in muscle was measured as glucose residues after enzymatic hydrolysis of tissue samples using a commercial kit.

2.4. Immunoblot analysis

Antibodies were obtained from the following sources: AMPKα, phospho-AMPKα Thr172, Nrf1 (Cell Signaling Technology, Danvers, MA, USA), CPT1ββand β-actin (Santa Cruz Biotechnology, Dallas, TX, USA), UCP2, PGC1α, TFAM and OPA1 (Abcam, Cambridge, UK). Conventional immunoblotting procedures were used to detect the target proteins: Gastrocnemius muscle tissues were collected to extract protein using RIPA buffer (Cell Signaling Technology). Tissue lysates were then cleared by centrifugation at 15,000 × g for 20 min. Total protein concentration was determined by Bradford assay. Equal amounts of protein were separated on 12% SDS/PAGE and the proteins were transferred to polyvinylidene difluoride membranes. The membranes were then blocked for 30 min in a PBS solution containing 3% BSA and 0.1% Tween-20 and then probed with primary antibody overnight in 0.5% BSA, 0.1% Tween-20 in PBS. After washing, membranes were incubated for 1 h with horseradish peroxidase-linked secondary antibodies (Sigma–Aldrich, St. Louis, MO, USA) in PBS solution containing 0.5% BSA and 0.1% Tween-20 in PBS. Finally, after three 10 min washes in 0.1% PBS/Tween-20, proteins were visualized by ImageQuant LAS 4000 (General Electric, Pittsburgh, PA, USA). Band intensities were quantified with ImageJ software (National Institute of Health, NIH Version v1.32j).

2.5. Immunofluorescence staining

Gastrocnemius muscles were extracted and saturated in 30% sucrose-PBS overnight. Frozen sections (5 μm) were cut in a cryostat on microscope slides, washed in PBS and permeabilized in PBS with 0.1% Triton X-100 for 10 min. Blocking was performed using a PBS with 5% normal goat serum for 30 min at room temperature, followed by incubation with OPA1 antibody (Abcam) diluted 1:100 at 4 °C overnight. Three consecutive washes with PBS for 5 min each were followed by sequential incubation with Alexa Fluor 488 goat anti-rabbit IgG secondary antibody (1:200) at room temperature for 1 h. The slides were washed three times with PBS and mounted using anti-fade mounting medium. Images were captured under the confocal microscope (Carl Zeiss AG; Oberkochen, Germany).

2.6. Real-time Q-PCR

The gastrocnemius muscle was homogenized in 1 mL of TRIzol reagent, and then total RNA was isolated according to the TRIzol protocol. Total RNA was reverse transcribed to cDNA using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster, CA) as described in the manufacturer protocol. cDNA was used as a template for the relative quantitation for the selected target genes with predesigned TaqMan gene expression assay kits. Each 20 μL reaction contained 100 ng cDNA, 2 × TaqMan Gene

Expression Mastermix, forward and reverse primers and TaqMan probe. All reactions were carried out in triplicate with an ABI 7500 system (Applied Biosystems, Foster, CA) using the following conditions: 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Results were normalized to GAPDH as an internal standard, and relative quantities of each gene were presented in terms of $2^{-\Delta\Delta Ct}$, calculated using the ΔCt and ΔΔCt values.

2.7. Statistical analyses

Data were analyzed by one-way ANOVA using the SAS software for Windows release 9.2 (SAS Institute Inc., Cary, NC) on the W32_VSHOME platform. One-way ANOVA with repeated measures was performed to assess mean differences between groups for body weight and endurance capacity over time. The least squares means option using a Tukey–Kramer adjustment was used for multiple comparisons among the experimental groups. Data are shown as the mean ± SE. *P* values of<0.05 are reported as statistically significant

3. Results

3.1. L-Carnitine enhances endurance capacity with a reduction of body fat in exercise-trained mice

Mice in the LC group showed the lowest body weights with a significant reduction of visceral fat depots compared with sedentary and exercised control mice, while no significant differences were observed in body weights and visceral fat between sedentary and exercised control mice (Fig. 1A and B). There were no significant differences in food intake during the experimental period (Fig. 1C).

