Pulmonary, Gastrointestinal and Urogenital Pharmacology

Beneficial effects of citrulline malate on skeletal muscle function in endotoxemic rat

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1. Introduction

Asthenia, more commonly known as pathological fatigue, is linked to an altered skeletal muscle performance in many infectious and metabolic diseases. Citrulline malate (CM; CAS 54940-97-5, Stimol®) is usually prescribed as an anti-asthenic treatment and studies performed in animal models have shown that CM ingestion reduces muscle fatigue and weakness. More particularly, it has been shown in endotoxemic rats (a model of muscle weakness) that CM treatment improves the running endurance in treadmill tests (Verleye et al., 1995) and increases the electrically-evoked force-generating capacity in isolated epitrochlearis muscle (Goubel et al., 1997). These beneficial effects have also been documented in humans (Bendahan et al., 2002).

Considering the potential role of muscle energetics in muscle fatigue (Allen et al., 2008; Allen and Westerblad, 2001; Sahlin et al., 1998) and given that fatigue reduction and improved muscle performance have been reported in asthenic patients and treated animals as a result of CM administration, one could hypothesize an effect of CM on muscle energetics. Fatigue is actually a complex mechanism (Allen et al., 2008; Fitts, 1994). Potential factors involved in its etiology fall into two broad categories: central factors, which would disturb neuromuscular transmission between the central nervous system and muscle membrane, and peripheral factors, which would lead to alteration within the muscle. During the last decades, the relative contributions of central and peripheral factors in fatigue development have been subject to controversy. However, it seems now established that fatigue would have, at least for a significant part, a peripheral origin, central factors contributing modestly (Kent-Braun, 1999; Westerblad et al., 1998). Regarding the metabolic origin of peripheral fatigue, intramuscular accumulation of by-products of ATP hydrolysis and limitation in ATP availability have been proposed, among others, as potential causative factors of fatigue development (Allen and Westerblad, 2001; Sahlin et al., 1998). In that respect, the beneficial effect of CM on mechanical performance might be linked to a direct contribution of both malate and citrulline at the muscle energy metabolism level as previously suggested (Goubel et al., 1997; Meneguello et al., 2003). On the one hand, malate is an intermediate of the tricarboxylic acid cycle (TCA) and its supplementation in endotoxemic animals prevented the basal phosphocreatine/ATP ratio reduction and normalized the intracellular pH (pH i) time-course during muscular activity as a sign of an effect at the muscle energetics level. In addition, CM treatment avoided the endotoxemia-induced decline in developed force. These results demonstrate the efficiency of CM for limiting skeletal muscle dysfunction in rats treated with bacterial endotoxin.

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muscle function is controversial given that the time to exhaustion following oral citrulline supplementation increases in rats (Meneguello et al., 2003) but decreases in humans (Hickner et al., 2006).

The aim of this double-blind study was to investigate in vivo the effect of oral ingestion of CM on muscle energetics and mechanical performance in a rat model of muscle weakness. Given the established beneficial effects of CM on muscle fatigue, we hypothesized that CM treatment can modify muscle energetics under endotoxemia. Muscle weakness was induced by intraperitoneal injections of bacterial endotoxins as done previously (Goubel et al., 1997; Verleye et al., 1995), and investigations were performed using $^{31}$P-MRS. The novelty of our approach was to repeat investigations of skeletal muscle function twice in the same animals before and during treatment, using an original experimental setup (Giannesini et al., 2005).

2. Materials and methods

2.1. Animal care and feeding

Nineteen virus-free male Wistar rats (Charles River Laboratories, L’Arbresle, France) weighing 300–325 g were used for these experiments, in agreement with the French guidelines for animal care and with the approval of the local Ethics Committee. Rats were housed in an environmentally controlled facility (12–12 h light–dark cycle, 22 °C) and received water and standard food ad libitum until the time of experiment.

