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Effect of carnitine, acetylcarnitine or propionylcarnitine supplementation on skeletal muscle composition and physical performance in mice

Running title: Oral carnitine and acylcarnitine supplementation and energy metabolism

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Abstract

Carnitine has key functions in energy metabolism. In most studies in animals or humans, carnitine supplementation did not influence muscle composition and function, but the effects of the carnitine esters acetylcarnitine or propionylcarnitine are not well characterized. We therefore investigated the influence of carnitine, acetylcarnitine and propionylcarnitine on body carnitine homeostasis, energy metabolism and physical performance in mice. Animals were orally supplemented with approximately 2 mmol/kg/d carnitine, acetylcarnitine or propionylcarnitine for four weeks, before being subjected to exhaustive exercise and analysis of skeletal muscle energy metabolism and morphology.

In supplemented groups, the total plasma and urine carnitine concentrations were significantly higher than in the control group, whereas the skeletal muscle carnitine content remained unchanged. The bioavailability of supplemented carnitine or acylcarnitines was in the range of 20 to 23% as determined by urinary excretion of total carnitine. The supplemented acylcarnitines were hydrolyzed in the intestine and in the liver before reaching the systemic circulation. Skeletal muscle morphology including fiber type composition was not affected by treatment with carnitine or acylcarnitines. Oxygen consumption by muscle fibers obtained from soleus or gastrocnemius was not different between the groups. The supplementation of carnitine or acylcarnitines had no significant impact on the running capacity and on key substrates of skeletal muscle energy metabolism such as glycogen, ATP, phosphocreatine or creatine.

In conclusion, oral supplementation of carnitine, acetylcarnitine or propionylcarnitine is associated with increased plasma concentrations of total carnitine and increased urinary excretion of carnitine, but does not affect the skeletal muscle carnitine content. Accordingly, physical performance, skeletal muscle energy metabolism, and muscle fiber type composition are not affected by high oral doses of carnitine or acylcarnitines in mice.

Keywords: carnitine, acetylcarnitine, propionylcarnitine, skeletal muscle composition & metabolism, exercise

Introduction

Carnitine is an amino acid derivative playing an essential role in cellular energy metabolism due to the acylation of its β-hydroxy group. The major carnitine pool, accounting for >95% of the total body stores, is in skeletal muscle (Krahenbuhl *et al.*, 2000; Spaniol *et al.*, 2001), where carnitine functions as a carrier of long-chain fatty acids (Fritz, 1955) and as a buffer of free coenzyme A (CoASH) (Brass *et al.*, 1980; Friolet *et al.*, 1994).

Carnitine is an obligatory intermediate for the transport of long-chain fatty acids into the mitochondria. In order to reach the mitochondrial matrix for subsequent β-oxidation, long-chain fatty acids have to be converted into the corresponding acyl-CoA and then into the acylcarnitine derivative. The second step is achieved by carnitine palmitoyltransferase 1 (CPT1), the rate-limiting enzyme of β-oxidation (Roepstorff *et al.*, 2005). As acylcarnitines, long-chain fatty acids are then transported across the inner mitochondrial membrane through the carnitine/acylcarnitine carrier (Indiveri *et al.*, 2011) into the mitochondrial matrix. There, they enter β-oxidation after reconversion into the acyl-CoA derivative by CPT2 (Bremer, 1983).

During high intensity exercise, acetyl-CoA generated by pyruvate oxidation or by β-oxidation overwhelms the capacity of the Krebs cycle. Accumulating acetyl-CoA would rapidly deplete the limited CoA pool, if carnitine could not function as a buffer of the acetyl groups. Carnitine acetyltransferase converts acetyl-CoA to acetylcarnitine, thereby ensuring the availability of CoASH (Brass *et al.*, 1980; Friolet *et al.*, 1994).

Taking into account these important functions of carnitine, it is not surprising that carnitine supplementation has been studied intensively as a potential performance enhancer (Brass, 2004). In *ex vivo* models it has been reported that an elevation of the carnitine muscle content increases skeletal muscle force and delays fatigue (Brass *et al.*, 1993; Dubelaar *et al.*, 1991). In humans, most studies failed to show a positive effect of carnitine supplementation on physical performance, a finding correlating with the fact that the muscle carnitine content could not be increased by oral or parenteral carnitine supplementation despite elevated plasma concentrations (Brass, 2000). High oral doses of carnitine during

several days or intravenous infusion for several hours before exercise failed to show an impact on the physical performance of healthy volunteers or athletes (Barnett *et al.*, 1994; Oyono-Enguelle *et al.*, 1988; Wachter *et al.*, 2002). Recently, Wall and colleagues reported an approximately 20% increase of the carnitine muscle content in human subjects ingesting 80 g carbohydrate and 2 g carnitine tartrate per day (Wall *et al.*, 2011). This increase in the skeletal muscle carnitine content was associated with an enhanced work output, maintained skeletal muscle glycogen stores and a reduced production of lactate.

In rodents, studies investigating the accumulation of carnitine in skeletal muscle associated with carnitine supplementation are controversial. An increase of the skeletal muscle carnitine content of supplemented animals was reported by several groups (Bacurau *et al.*, 2003; Negrao *et al.*, 1987), while in other investigations, no such increase could be detected (Lambert *et al.*, 2009; Primassin *et al.*, 2008).

Whereas the dose und duration of carnitine supplementation seems to influence the carnitine accumulation in skeletal muscle of rodents, little is known about the effect of supplementation with carnitine derivatives, e.g. acetylcarnitine or propionylcarnitine, on the skeletal muscle carnitine content and physical performance. Elevated carnitine plasma concentrations are not necessarily associated with an increase in the skeletal muscle carnitine content due to saturation of the carnitine transport system into skeletal muscle at normal carnitine plasma concentrations (Berardi *et al.*, 2000) and due to a large concentration gradient between plasma and skeletal muscle (Brass, 2000; Friolet *et al.*, 1994). In comparison to carnitine, skeletal muscle concentrations of acetylcarnitine and propionylcarnitine are much lower under resting conditions. Similar to plasma, the acetylcarnitine concentration in skeletal muscle is in the range of 20% of the free carnitine concentrations (Friolet *et al.*, 1994; Minkler *et al.*, 2008), whereas, to the best of our knowledge, skeletal muscle propionylcarnitine concentrations have so far not been reported. A favorable gradient between plasma and skeletal muscle after supplementation could therefore facilitate transport of the amphiphilic acetyl- and propionylcarnitine into skeletal muscle by passive diffusion.

In this study we therefore aimed to investigate the potential benefits of oral acetylcarnitine or propionylcarnitine supplementation on the skeletal muscle carnitine content and working capacity of mice and compare the findings with carnitine supplementation. After oral supplementation of carnitine or acylcarnitines during four weeks, mice were subjected to exhaustive exercise and the plasma and skeletal muscle carnitine pools as well as metabolic markers in muscle and plasma were analyzed.

Material and methods

Animals

The experiments were performed in agreement with the guidelines for the care and use of laboratory animals and were approved by the cantonal veterinary authority (License 2509). The animals were adult male C57BL/6 mice (Janvier, Le Genest-Saint-Isle, France), housed in a standard facility with a 12h light-dark cycles and controlled temperature (21-22°C). The mice were fed with a standard pellet chow and water *ad libitum*. After seven days of acclimatization, the mice were divided into four groups of 12 animals each: 1) Control group (Ctrl), 2) Carnitine group (Cn), 3) Acetylcarnitine group (Acetyl-Cn), and 4) Propionylcarnitine group (Propionyl-Cn). Half of the animals of each group (n=6) were housed for 24 h in the metabolic cage and performed exhaustive exercise before sacrifice (see below).