Endurance capacities of exercise-trained mice were determined by the maximum running time recorded on the treadmill. The maximum running time was increased significantly in the LC group compared with control mice at Week 1 and Week 3 (Fig. 1D). LC administration induced an average 25% increase in maximum running time of mice at Week 1 compared with their baseline.

3.2. L-Carnitine promotes fatty acid oxidative metabolism in skeletal muscle during exercise

To elucidate whether LC influences lipid and energy expenditure metabolism during exercise, we analyzed the muscle gene and protein expression levels of key enzymes involved in fatty acid uptake and β-oxidation as well as serum and muscle concentrations of major metabolic substrates (Fig. 2 and Table 1). In the exercise group, serum and muscle samples were collected immediately after 25 min of endurance exercise. LC administration increased muscle mRNA expressions of fatty acid translocase (*FAT/CD36*) and fatty acid binding protein 3 (*FABP3*), which are key enzymes in fatty acid uptake (Fig. 2A). In addition, muscle mRNA and/or protein expressions of carnitine palmitoyl transferase 1β (*CPT1β*), uncoupling protein 2 (*UCP2*), hormone-sensitive lipase (*HSL*), and peroxisome proliferator-activated receptor δ (*PPARδ*), which are key regulators in mitochondrial β-oxidation and energy expenditure, were up-regulated in the LC group compared with those in the exercised control group (Fig. 2A and B). However, LC did not alter AMP-activated protein kinase (AMPK) phosphorylation induced by exercise training (Fig. 2B).

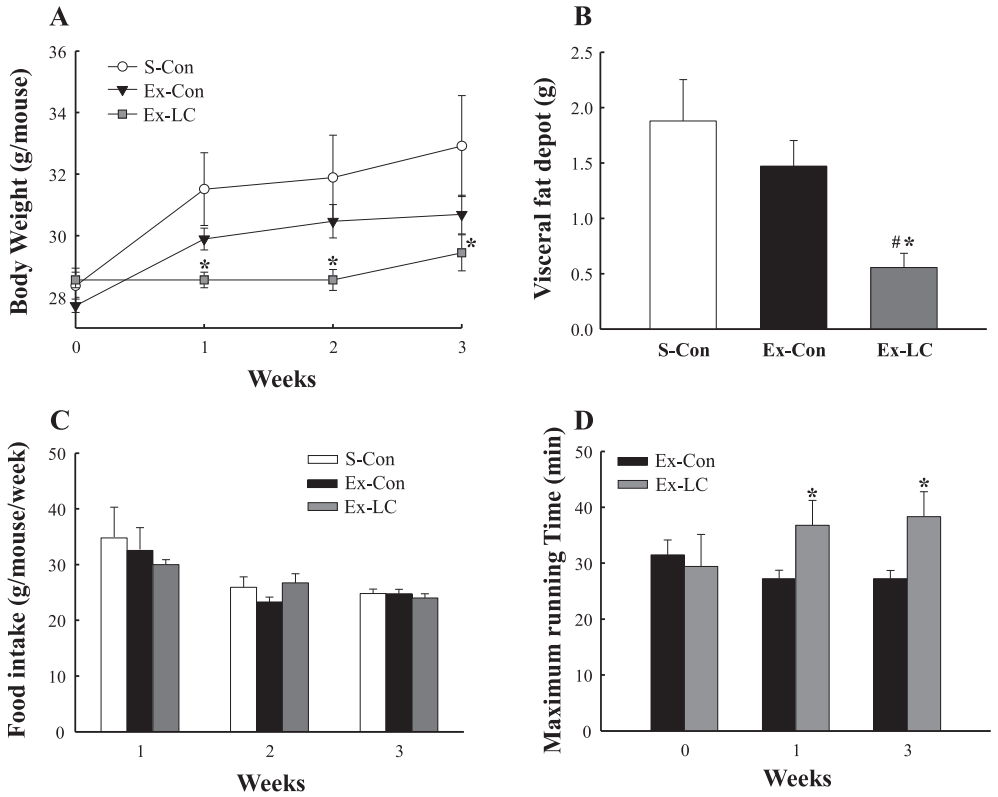

Fig. 1. L-Carnitine enhances endurance capacity with a reduction of body fat in exercise-trained mice. Mice were fed a high-fat diet and received daily oral administrations of vehicle or LC for 3 weeks. (A) Body weight, (B) visceral fat depot including epididymal, mesenteric, and retroperitoneal fat, (C) food intake and (D) maximum running time. Endurance capacity was determined by measuring maximum running time until exhaustion. S-Con, sedentary control; Ex-Con, exercised control; Ex-LC, exercised L-Carnitine. Values represent means ± SE (n = 10). #Significantly different from the S-Con group (P < 0.05). *Significantly different from the Ex-Con group (P < 0.05).