2.2. Experimental design

Animals were randomly assigned to two groups. For each group, animals received two intraperitoneal injections of a lipopolysaccharides (LPS) suspension at 3 mg/kg body weight, at $t_0$ and at $t_0+24$ h. For this purpose, lyophilized LPS from Klebsiella pneumoniae (ref L4268; Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany) was dissolved in a sterile physiological saline (0.9% NaCl w/v). The first group (CM: $n=9$) orally received CM treatment (1 g/kg) three times a day during 48 h after the first LPS injection. The last dose was administered 1 h before the muscle function investigation. The second group (Vehicle: $n=10$) received equivalent volumes of vehicle.

2.3. Animal preparation

For each animal, muscle function was investigated at two times, at $t_0−24$ h (before treatment) and at $t_0+48$ h (during treatment). Basal body weight and body temperature were measured at rest immediately before each investigation.

Rats were initially anaesthetized in an induction chamber with 4% isoflurane (Forene®; Abbott France, Rungis, France) mixed in 33% oxygen ($O_2$: 0.5 L/min) and 66% Nitrous oxide ($N_2O$: 1 L/min). Once the right lower hindlimb was shaved, electrode cream for electromyogram was applied at knee and heel levels in order to optimize electrical stimulation. Anaesthetized rat was placed supine in a home-built cradle especially designed for the strictly non-invasive functional investigation of the right gastrocnemius muscle (Giannesini et al., 2005). This cradle integrates a hydraulic ergometer and two rod-shaped transcutaneous electrodes (located above the knee and under the heel respectively) connected to an electrical stimulator (Stimu- lator I series, Hugo Sachs Elektronik, Harvard Apparatus, USA). The foot was positioned on the ergometer pedal and the hindlimb was immobilized in the cradle. In this position, the belly of the gastrocnemius muscle was located above an elliptic (10×16 mm) $^{31}$P-MRS surface coil. The pedal position was adjusted in order to modify the angle between the foot and the lower hindlimb so that the gastrocnemius muscle was passively stretched at rest and produced a maximum isometric twitch tension in response to supramaximal square wave pulses (6–8 mA, 1 ms duration).

Throughout the experiment, anesthesia was maintained by gas inhalation with a facemask continuously supplied with 2.5% isoflurane in 33% $O_2$ (0.4 L/min) and 66% $N_2O$ (0.8 L/min). The facemask was connected to an open-circuit gas anesthesia machine (Isotec 3; Ohmeda Medical, Herts, UK). Exhaled and excess gases were removed through a canister filled with activated charcoal mounted on an electrical pump extractor (Equipement Vétérinaire Minerve, Esterney, France). Corneas were protected from drying by application of ophthalmic cream (Lacrigel®; Europhta, Monaco). During anesthesia, animal body temperature was maintained through a feedback loop including an electrical heating blanket (Prang+Partner AG, Pfunz, Switzerland), a temperature control unit (ref 507137; Harvard Apparatus, Holliston, Massachusetts, USA) and a rectal probe (ref 507145; Harvard Apparatus).

2.4. Stimulation protocol and force measurement

Muscle contractions were electrically induced with square-wave pulses (6–8 mA, 1 ms duration). The stimulation protocol consisted in 5.7 min of repeated isometric contractions at a frequency of 3.3 Hz. Electrical signal coming out from the pressure transducer was amplified (reference: 13-4515-50, Gould, USA), converted to a digital signal and processed on a personal computer using ATS software (SYSMA, Aix-en-Provence, France). Isometric force production was calculated for each 14.25 s period of stimulation by integrating isometric tension (N) with respect to time (s).

2.5. MR data acquisition data processing

Investigations were performed in a 4.7 Tesla horizontal superconducting magnet (47/30 Biospec Avance, Bruker, Germany). $^{31}$P-MR spectra (16 accumulations: 1.8 s repetition time; 8 kHz spectral width, 512 data points) from the gastrocnemius muscle region were continuously acquired in 28.5-s blocks throughout the experimental protocol, i.e., 5.7 min of rest, 5.7 min of stimulation and 16.6 min of post-stimulation recovery. MR data acquisition was gated to muscle stimulation in order to reduce potential motion artifacts due to contraction.