Carnitine or acylcarnitines supplementation

L-carnitine and acetyl-L-carnitine HCl were purchased from Sigma (St. Louis, MO, USA). Propionyl-L-carnitine HCl was a kind gift of Sigma Tau (Pomezia, Rome, Italy). The solutions of carnitine, acetylcarnitine and propionylcarnitine were made in tap water once a week and were kept at 4°C until use. After one week of acclimatization, the mice were supplemented with carnitine, acetylcarnitine or propionylcarnitine *via* drinking water for 4 weeks. The concentration of carnitine and carnitine derivatives in tap water was 10 mmol/L so that mice of about 25 g drinking 5 mL/d would be exposed to approximately 2 mmol/kg/d of each

compound. Water consumption was monitored daily. The carnitine content in the standard chow and in the drinking water was evaluated with a LC-MS/MS method previously described (Morand, 2012).

Protocol

Between week 1 and 2 of supplementation, the mice spent 24h individually in metabolic cages with supplemented water and food *ad libitum*. During this period, 24h urine was collected for the assessment of the excretion of carnitine and acylcarnitines.

After 4 weeks of supplementation, the mice were submitted to an exhaustive exercise on a treadmill with previous acclimatization to the apparatus (Exer-3/6, Columbus Instruments, Columbus, OH, USA). Two days and one day before initiation of the study, each mouse underwent a five-minute training session at a speed of 5 m/min. On the day of sacrifice, the starting speed was 5 m/min; after 5 minutes, the speed was increased by 1 m/min every minute until the maximal speed of 20 m/min was reached. The rear of the treadmill was equipped with low-voltage, electric stimulating grids, to encourage the mice to run. The grid delivered 0.4 mA at a frequency of 2 Hz, which caused an uncomfortable shock without injuring the animal. Exhaustion was defined as immobility for more than 5 seconds on the end lane grid despite electric stimulation.

The physical performance of the mice was calculated from the distance and the duration of the run according to the following equation:

$$(1) \qquad \text{Performance}\,(\text{W}) = \frac{\text{m}\,(\text{kg}) \times \text{a}\,(\text{m/s}^2) \times \text{d}\,(\text{m})}{\text{t}\,(\text{s})}$$

with m being the mass of the animal, a the gravitational acceleration, d the run distance, and t the running time.

Biological sample collection

After exhaustion the mice were anaesthetized with an intraperitoneal application of ketamine (100 mg/kg) and xylazine (10 mg/kg). The soleus and the superficial part of the gastrocnemius muscles were excised and conserved in ice cold BIOPS buffer (10 mmol/L Ca-EGTA buffer, 0.1 µmol/L free calcium, 5.77 mmol/L ATP, 6.56 mmol/L $MgCl_2$, 20 mmol/L taurine, 15 mmol/L phosphocreatine, 0.5 mmol/L dithiothreitol, and 50 mmol/L K-MES, pH 7.1) until analysis. Blood was collected into heparin-coated tubes by an intracardiac puncture or a tail incision. Plasma was separated by centrifugation at 3000 g for 15 minutes.
For chemical analysis muscle samples -red and white quadriceps biopsies- and liver samples were frozen in liquid nitrogen immediately after excision. Plasma, urine, liver and muscle samples were kept at -80°C until analysis.

High resolution respirometry

Mitochondrial respiration measured *in situ* allows the characterization of functional mitochondria in their normal intracellular position and assembly, preserving essential interactions with other organelles. Mitochondrial oxygen consumption was studied in saponin-skinned fibers (Kuznetsov, 2008). Fibers were separated under a binocular microscope in BIOPS buffer at 4°C. After dissection, fibers were transferred into BIOPS buffer containing 50 µg/mL saponin and incubated at 4°C for 30 minutes while shaking to complete permeabilization of the sarcolemma. Permeabilized fibers were then washed in BIOPS buffer for 10 minutes under intense shaking to completely remove saponin. Before oxygraphic measurements, the fibers were washed twice for 5 minutes in MiR05 buffer (EGTA 0.5 mmol/L, $MgCl_2$ 3 mmol/L, taurine 20 mmol/L, KH_2PO_4 10 mmol/L, HEPES 20 mmol/L, D-sucrose 110 mmol/L, BSA essentially fatty acid free 1 g/L, and lactobionic acid 60 mmol/L) to remove any trace amount of high-energy phosphates. All oxygen measurements were performed at 37°C with an Oxygraph 2k apparatus equipped with the Datlab software (OROBOROS, Innsbruck, Austria) (Pesta *et al.*, 2012). Per respiration chamber, 1-2 mg of permeabilized fibers was added to 2.0 mL of MiR05 buffer.
The following protocol was used to evaluate the activity of the different mitochondrial

complexes. The rate of basal respiration was measured with the complex I substrates glutamate (10 mM) and malate (2 mM). Active respiration was induced with 2 mM ADP. After inhibition of complex I with 0.5 µM rotenone, the respiration was restored by addition of complex II substrate succinate (10 mM). Inhibition of complex II with 20 mmol/L malonate was followed by the addition of the artificial substrates for complex IV TMPD (0.5 mM) and ascorbate (2 mM). To verify the integrity of the outer mitochondrial membrane, cytochrome c (10 µmol/L) was added at the end of the respiration protocol. Respiration rates are expressed in picomoles O_2 per second per gram wet weight.

Histology

Muscle biopsies for histological imaging were frozen in isopentane cooled in liquid nitrogen. Ten-micrometer serial sections were cut from the soleus and gastrocnemius muscle on a cryostat microtome (HYRAX C60, Carl Zeiss Microscopy, Jena, Germany) and mounted on glass slides. The histological structure of muscular fibers was determined by a routine hematoxylin/eosin staining. Soleus and gastrocnemius fiber type composition was determined by histochemical staining of the myosine ATPase activity (Bancroft 2008). Stained fibers at pH 9.4 were counted from two non-overlapping visual fields using a microscope (Olympus BX43, Olympus corporation, Tokyo, Japan) at a 100-fold magnification. Quantification was performed with an in-house software recognizing dark from light fibers.

Urinary carnitine concentrations

A LC-MS/MS method previously described was adapted for the determination of carnitine and acylcarnitines in urine (Morand, 2012). The method was extended to the analysis of propionylcarnitine (transitions 218/85 and 218/159) and creatinine (114/44 and 114/86). Hydrolysis of total carnitine was achieved with 0.5 mol/L potassium hydroxide, neutralization and dilution with 0.1% formic acid.

Plasma parameters

Plasma concentrations of carnitine were determined as previously described with adaptation to propionylcarnitine (Morand, 2012). Venous lactate concentrations were analyzed with an enzymatic assay (Olsen, 1971). Creatine kinase activity in venous plasma was determined before and after exhaustive exercise with a kit according to the supplier's instructions (BioAssay Systems, Hayward, CA, USA).