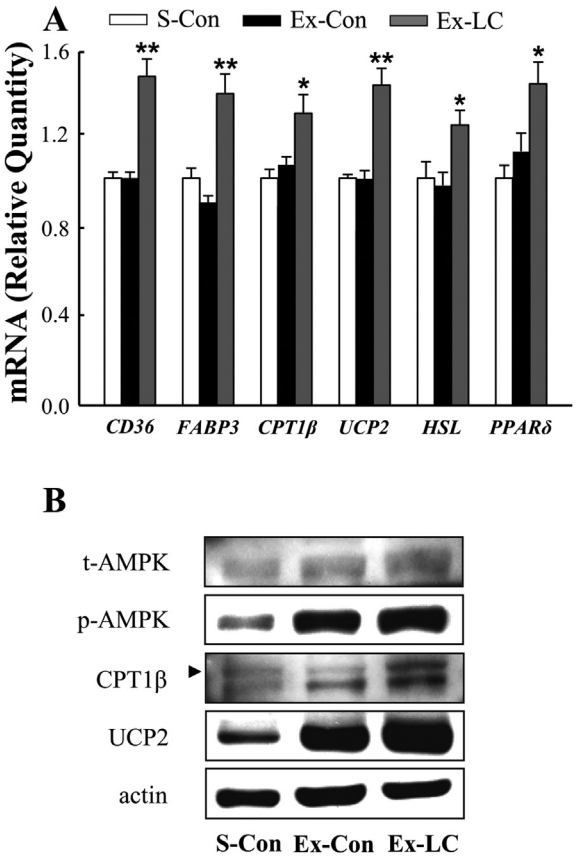

Fig. 2. L-Carnitine promotes fatty acid oxidative metabolism in skeletal muscle. (A) mRNA expressions of cluster of differentiation 36 (*CD36*), fatty acid binding protein 3 (*FABP3*), carnitine palmitoyl transferase 1β (*CPT1β*), uncoupling protein 2 (*UCP2*), hormone-sensitive lipase (*HSL*), peroxisome proliferator-activated receptor δ (*PPARδ*) in skeletal muscle (n = 10). (B) Muscle protein expressions of total AMPK (t-AMPK), phosphorylated AMPK (p-AMPK), CPT1β and UCP2 were measured by western blotting (n = 4). S-Con, sedentary control; Ex-Con, exercised control; Ex-LC, exercised L-Carnitine. Values represent means ± SE. *Significantly different from the Ex-Con group (*P < 0.05, **P < 0.01).

The serum levels of TG, NEFA, and urea nitrogen in the LC group were significantly lower than those in the exercised control group, while serum ketone body levels were higher in the LC group (Table 1). Consistent with the changes in serum parameters, LC-administered mice showed reduced muscle TG content, concomitant with an increase in glycogen content. Taken together, these results indicate that LC administration promotes fatty acid oxidative metabolism, resulting in a reduction of muscle glycogen consumption during exercise.

Table 1
Biochemical parameters.

	Treatment	
	Ex-Con	Ex-LC
Serum		
Triglyceride, mg/dL	158.3 ± 5.8	128.7 ± 7.8*
Glucose, mg/dL	99.3 ± 16.3	92.5 ± 14.7
NEFA, mEq/L	1.14 ± 0.11	0.75 ± 0.07*
Ketone body, mM	1.39 ± 0.30	2.25 ± 0.32*
Urea nitrogen, mg/dL	13.4 ± 0.4	6.3 ± 0.4*
Muscle		
Triglyceride, mg/g tissue	17.65 ± 2.91	9.95 ± 1.29*
Glycogen, mg/g tissue	0.13 ± 0.02	0.20 ± 0.02*

At the end of study, the mice ran for 25 min according to the endurance protocol and were immediately euthanized by an overdose of avertin. Ex-Con, exercised control; Ex-LC, exercised L-Carnitine. Values represent means ± SE (n = 10). *Significantly different from the Ex-Con group ($P < 0.05$).