MR data were processed using a proprietary software developed using IDL (Interactive Data Language, Research System Inc., Boulder, Colorado, USA). Relative concentrations of phosphorylated compounds were obtained by a time-domain fitting routine using the AMARES-MRUI Fortran code (Vanhamme et al., 1997). Signal areas were corrected for magnetic saturation using fully relaxed spectra collected at rest with a repetition time of 20 s. Absolute concentrations of phosphorylated compounds were expressed relative to a resting ATP concentration (5.1 mM) determined from HPLC assays in extracts of freeze-clamped gastrocnemius muscle samples (Giannesini et al., 2007). We calculated pH from the chemical shift difference between $P_i$ and phosphocreatine (PCr) peaks (Arnold et al., 1984). Time-points for the time course of phosphorylated metabolite concentrations and pH were assigned to the midpoint of the acquisition interval. The rate constant ($k_{rec}$) of PCr resynthesis during the post-stimulation period was determined by fitting the PCr time-dependent changes during this period to a single exponential curve described by the equation: $[PCr]=|PCr|_{rest}−|PCr|_{cons}e^{−k_{rec}t}$, where $[PCr]_{rest}$ and $|PCr|_{cons}$ are respectively the concentration of PCr measured at rest and the difference between $|PCr|_{rest}$ and the PCr concentration measured at end of the stimulation period.

2.6. Statistical analysis

For variables changing with respect to time during the stimulation period (isometric force, metabolite concentrations and pH), the effect of LPS treatment on the overall time-course was analyzed with repeated-measurements ANOVAs using JMP software (SAS Institute
Inc., Cary, North Carolina, USA). Other variables were compared with Student’s *t*-test. Values are means±S.E.M. In all statistical analyses, the 0.05 level of significance was used.

### 3. Results

#### 3.1. Clinical outcomes

Endotoxin treatment induced a number of pathological features such as decreased sensitivity to touch, piloerection, behavioral depression, lethargy and diarrhea. Body temperature and body mass did not differ between both groups before treatment (Table 1). LPS injections were associated to a significant increase in body temperature (+0.7±0.1 °C and +0.4±0.1 °C in vehicle and CM group, respectively) and a reduction of body mass (−6.1±0.6% and −8.3±0.7% in vehicle and CM group, respectively).

#### 3.2. Muscle force measurements

Before the endotoxin injections, one-way repeated-measurements ANOVA indicated that the time-course of force production did not differ between both groups (*P*=0.74) throughout the whole stimulation period (Fig. 1). For each group, force decreased throughout the stimulation period as a sign of fatigue. At the end of the stimulation period, the force output did not differ between groups, reaching 59.1±6.1% and 55.1±6.3% for vehicle and CM group respectively. In both groups, LPS injections did not alter the extent of relative force at the end of the stimulation period. However, two-way repeated-measurements ANOVA indicated that the time-course of force production throughout the stimulation period was reduced in the vehicle group (*P*=0.025) whereas it was not altered in the CM group (*P*=0.054).

#### 3.3. Muscle energy metabolism

Before endotoxemia, pH$_i$ and PCr/ATP ratio at rest did not differ between groups (Table 2). During the stimulation period, there were no differences between both groups regarding the time-courses in [PCr] (*P*=0.372; Fig. 2A), [ATP] (*P*=0.690; Fig. 2B) and pH$_i$ (*P*=0.533; Fig. 2C). PCr was rapidly consumed at the onset of the stimulation period and reached a steady-state after 1.5 min of stimulation (Fig. 2A). This level was maintained until the end of the stimulation period, when PCr levels were 45.8±3.6% and 45.6±2.4% of their basal value for the vehicle and CM groups respectively. In addition, the rate constant of PCr recovery ($k_{rec}$) was similar between both groups (Table 2). At the same time, [ATP] decreased slightly throughout the stimulation period (Fig. 2B). For each group, pH$_i$ fell rapidly in the early stage of the stimulation period and reached a minimum value 3 min after the onset of stimulation (Fig. 2C). Then, pH$_i$ slowly increased and reached, at end

### Table 1

<table>
<thead>
<tr>
<th></th>
<th>Vehicle before treatment</th>
<th>Vehicle + LPS</th>
<th>CM before treatment</th>
<th>CM + LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body temperature (°C)</td>
<td>36.5±0.1</td>
<td>37.2±0.1</td>
<td>36.7±0.1</td>
<td>37.2±0.1</td>
</tr>
<tr>
<td>Body mass (g)</td>
<td>306±4</td>
<td>287±5</td>
<td>322±7</td>
<td>290±5</td>
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</tbody>
</table>

Values are means±S.E.M.  
*P*<0.05 versus vehicle before treatment.  
*P*<0.05 versus CM before treatment.