Muscle and liver parameters

Muscle tissue was homogenized with a Micro-dismembrator during 1 minute at 2000 rpm (Sartorius Stedim Biotech, Göttingen, Germany). 50 mg muscle was extracted with 1 mL of extracting solvent depending on the metabolites determined as described below. Liver tissue was homogenized during 30 seconds at 2000 rpm and then extracted with 1 mL methanol per 100 mg tissue.

Phosphocreatine, creatine and ATP were determined photometrically in an acidic extract (perchloric acid 0.5 mol/L, EDTA 1 mmol/L) of muscle powder as described (Harris *et al.*, 1974). Muscle glycogen content was analyzed in alkaline (NaOH 0.1 mmol/L) muscle extracts according to Harris (Harris *et al.*, 1974). Carnitine, acylcarnitines and total carnitine were determined in aqueous muscle extracts with an established LC-MS/MS (Morand, 2012).

Statistical analysis

Data is expressed as mean ± SEM. Statistical analyses were performed using unpaired two-tailed Student's t-test, one-way or two-way ANOVA followed by a Dunnett post-test with the software Prism version 5 (Graph Pad Software, San Diego, CA). Statistical significance was set at *p <0.05 and **p <0.01.

Results

Characterization of the animals

During the study, food intake was similar in all groups and we observed no weight differences between the groups (Figure 1A). The daily water intake was increased in all groups treated with carnitine or acylcarnitines ($p<0.05$) compared to the control group (Figure 1B). The control animals (n=12) drank 4.1 ± 0.2 mL/d, whereas the animals of the carnitine, acetylcarnitine and propionylcarnitine group ingested 5.2 ± 0.1 mL/d, 5.7 ± 0.3 mL/d, and 5.6 ± 0.1 mL/d, respectively (mean ± SEM; no difference between treated animals). The mean daily exposure to exogenous carnitine, acetylcarnitine or propionylcarnitine was 2.2 mmol/d/kg (range 1.9-2.7 mmol/d/kg) in the treated groups and below 10 µmol/kg/d in the control group, taking into account the carnitine ingested in the drinking water and in the food (Figure 1C). The carnitine content of the food was 60 nmol/g. The concentrations of carnitine, acetylcarnitine, and propionylcarnitine in the drinking solutions remained stable during their storage at 4°C and in the water bottles at room temperature (data not shown).

Carnitine and acylcarnitines pools in plasma, liver, and skeletal muscle and renal carnitine excretion

In plasma, total carnitine concentrations were significantly higher in the treated groups compared to control ($p<0.01$ for Cn and Acetyl-Cn groups, $p<0.05$ for Propionyl-Cn) due to higher free carnitine concentrations (Table 1). Acetylcarnitine, propionylcarnitine, and palmitoylcarnitine plasma concentrations were similar between the groups.

In liver samples of propionylcarnitine treated animals, carnitine and propionylcarnitine concentrations were significantly elevated ($p<0.05$) compared to control animals (Supplementary Table 2).

In red or white skeletal muscle, we observed no difference in the carnitine pools between the groups treated with carnitine or acylcarnitines and the control group. The acetylcarnitine concentrations accounted for 20% of the total pool in red fibers and for 15% in the white fibers. Total carnitine, free carnitine, acetylcarnitine, and palmitoylcarnitine concentrations were by trend lower in the white compared to red quadriceps. Propionylcarnitine

concentrations in skeletal muscle were below detection limits (<0.5 μM), also in mice treated with propionylcarnitine (Table 1).

Treated animals excreted about 17 times more total carnitine than controls animals as reported in the supplementary Table 1. The excreted carnitine was found principally in the form of free carnitine and acetylcarnitine (Figure 2). In all treated groups, independently of the supplemented form of carnitine, the excretion of carnitine and acetylcarnitine was significantly higher ($p<0.01$) compared to control animals. Urinary excretion of propionylcarnitine could only be detected in the three supplemented groups, whereas the propionylcarnitine concentration was below the limit of detection in the urine of control mice. The highest urinary propionylcarnitine concentration was found in the propionylcarnitine group. Due to the high variability, this difference did not reach statistical significance between the treated groups (Supplementary Table 1).

Carnitine balance under resting conditions

Total carnitine ingestion and excretion in control and treated animals are presented in Table 2. The total intake of carnitine was 200 times higher in animals supplemented with carnitine, acetylcarnitine or propionylcarnitine as compared to control animals. The carnitine tissue accumulation, which was calculated from the mean total carnitine concentration in red and white muscles and from the estimated muscle weight gain during the observation period of one month, was not different between the groups and was quantitatively negligible compared to oral ingestion of carnitine. Excretion of total carnitine was, as discussed above, 10 to 20 times higher in the treated groups as compared to the control group. The carnitine balance, i.e. the difference between carnitine intake plus tissue accumulation and carnitine excretion, was negative in control mice and positive in all treated groups. The negative value in control mice mainly represents carnitine biosynthesis; the value is in agreement with reported values in rats assessed by a similar method (Krahenbuhl *et al.*, 2000). The positive value in treated animals is mainly explained by incomplete bioavailability of carnitine and acylcarnitines.

Bioavailability, as estimated from carnitine ingestion and urinary excretion, ranged between 20% and 23% without a difference between the groups supplemented with carnitine.

High resolution respirometry

As expected, the maximal respiration rate in the soleus muscle, which is mainly composed of oxidative fibers, was higher than in the gastrocnemius, which consists mainly of glycolytic fibers. For the soleus and the gastrocnemius muscle biopsies, we observed no significant difference in oxygen uptake for the different conditions tested between the groups. These findings indicate that mitochondrial content and capacity remained unchanged in the supplemented compared to the control group (Figure 3).

Histology

As reported in Figure 4, we observed no difference in the muscle fiber type composition in the soleus and gastrocnemius muscles of treated as compared to control animals. In soleus muscle, type I fibers represented about 70% of the fibers in all the groups studied (Fig. 4B).

Exhaustive exercise

As shown in Figure 5, the mean running distance until exhaustion of untreated mice was 561 ± 150 m (n=6). The distance covered by treated mice was by trend increased in all the groups but no statistical significance was observed because of high interindividual variability (carnitine 699 ± 265 m, acetylcarnitine 772 ± 320 m, propionylcarnitine 820 ± 525 m). The performance of the animals was in the range of 67 – 69 mW and was not different between the groups.

Energy parameters in plasma and skeletal muscle in resting mice and mice after exhaustive exercise

As shown in Table 3, plasma lactate levels increased significantly with exercise in all the groups ($p<0.01$). By trend, lactate concentrations after exercise were lower in the three

supplemented groups as compared to the control group, though not reaching statistical significance. Creatine kinase activity was not different between supplemented and control animals and showed a slight elevation with exercise compared to resting conditions (results not shown).

Energy parameters were determined in separate samples of white and red quadriceps femoris (Table 3). The creatine and phosphocreatine concentrations were not different between supplemented and control groups and were not significantly influenced by exercise. Similarly, the skeletal muscle ATP content was not affected by exercise. There were no significant differences between control and supplemented groups, although there was a trend for a higher ATP content in red fibers from supplemented mice. The glycogen content was higher in white skeletal muscle fibers compared to red fibers. After exercise, a significant decrease of the glycogen content was observed in white fibers, while no difference was noticed in red fibers. The glycogen content after exercise was by trend higher in treated groups, but again without reaching statistical difference.