3.3. L-Carnitine increases markers of mitochondrial biogenesis in skeletal muscle

Since the repeated bouts of endurance exercise stimulate mitochondrial biogenesis to fulfill the new energetic status in skeletal muscle [21], we next measured the protein expressions of key regulators involved in mitochondrial biogenesis in gastrocnemius muscle (Fig. 3). As expected, exercise training increased the protein expressions of peroxisome proliferator-activated receptor γ coactivator 1α (PGC1α), a master regulator of mitochondrial biogenesis, and its downstream transcription factors including nuclear respiratory factor 1 (NRF1) and mitochondrial transcription factor A (TFAM) compared to the sedentary control (Fig. 3A). In addition, exercise stimulated muscle expression of optic atrophy 1 (OPA1) that plays a key role in the mitochondrial fusion–fission mechanism, as reflected by the western blotting and immunofluorescence (Fig. 3A and B). Importantly, the stimulations of these protein expressions in exercised mice were more pronounced with LC administration. Thus, LC administration may enhance exercise-mediated mitochondrial biogenesis in skeletal muscle.

4. Discussion

During exercise there is a dramatic increase in energy requirements because of the metabolic needs of working muscles; the oxidation rates of fat and glucose are increased 5–10-fold during prolonged mild or moderate-intensity exercise [2]. There has been much evidence that increasing fat oxidation could be an

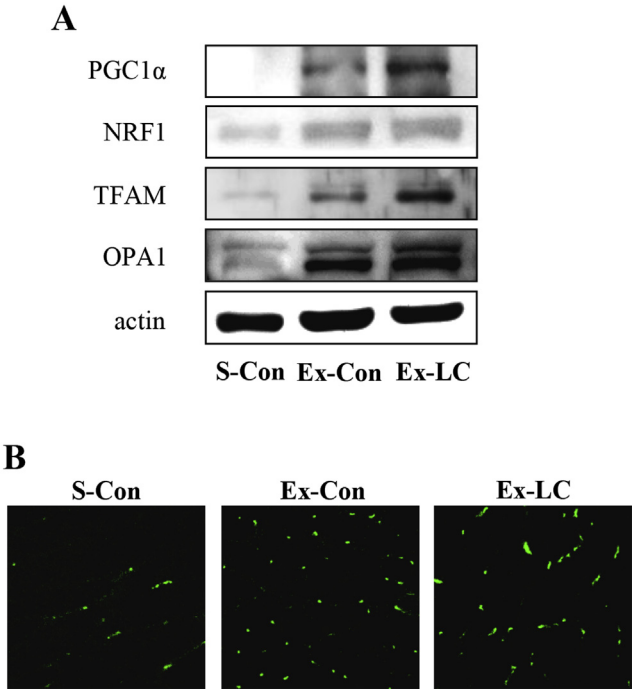

Fig. 3. L-Carnitine increases markers of mitochondrial biogenesis in skeletal muscle. (A) Muscle protein expressions of PPARγ coactivator 1α (PGC1α), nuclear respiratory factor 1 (NRF1), mitochondrial transcription factor A (TFAM) and optic atrophy 1 (OPA1) were measured by western blotting (n = 4). (B) Representative skeletal muscle sections showing immunofluorescent staining for OPA1. S-Con, sedentary control; Ex-Con, exercised control; Ex-LC, exercised L-Carnitine.

important strategy to enhance endurance capacity while sparing muscle glycogen as a substrate for energy production [22,23]. These reports support our current data showing that LC administration enhances the running endurance capacity of mice by increasing fat oxidation and reducing the consumption of stored glycogen in skeletal muscle during prolonged exercise.