### Table 2

<table>
<thead>
<tr>
<th></th>
<th>Vehicle before treatment</th>
<th>Vehicle + LPS</th>
<th>CM before treatment</th>
<th>CM + LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal pH$_i$</td>
<td>7.04±0.01</td>
<td>7.04±0.01</td>
<td>7.05±0.01</td>
<td>7.04±0.01</td>
</tr>
<tr>
<td>Basal [PCr]/[ATP]</td>
<td>3.68±0.06</td>
<td>3.22±0.09</td>
<td>3.51±0.12</td>
<td>3.20±0.12</td>
</tr>
<tr>
<td>$k_{rec}$ (min$^{-1}$)</td>
<td>0.35±0.02</td>
<td>0.43±0.03</td>
<td>0.36±0.02</td>
<td>0.35±0.03</td>
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Values are means±S.E.M. $k_{rec}$, rate constant of PCr resynthesis during the post-stimulation period.  
*P*<0.05 versus vehicle before treatment.
of the stimulation period, a value of 6.45±0.03 in the vehicle group and 6.48±0.04 in the CM group.

Endotoxin injections did not disturb the basal pH$_i$ value whatever the group but led to a significant reduction in the PCR/ATP ratio in the vehicle group (Table 2). On the contrary, the basal PCR/ATP ratio remained unchanged when the LPS treatment was accompanied by an oral administration of CM. Throughout the stimulation period, endotoxemia did not affect the [ATP] time-course whatever the group ($P=0.153$ and $P=0.501$ for vehicle and CM groups respectively) (Fig. 2B). On the contrary, the [PCR] time-course was significantly altered by LPS injections in both groups ($P=0.0015$ and $P=0.011$ for vehicle and CM groups respectively) (Fig. 2A). At the end of the stimulation period, PCR levels were $37.2±1.2\%$ and $37.1±2.1\%$ in the vehicle and CM group respectively. In addition, $k_{rec}$ was reduced in the vehicle group but remained unchanged in CM-administered animals (Table 2). Endotoxemia strongly affected the pH$_i$ time-course in the vehicle group ($P=0.0005$; Fig. 2C). In this group, the pH$_i$ recovery recorded during the second part of the stimulation session was enhanced when compared to the pre-endotoxemia time-course so that the end of the stimulation value was $6.68±0.03$. On the contrary, the pH$_i$ kinetics was not affected by endotoxemia in CM animals ($P=0.142$) and the end-of-stimulation pH$_i$ value was $6.63±0.05$.

4. Discussion

The present work reported for the first time results related to repeated investigations of muscle function in contracting rat gastrocnemius muscle using $^{31}$P-MRS. We showed that CM supplementation in endotoxemic rats prevented the basal PCR/ATP ratio reduction and normalized the pH$_i$ time-course during muscular activity as a sign of an effect at the muscle energetics level. In addition, CM treatment avoided the endotoxemia-induced decline in developed force.

4.1. CM effect in resting muscle

The increased body temperature and the body mass reduction we reported in the vehicle group as a result of endotoxemia is comparable with results of experiments conducted in animal models of sepsis and endotoxemia (Angeras et al., 1991; Brealey et al., 2004; Mizobata et al., 1995; Verleye et al., 1995). It is noteworthy that CM supplementation had no protective effect on these parameters, as previously reported in endotoxemic rats (Verleye et al., 1995).

In agreement with previous studies, we measured a significant reduction in basal PCR/ATP ratio resulting from LPS injections in the vehicle group (Giannesini et al., 2007; Jacobs et al., 1991; Jacobs et al., 1988; Lara et al., 1998; Mizobata et al., 1995). This alteration in basal energy metabolism could be due to a failure in oxygen supply. Cellular hypoxia and abnormal microvascular control of oxygenation have indeed been reported in endotoxemic rats (Anning et al., 1999; Sair et al., 1996). However, similarly to previous in vivo $^{31}$P-MRS studies in septic rats (Jacobs et al., 1991; Jacobs et al., 1988; Mizobata et al., 1995), we did not measure at rest any intracellular acidosis, a marker of cellular hypoxia. Thus, we can dismiss an impaired oxygen supply as an accounting factor of the reduced PCr/ATP ratio.