Discussion

In the current study we investigated the effects of an oral supplementation of carnitine, acetylcarnitine, or propionylcarnitine on the body carnitine balance, skeletal muscle composition and exercise capacity of mice. We could show that carnitine, acetylcarnitine, and propionylcarnitine supplementation did not influence the skeletal muscle carnitine pool when administered orally for 4 weeks at a dose of 2 mmol/kg/d. Accordingly, acetylcarnitine or propionylcarnitine administered orally had no significant impact on skeletal muscle composition or physical performance.

Other animal studies also reported that there is no impact of carnitine supplementation on the skeletal muscle carnitine content. In rats, low oral doses of carnitine or acetylcarnitine (200 µmol/kg/d) for 14 days did not increase the skeletal muscle carnitine pool (Lambert *et al.*, 2009). In mice, a higher carnitine dose (200 mg/kg/d or approximately 1 mmol/kg/d) for

five weeks also failed to show an effect on the skeletal muscle carnitine pool (Primassin *et al.*, 2008). To the best of our knowledge, such data were so far lacking for propionylcarnitine. The few animal studies performed in rats or mice reporting a positive effect of carnitine supplementation on the skeletal muscle carnitine pool either used high parenteral doses during a relatively long period of time (4 mmol/kg/d during 8 weeks) (Negrao *et al.*, 1987) or determined the carnitine content only in soleus muscle (Bacurau *et al.*, 2003). In our study, we could not confirm this finding in deep quadriceps biopsies, which mainly contain red fibers similar to the soleus muscle. In agreement to our findings, supplementation of high doses of carnitine or acylcarnitines had no influence on the muscle fiber type composition also in other studies (Cassano *et al.*, 2010). This is in agreement with our observation that treatment with carnitine or acylcarnitines had no significant impact on the oxygen consumption profiles by skeletal muscle fibers.

Similar to our study, also most studies in humans failed to show an impact of carnitine supplementation on total muscle carnitine content and on physical performance (Barnett *et al.*, 1994; Wachter *et al.*, 2002). Recently, however, increased carnitine skeletal muscle concentrations in humans treated with high amounts of carbohydrates to induce hyperinsulinemia have been reported (Wall *et al.*, 2011). It was hypothesized that insulin enhances carnitine transport *via* stimulation of the Na^+/K^+ ATPase, leading to the formation of a higher Na^+ gradient to drive the sodium-dependent carnitine transporter OCTN2. In a similar study, a higher expression of OCTN2 five hours after an infusion of carnitine and insulin was demonstrated, offering a second possible explanation for the observed increase in the skeletal muscle carnitine content under hyperinsulinemic conditions (Stephens *et al.*, 2006). In the study of Wall et al. the increase of the skeletal muscle carnitine content was observed only in the group treated for 24 weeks and not in the group treated for 12 weeks, suggesting that long supplementation periods are required to increase the muscle carnitine pool (Wall *et al.*, 2011). In this context, it has to be pointed out that the transport mechanisms of carnitine into skeletal muscle are not fully characterized. It is well established that the sodium-dependent carnitine carrier OCTN2 is expressed in skeletal muscle (Nezu *et al.*,

1999; Spaniol *et al.*, 2001; Stephens *et al.*, 2006; Tamai *et al.*, 1998; Tamai *et al.*, 2000). On the other hand, the study of Berardi et al. (Berardi *et al.*, 2000) using rat skeletal muscle membrane vesicles revealed carnitine transport characteristics which are not completely compatible with the properties of OCTN2. It is therefore currently unclear whether the transport of carnitine into skeletal muscle can fully be explained by OCTN2 or whether additional carnitine carriers exist.

After exercise, we did not observe significant differences in the skeletal muscle phosphocreatine content compared with resting conditions, suggesting that exercise was not intensive enough, although the mice were exhausted according to our criteria. Exhaustive exercise is supported, however, by the observed increase in the plasma lactate concentrations in exercising mice and in the decrease of the glycogen content in skeletal muscle. Even though the interval between end of exercise and sacrifice of the mice was in no case longer than 15 minutes, this period may have been long enough to allow an at least partial recovery of the phosphocreatine pool.

In mice, the bioavailability of low doses of carnitine (250 ng/kg) was reported to be 67% (Yokogawa *et al.*, 1999), which is clearly higher than the values found in the current study. For acetylcarnitine and propionylcarnitine, the absorption profile in mice is not known. In humans, although it was suggested that acetylcarnitine is partly hydrolyzed in enterocytes, oral doses of acetylcarnitine increased the corresponding plasma concentrations by 40% (Rebouche, 2004). The bioavailability of propionylcarnitine has not been determined specifically in mice, but has been estimated to be less than 20% (Mancinelli *et al.*, 2005). Our study was not designed as a pharmacokinetic study, but nevertheless allowed to estimate some kinetic parameters of the administered compounds. As shown in Table 2, the bioavailability of carnitine with carnitine, acetylcarnitine or propionylcarnitine is in the range of 20 – 24% irrespectively of the compound administered and based on urinary excretion of carnitine after oral ingestion of the compounds. The comparison with the study of Yokogawa et al. therefore suggests that carnitine absorption is concentration-dependent (Yokogawa *et al.*, 1999). Furthermore, the current study indicates that orally ingested acylcarnitines

undergo hydrolysis both in the intestinal tract and in the liver. This interpretation is based on our findings that we did not observe increased concentrations of acylcarnitines in the systemic circulation and that we found elevated concentrations of carnitine and propionylcarnitine in the liver after oral ingestion of propionylcarnitine.

As mentioned above, the supplemented carnitine, acetylcarnitine or propionylcarnitine were excreted mainly as carnitine and to a lesser extent as acetylcarnitine, independently of the supplemented carnitine derivative. Whereas propionylcarnitine was below the limit of quantification in the urine of control mice, it was present in the urine of all treated animals, also of the mice supplemented with carnitine or acetylcarnitine. Similarly, in humans treated with oral carnitine, elevated concentrations of acetylcarnitine and propionylcarnitine have been observed in plasma and urine (Cao *et al.*, 2009). Furthermore, in patients with end-stage renal failure undergoing hemodialysis, intravenous administration of carnitine was associated with increased plasma concentrations of many acylcarnitines and increased elimination of acylcarnitines by dialysis (Vernez *et al.*, 2006). These findings suggest that the circulating plasma carnitine exchanges with tissue where it can acylated, transported back into plasma and eventually be excreted via the urine.

In conclusion, oral supplementation of carnitine, acetylcarnitine or propionylcarnitine was associated with increased plasma concentrations of total carnitine and increased urinary excretion of carnitine, but did not affect the skeletal muscle carnitine content. Accordingly, physical performance and skeletal muscle energy metabolism were not affected by carnitine supplementation. Further studies assessing the effect of acylcarnitines on the skeletal muscle carnitine content and physical performance would have to be carried out with parenteral administration, thus circumventing precocious hydrolysis.

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Figures legends

Figure 1

Characterization of the animals. The weight gain (in grams) was similar in the treatment groups compared to control (A). Water consumption (in mL/mouse/d) was significantly higher in the treated groups (B). Daily consumption of carnitine or acylcarnitines was below 10 µmol/kg/d in the control group and in the range of 2000 µmol/kg/d in the supplemented groups (C). Data is represented as mean ± SEM of 12 animals per group.