The mitochondrial carnitine system plays an essential role in β-oxidation of long-chain fatty acids by catalyzing their transport into the mitochondrial matrix [24]. In the present study, LC administration up-regulated muscle mRNA expressions of *CD36* and *FABP3*, which serve as transmembrane fatty acid transporter and a cytosolic fatty acid carrier to the mitochondria, respectively. In addition, the mRNA and protein expressions of CPT1β and UCP2 in muscle were significantly up-regulated by LC administration. CPT1β is a rate-limiting enzyme of mitochondrial fatty acid β-oxidation, and UCP2 has been shown to be associated with energy expenditure by dissipating the chemiosmotic gradient in mitochondria [25]. It is important to note that LC increased mRNA expression of *PPARδ* which is one of the primary downstream targets of AMPK, although we did not observe the change in AMPK phosphorylation by LC in the exercised group. PPARδ acts as a key regulator of lipid and carbohydrate metabolism in the muscle during exercise [26]. Indeed, it was demonstrated that a PPARδ agonist and exercise training show synergistic activity of increasing oxidative myofibers and running endurance in mice [27]. Therefore, these results suggest that the LC regulates energy metabolism beneficially for endurance by enhancing fatty acid uptake and β-oxidation in muscle during exercise.

Consistent with the increased gene and/or protein expressions of muscle oxidative enzymes, LC-administered mice showed reduced muscle TG content and increased serum levels of ketone bodies, indicating enhanced fat utilization for energy production. Moreover, our data showed that LC administration reduced the serum level of urea nitrogen, which is an important index for evaluating the body endurance ability with stored glycogen content [28]. Proteins can be catabolized to a greater degree when the body cannot obtain enough energy from carbohydrate and fat sources; accordingly, urea nitrogen concentrations increase substantially following sustained exercise [28]. Thus, in the present study, lower serum levels of urea nitrogen and higher muscle glycogen content with LC administration reflect the preservation of both protein and carbohydrate consumption for energy, which are indicative of enhanced endurance.

Mitochondrial biogenesis is a critical metabolic adaptation to aerobic exercise, and an increased mitochondrial biogenesis may delay skeletal muscle fatigue and enhance endurance exercise performance [29]. One of interesting results here is that LC increased muscle protein expressions of key regulators involved in mitochondrial biogenesis, such as PGC1α, NRF1, TFAM, and OPA1. AS a master regulator of mitochondrial biogenesis, PGC1α strongly induces the expressions of downstream transcription factors including NRF1 and TFAM, which are responsible for the induction of mitochondrial genes. TFAM activates transcription and replication of the mitochondrial genome during biogenesis, and OPA1 plays an important role in the fusion–fission cycles for the synthesis of new mitochondria [30,31]. Recently, Civiletto et al. [32] demonstrated that OPA1 overexpression induced a dramatic increase in endurance capacity with an improved phenotype in mitochondrial disease mouse models. Therefore, it would be reasonable that LC could enhance endurance capacity in part by stimulating muscle mitochondrial biogenesis.

Since LC is a carrier molecule that transports long-chain fatty acids through the inner mitochondrial membrane, additional fat supplementation with LC may boost ATP production with increased fat oxidation. Indeed, Pandareesh and Anand [11] reported that LC supplementation with a 10 or 15% fat diet enhanced endurance with increased tissue levels of ATP and glycogen in rats, but not with a 5% fat diet. Similarly, our study also showed that LC with HFD (45% fat from calories) feeding improved energy metabolism and endurance, although we did not observe those changes with a low-fat diet. On the other hand, in a clinical study with trained athletes, chronic ingestion of LC and carbohydrate induced muscle glycogen sparing and increased muscle carnitine content, resulting in improved exercise performance compared with the carbohydrate-only control [33]. These previous studies, along with our current observations, suggest that dietary LC with fat or carbohydrate supplementation improves energy metabolism for endurance, and further studies are needed to determine the optimal dietary composition with LC for improvement of endurance capacity.

In conclusion, the present study demonstrated that LC administration increased mRNA and/or protein expressions of key regulators involved in fatty acid oxidation and mitochondrial biogenesis in skeletal muscle of exercise-trained mice, resulting in enhanced exercise endurance. We also found the reduced serum levels of urea nitrogen and increased muscle glycogen content with LC administration in exercised mice, indicating the preservation of both protein and carbohydrate consumption during exercise. These findings suggest that LC administration could enhance the running endurance capacity of mice by promoting fat utilization and mitochondrial biogenesis. This study provide a better understanding for the application of LC as a promising ergogenic aid.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Transparency document

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