Interestingly, CM supplementation did not affect muscle oxidative activity thereby indicating that, in LPS-treated animals, malate supply did not accelerate the TCA intermediate pool increases very quickly and several fold at the start of exercise in skeletal muscle (Gibala et al., 1997). If the TCA intermediates pool would not increase, the flux through the TCA cycle would decrease, thereby contributing to muscle fatigue development as previously suggested (Sahlin et al., 1990; Wagenmakers, 1998). However, we clearly showed that CM supplementation did not affect muscle oxidative activity thereby indicating that, in LPS-treated animals, malate supply did not accelerate the TCA cycle energy production. Then, the limitation of force decline in CM group cannot be linked to any increase in oxidative ATP supply.

Malate is a TCA intermediate and its supply can affect oxidative ATP production through anaplerotic reactions (Gibala et al., 2000) allowing the replenishment of the TCA intermediates pool (Sahlin et al., 1990). Studies in humans and animals have shown that the total TCA intermediate pool increases very quickly and several fold at the start of exercise in skeletal muscle (Gibala et al., 1997). If the TCA intermediates pool would not increase, the flux through the TCA cycle would decrease, thereby contributing to muscle fatigue development as previously suggested (Sahlin et al., 1990; Wagenmakers, 1998). Interestingly, among the TCA intermediates, malate shows the largest relative and absolute change during exercise likely in order to allow high rates of ATP production through the TCA cycle (Sahlin et al., 1990; Wagenmakers, 1998). However, we clearly showed that CM supplementation did not affect muscle oxidative activity thereby indicating that, in LPS-treated animals, malate supply did not accelerate the TCA cycle energy production. Then, the limitation of force decline in CM group cannot be linked to any increase in oxidative ATP supply.

Citrulline is involved in the pathway of nitric oxide (NO) synthesis, which is linked to the urea cycle metabolism via the synthesis of L-citrulline from L-arginine. Oral ingestion of citrulline is known to increase NO production (Hayashi et al., 2005) and interestingly, an enhanced NO production has been shown to accelerate glucose uptake into skeletal muscle cells during exercise (Kingwell et al., 2002). Such an uptake could affect muscle energy metabolism and explain the metabolic events we observed in the CM-treated animals under endotoxemia. In the vehicle group, endotoxemia reduced acidosis during the stimulation period. Yet, the pH drop during muscular contraction is linked to an increased glycolytic activity (Hochachka and Sommense, 1983). Then, considering that glycolytic activity depends on available glycogen content, we could hypothesize that the reduced acidosis in vehicle would be linked to a decreased
intramuscular glycogen store due to endotoxia as previously suggested (Giannesini et al., 2007). Indeed, in endotoxemic animals, a raised epinephrine production would enhance sarcolemmal Na⁺-
K⁺-ATPase activity thereby accelerating the basal glycolytic flux and leading to a glycogen store reduction (L’Her and Sebert, 2004; Levy et al., 2005; Virkamaki and Yki-Jarvinen, 1994).

On the contrary, in the CM-treated group, endotoxia did not affect the time-course of pH. This normalizing effect of CM on pH indicates that CM would protect skeletal muscle from basal glycogen depletion due to endotoxia. The corresponding mechanism might be linked to a limitation of the sarcolemmal Na⁺-K⁺-ATPase activity. Another possibility would be that CM supplementation would increase glucose uptake, thereby leading to an increased glycolytic activity and a corresponding larger stimulation-induced acidosis.

4.3. Conclusion

CM supplementation prevents the decline in muscle performance and normalizes energy metabolism in LPS-treated animals thereby illustrating the inhibition of the deleterious effect of endotoxia on muscle function. Although the exact mechanism has still to be elucidated, our results demonstrate that CM administration efficiently limits muscle dysfunction under asthma.

Acknowledgments

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