Figure 2

Mean urinary excretion of carnitine, acetylcarnitine and total carnitine. Mice supplemented with carnitine, acetylcarnitine or propionylcarnitine excreted significantly higher amounts of carnitine and acetylcarnitine than the control group. Data is expressed as mmol/kg/d (mean ± SEM of 6 animals per group)

Figure 3

Oxygen consumption of isolated muscle fibers. We observed no differences between carnitine or acylcarnitine supplemented mice and control mice. Data is represented as mean in pmol O_2/s/mg protein ± SEM of six animals.

Figure 4

Histochemical determination of muscle fibers composition. (A) Representative ATPase and hematoxylin/eosin (H/E) staining of soleus and gastocnemius (GC) biopsies. (B) In soleus

muscle we observed no difference in muscle fibers composition between treated and control animals. Mean values ± SEM of six animals are represented.

Figure 5

Running capacity of mice completing a treadmill exercise until exhaustion. We observed a slight upward trend for the running distance (A) in treated groups. Performance expressed in mW (B) did not differ between the groups. Mean values ± SEM of six animals are represented.

Table 1

Carnitine and acylcarnitines concentrations in plasma and skeletal muscle under resting conditions. Mean values ± SEM of six animals are represented. **: $p<0.01$ compared to control, * $p<0.05$ compared to control.

	Control	Carnitine	Acetylcarnitine	Propionylcarnitine
Plasma concentrations (µmol/L)				
Carnitine	31.0±0.5	37.2±2.2	36.1±1.4	35.6±3.4
Acetylcarnitine	7.8±0.8	8.5±1.3	9.3±0.6	7.6±0.7
Propionylcarnitine	0.30±0.04	0.24±0.07	0.27±0.04	0.76±0.38
Palmitoylcarnitine	0.25±0.01	0.29±0.04	0.25±0.02	0.30±0.03
Total carnitine	36.4±1.4	47.7±1.8**	47.6±1.6**	42.7±1.6*
Skeletal muscle concentrations (µmol/kg wet weight)				
Red quadriceps				
Free carnitine	205±29	272±32	216±25	189±14
Acetylcarnitine	66±7	69±11	70±3	62±9
Propionylcarnitine	<0.5	<0.5	<0.5	<0.5
Palmitoylcarnitine	44±8	55±6	32±12	28±5
Total carnitine	291±31	309±59	359±56	249±43
White quadriceps				
Free carnitine	110±7	115±10	127±11	130±11
Acetylcarnitine	39±5	37±10	26±3	28±5
Propionylcarnitine	<0.5	<0.5	<0.5	<0.5
Palmitoylcarnitine	15±5	20±3	14±3	19±5
Total carnitine	195±17	207±14	169±20	152±14

Table 2

Total carnitine balance under resting conditions. Intake by water and food was determined as described in Methods. Carnitine tissue accumulation was calculated based on the carnitine content in skeletal muscle and the estimated increase in skeletal muscle weight. Carnitine excretion was determined in 24-hour urines of the animals. The carnitine balance was calculated as the difference between carnitine intake plus tissue accumulation and carnitine excretion. Values are given as µmol/kg/d ± SEM.

Treatment group (n = 6)	Intake		Tissue accumulation	Excretion	Balance
	Water	Food			
Control	0±0	0.32±0.06	9.5±0.1	31±4	-22±4
Carnitine	2072±27	0.40±0.10	9.6±0.2	472±152	1609±155
Acetylcarnitine	2254±114	0.30±0.06	9.6±0.2	451±80	1812±140
Propionylcarnitine	2259±48	0.44±0.08	9.6±0.2	501±40	1767±63

Table 3

Effect of exercise on skeletal muscle and plasma parameters. Mean values ± SEM of six animals are presented. ** $p<0.01$ compared to resting values, * $p<0.05$ compared to resting values. There were no significant differences between control and carnitine or acylcarnitines treated animals.

	CTRL	**Cn**	**Acetyl-Cn**	**Propionyl-Cn**	**CTRL**	**Cn**	**Acetyl-Cn**	**Propionyl-Cn**
				Red quadriceps				
	Resting conditions				Exhaustive exercise			
Creatine (µmol/kg w.w.)	25.4±1.5	23.6±1.3	28.2±0.8	25.2±2.1	25.8±1.2	26.6±1.3	24.8±1.8	28.3±1.4
Phosphocreatine (µmol/kg w.w.)	3.1±1.2	7.5±1.8	7.1±1.0	6.1±1.8	4.4±1.1	7.7±1.8	5.5±1.4	7.0±1.2
ATP (µmol/kg w.w.)	4.3±0.6	8.6±1.7	9.9±0.9	8.5±1.4	6.6±1.2	11.0±1.6	8.8±1.0	9.3±1.2
Glycogen (mmol/kg w.w.)	9.5±1.2	11.8±1.0	12.8±1.0	11.5±2.9	7.3±1.7	14.7±2.3	11.1±0.8	8.5±1.9
				White quadriceps				
	Resting conditions				Exhaustive exercise			
Creatine (µmol/kg w.w.)	23.7±2.5	21.8±2.5	21.8±2.3	21.6±1.5	26.1±2.0	26.1±1.4	23.0±2.1	24.5±1.2
Phosphocreatine (µmol/kg w.w.)	11.8±2.0	12.1±0.4	10.9±2.5	10.3±2.0	8.1±1.3	7.7±2.2	12.1±0.9	9.3±1.3
ATP (µmol/kg w.w.)	8.2±1.0	9.7±1.0	9.6±0.6	9.2±0.9	9.4±1.0	9.1±2.0	10.9±1.1	9.3±0.7
Glycogen (mmol/kg w.w.)	17.0±2.9	13.6±1.5	20.9±1.7	15.1±1.4	5.6±2.0*	9.7±1.4	11.3±1.1*	12.6±2.5
				Plasma				
	Resting conditions				Exhaustive exercise			
Lactate (mmol/L)	2.2±0.2	2.2±0.2	2.1±0.2	1.9±0.1	6.2±0.5**	4.8±0.3**	5.0±0.2**	5.2±0.5**

Supplementary Table 1

Urinary excretion of carnitine, acetylcarnitine (Acetyl-Cn), propionylcarnitine (Propionyl-Cn), and total carnitine. Excreted amounts are mean values ± SEM of six animals, given in µmol/kg/d. **: $p<0.01$ compared to control group.

	CTRL	**Carnitine**	**Acetyl-Cn**	**Propionyl-Cn**
Free carnitine	19±2	356±71**	335±68**	360±34**
Acetylcarnitine	2±0	41±10**	54±13**	46±6**
Propionylcarnitine	<0.5	1.0±0.3**	1.1±0.3**	3.3±1.1**
Total carnitine	24±4	417±143**	390±76**	448±36**

Supplementary Table 2

Liver concentrations of carnitine, acetylcarnitine and propionylcarnitine. Values ± SEM of six animals of the control and propionylcarnitine (Propionyl-Cn) groups are given in µmol/kg wet weight. * $p<0.05$ compared to control group.

	CTRL	**Propionyl-Cn**
Free carnitine	314±16	449±46*
Acetylcarnitine	195±8	231±22
Propionylcarnitine	3.1±0.2	4.7±0.5*

Fig. 1

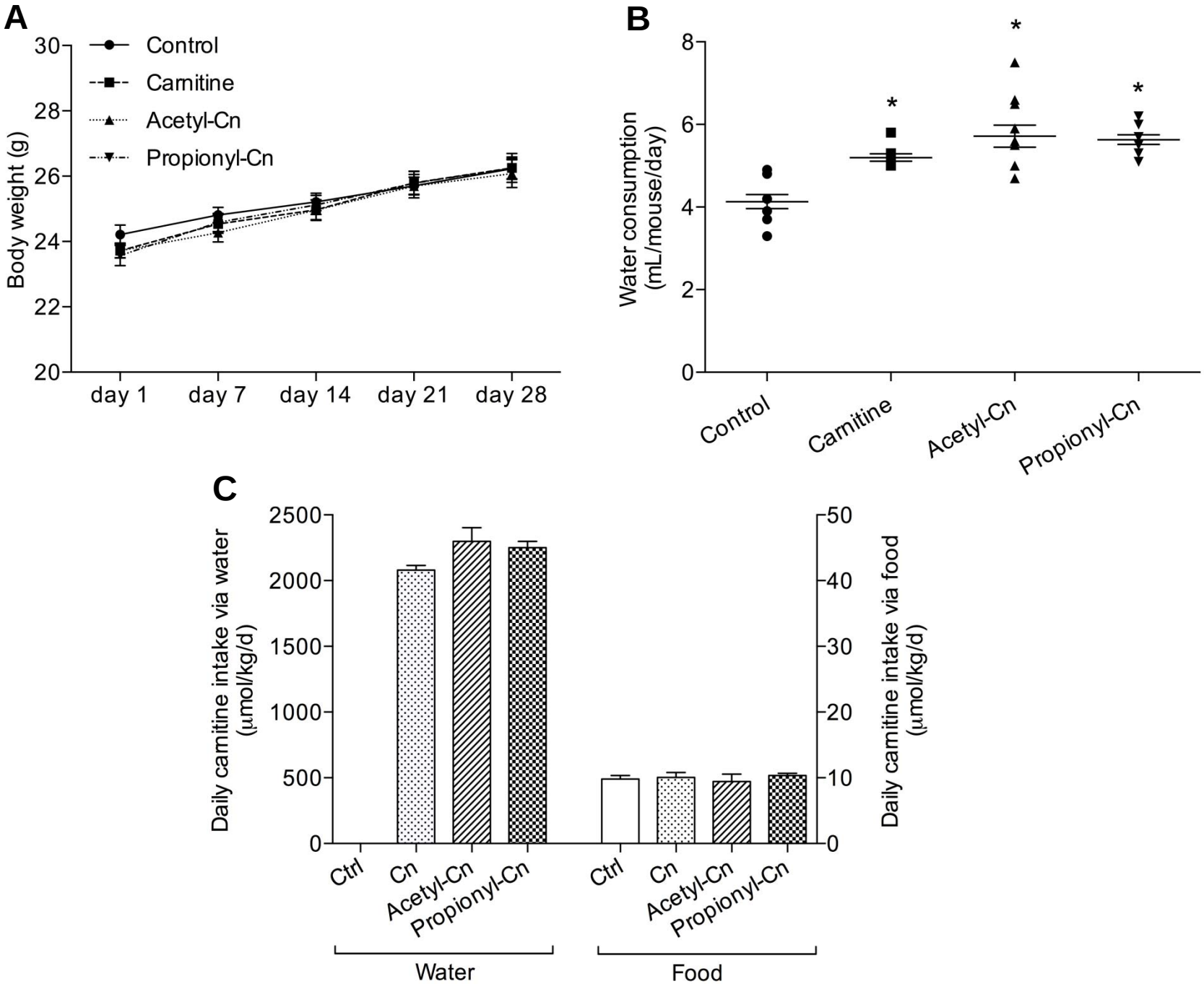

A
Control
Carnitine
Acetyl-Cn
Propionyl-Cn
Body weight (g)
30
28
26
24
22
20
day 1
day 7
day 14
day 21
day 28
B
Water consumption (mL/mouse/day)
8
6
4
2
0
*
*
*
Control
Carnitine
Acetyl-Cn
Propionyl-Cn
C
Daily carnitine intake via water (μmol/kg/d)
2500
2000
1500
1000
500
0
Daily carnitine intake via food (μmol/kg/d)
50
40
30
20
10
0
Ctrl
Cn
Acetyl-Cn
Propionyl-Cn
Ctrl
Cn
Acetyl-Cn
Propionyl-Cn
Water
Food

Fig. 2

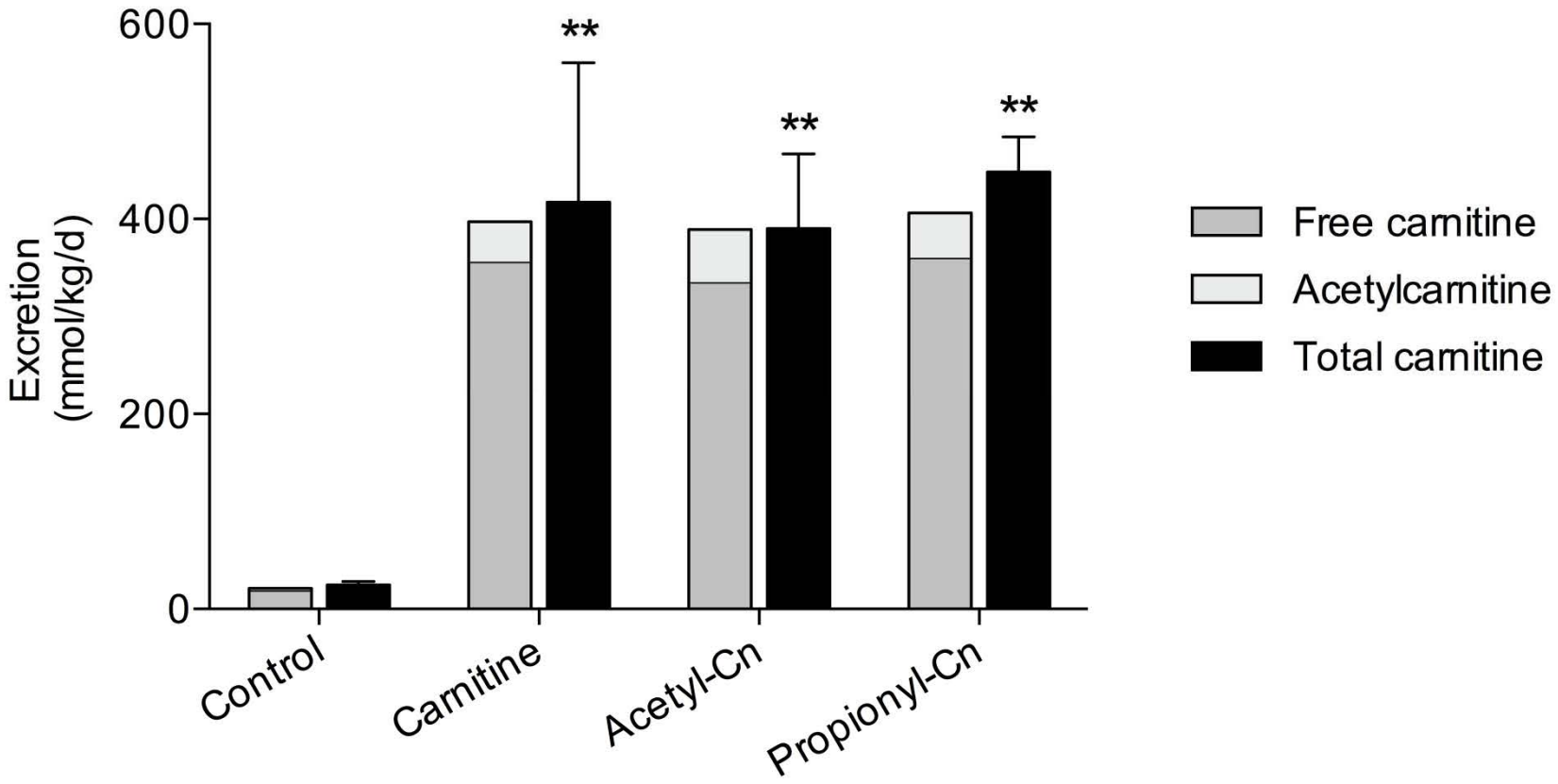

600
400
200
0
Excretion
(mmol/kg/d)
**
**
**
Control
Carnitine
Acetyl-Cn
Propionyl-Cn
Free carnitine
Acetylcarnitine
Total carnitine

Fig. 3

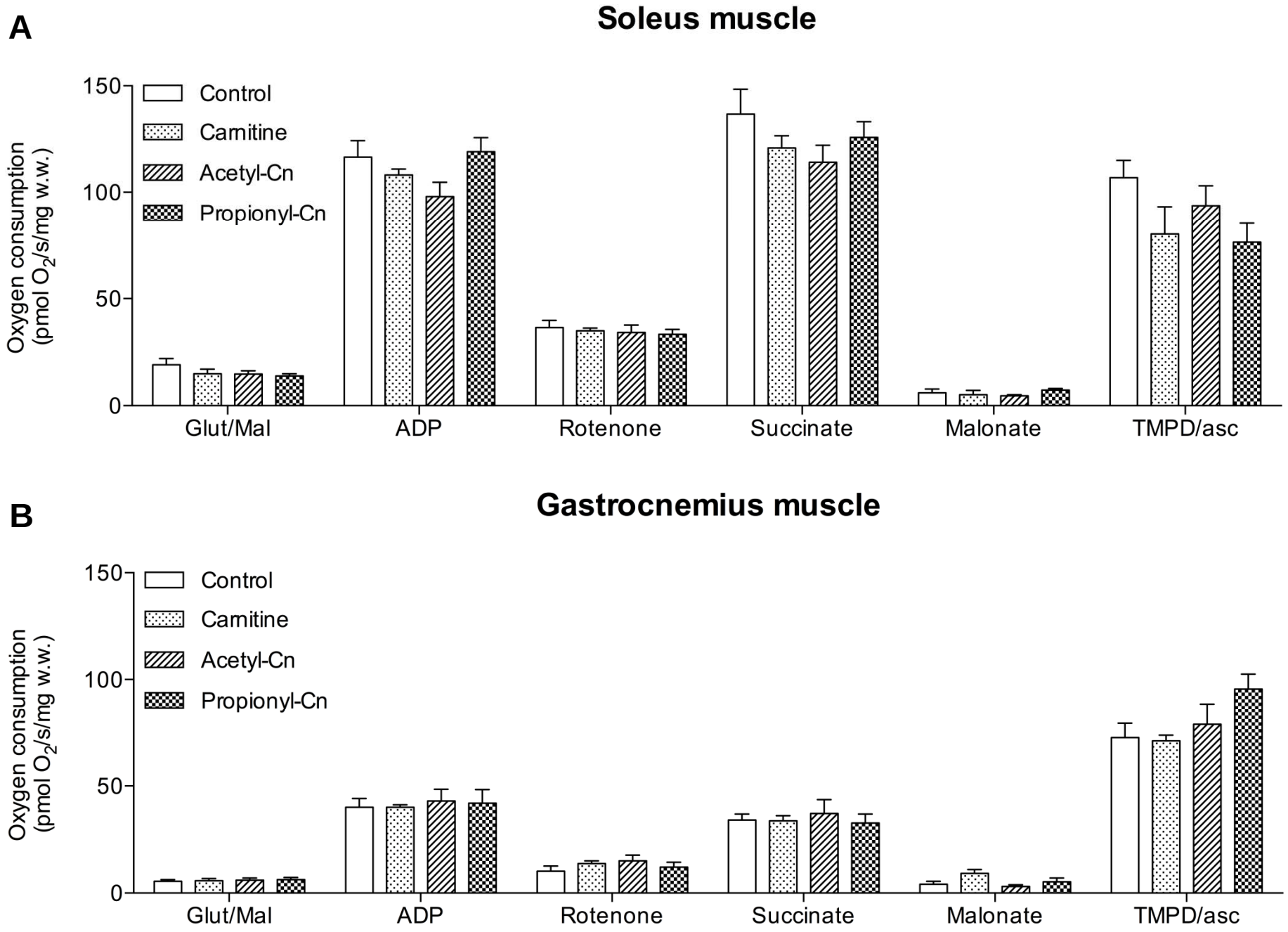
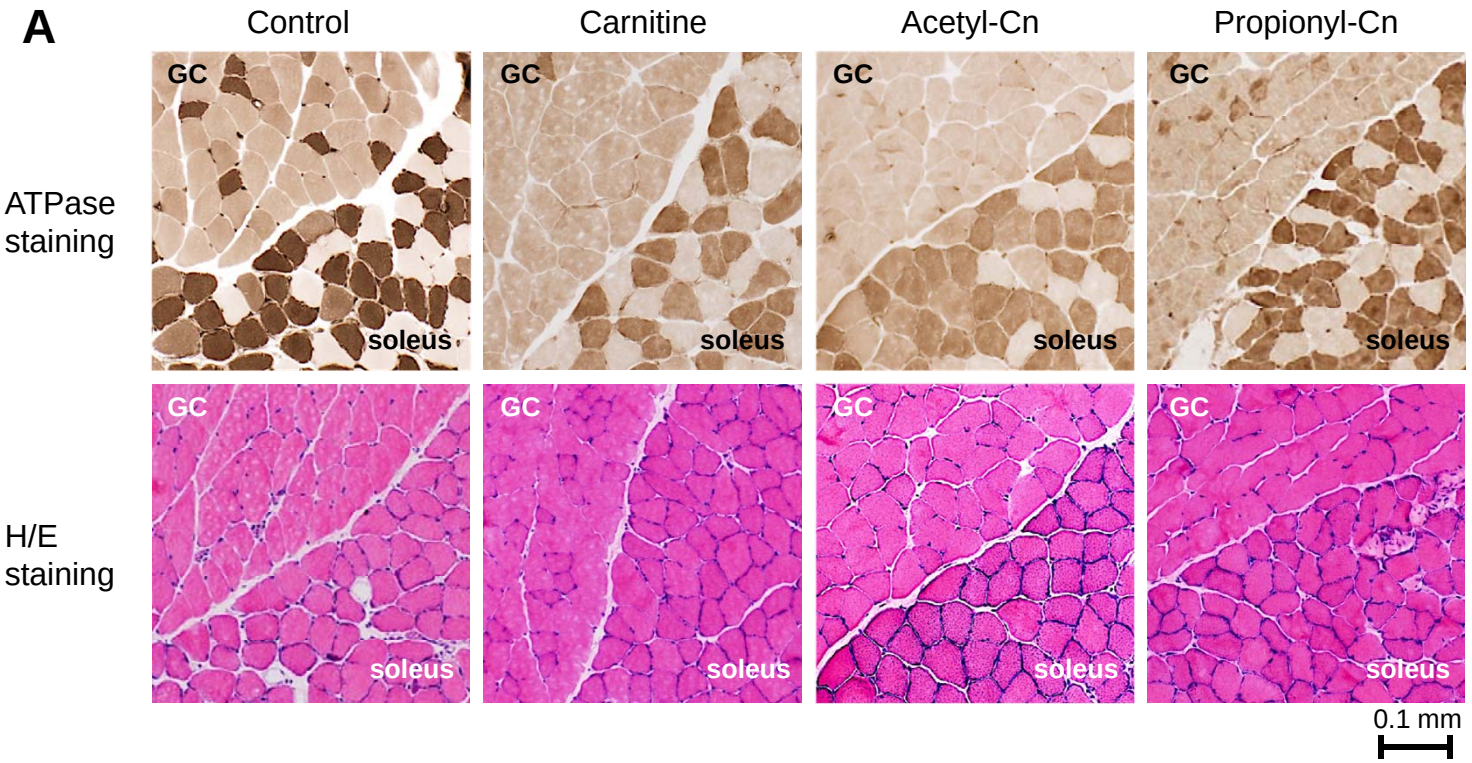

A
Soleus muscle
Oxygen consumption (pmol O_2/s/mg w.w.)
150
100
50
0
Control
Carnitine
Acetyl-Cn
Propionyl-Cn
Glut/Mal
ADP
Rotenone
Succinate
Malonate
TMPD/asc
B
Gastrocnemius muscle
Oxygen consumption (pmol O_2/s/mg w.w.)
150
100
50
0
Control
Carnitine
Acetyl-Cn
Propionyl-Cn
Glut/Mal
ADP
Rotenone
Succinate
Malonate
TMPD/asc

Fig. 4


A
Control
Carnitine
Acetyl-Cn
Propionyl-Cn
ATPase staining
H/E staining
GC
soleus
0.1 mm

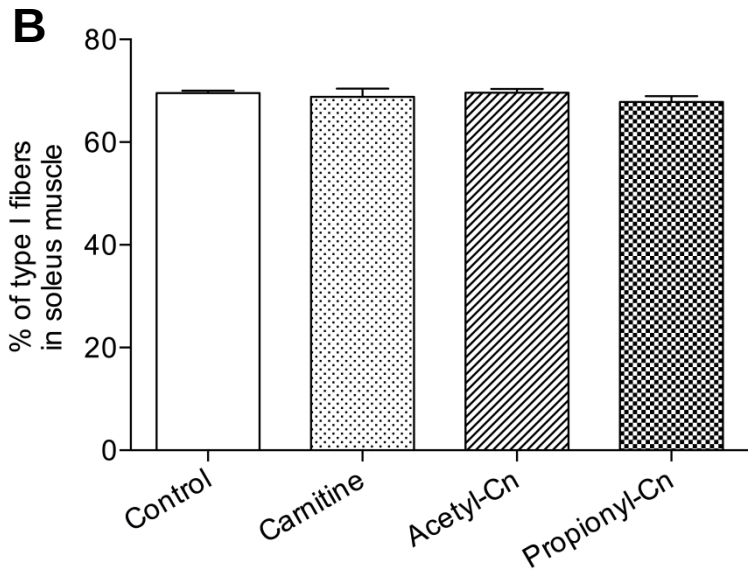
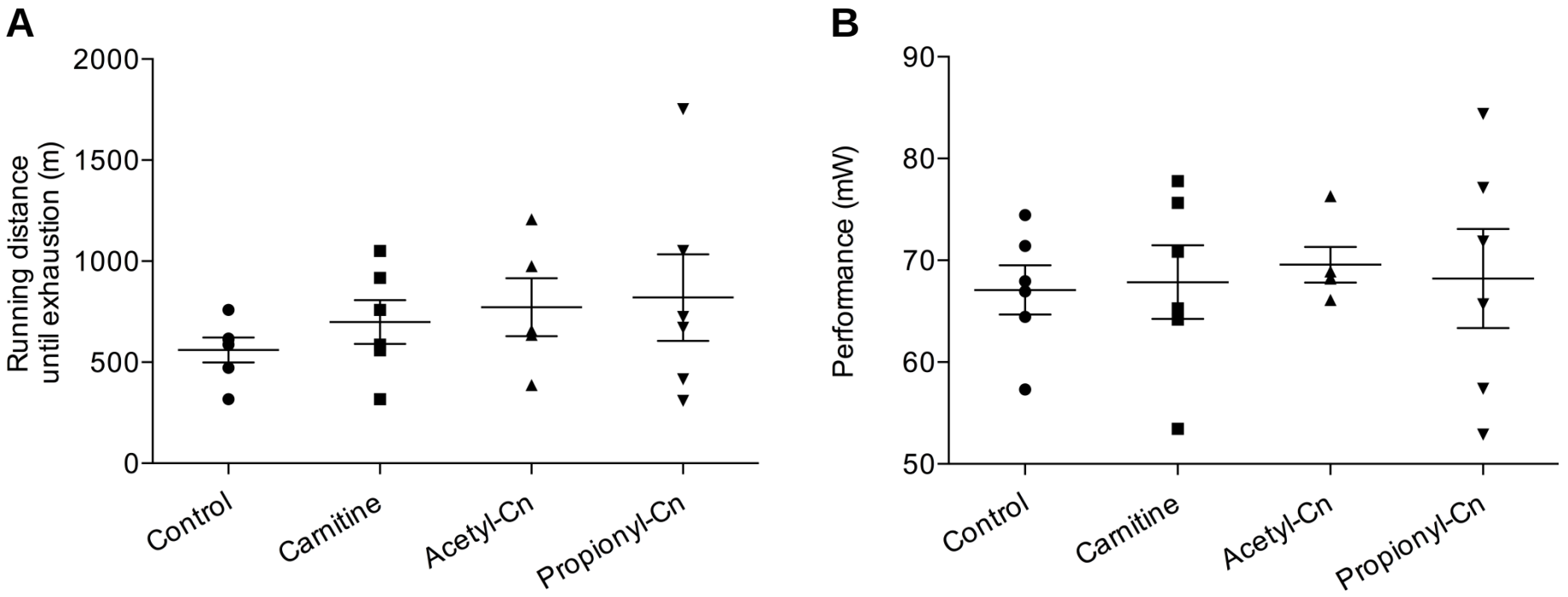

B
% of type I fibers in soleus muscle
80
60
40
20
0
Control
Carnitine
Acetyl-Cn
Propionyl-Cn

Fig. 5


A
Running distance until exhaustion (m)
2000
1500
1000
500
0
Control
Carnitine
Acetyl-Cn
Propionyl-Cn
B
Perfomance (mW)
90
80
70
60
50
Control
Carnitine
Acetyl-Cn
Propionyl-Cn