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Effect of Lipid Infusion on Metabolism and Force of Rat Skeletal Muscles During Intense Contractions

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Key Words

Skeletal muscle • Free fatty acids • UCP-3 and glycolysis

Abstract

The hypothesis that during intense muscle contraction induced by electrical stimulation, long chain fatty acids (LCFA) might reduce mitochondrial ATP/ADP ratio, raising the contribution of glycolysis for ATP production was examined. The effect of a lipid infusion (Lipovenus emulsion) on UCP-3 mRNA level, lactate, glucose-6-phosphate (G-6P) and glycogen content was investigated in rat. Blood samples for determination of free fatty acids and lactate were collected at 0, 30 and 60 min during rest and at 0, 10 and 20 min during muscle contraction. The content of lactate, glycogen and G-6P was also determined in soleus (SO), red gastrocnemius (RG) and white gastrocnemius (WG) muscles collected immediately after muscle contraction period. In addition, the force level was determined during muscle contractions. The effect of Lipovenus emulsion on respiration of mitochondria isolated from rat skeletal muscle, and content of UCP-3 and lactate in cultured skeletal muscle cells was also determined. The *in vivo* experiments showed that Lipovenus induced a significant increase of UCP-3 mRNA levels. After Lipovenus infusion, lactate level was increased in RG muscle only, whereas the contents of glycogen and G-6P were decreased in both RG and WG muscles ($P < 0.05$). Lipovenus infusion failed to exert any effect on muscle force performance ($P > 0.05$). The *in vitro* experiments showed that Lipovenus infusion induced a significant increase in mitochondrial respiration, but had no effect on UCP-3 content. Lactate concentration was significantly increased in the culture medium of stimulated cells in the control and Lipovenus groups compared with the respective not-stimulated cells ($P< 0.05$). We concluded that as mitochondrial function becomes limited by the FFA-uncoupling effect, the ATP demand is mainly supplied by anaerobic glucose metabolism preventing an expected decrease in muscle contraction performance.

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Introduction

In the classical glucose-fatty acid cycle, Randle and colleagues [1, 2] postulated that an increase in fatty acid availability and oxidation decreases glucose oxidation in cardiac and skeletal muscles. This mechanism was supported by an elevated acetyl-CoA content as a consequence of high rate of fatty acid oxidation followed by an increase in the intracellular contents of citrate and glucose-6 phosphate (G-6P). Acetyl-CoA inhibits pyruvate dehydrogenase complex (PDH) activity via activation of PDH kinase, the enzyme responsible for phosphorylation and inactivation of PDH. On the other hand, citrate inhibits the activity of the rate-limiting enzyme of glycolysis phosphofructokinase (PFK). This latter effect increases the G6-P/fructose-1,6-bisphosphate (F1,6-P2) ratio inhibiting hexokinase, the first enzyme of the glycolytic pathway.

The above mechanism may explain the fact that during low to moderate exercise skeletal muscle utilizes mainly fatty acids as fuel with low carbohydrate oxidation. Under this condition, the rate of lipolysis and free fatty acid (FFA) availability are elevated favoring fatty acid oxidation [3-7]. In contrast, during high-intensity exercise, glucose is the major fuel [5, 8, 9, 10]. In cyclists, the rate of fatty acid oxidation measured by constant infusion of trace amounts of a long chain fatty acid (1-^{13}C-palmitate) vs. medium chain fatty acid (1-^{13}C-octonoate) showed that elevated glycolytic flux reduces palmitate oxidation with no effect on octonoate [11]. The reduced oxidation of long chain fatty acids (LCFA) seems to be associated with acetyl-CoA carboxylase (ACC) activation raising malonyl-CoA formation, which is a known inhibitor of carnitine palmitoyltransferase-I (CPT-I) activity, with no effect on medium chain fatty acid transport into mitochondria [3, 8, 11]. In addition, an altered recruitment of slow-twitch oxidative to fast-twitch glycolytic fibers observed during intense exercise favors lactate accumulation, an inhibitor of fatty acid oxidation [12-14]. Interestingly, when fatty acid availability is artificially increased during intense exercise its oxidation remains lower compared with low and moderate exercise [15]. Therefore, during high energy demand, carbohydrate seems to be the major substrate for skeletal muscle improving energy generation efficiency [16]. Thus, fatty acid availability during intense muscle contraction may lead to a substantial accumulation of this metabolite in the skeletal muscle [11]. A high fatty acid supply is also recognized to increase uncoupling protein- 3 (UCP-3) activity in skeletal muscle [17, 18]. UCP-3 is a mitochondrial uncoupling protein being predominantly expressed in type-II skeletal muscle fibers in both rodents and humans [19]. However, its physiological role in skeletal muscle remains to be established. This protein seems to play a role in energy net production by uncoupling oxidative phosphorylation, resulting in a reduced oxidative ATP synthesis [20, 21] and increased fuel requirement [22, 23]. Early studies in which UCP-3 was overexpressed in cultured cells or yeast supported the proposed uncoupling function, showing a lowered mitochondrial membrane potential (1) and an elevated basal oxygen consumption in state 4. However, under these conditions, mitochondrial respiration is not completely inhibited by purine nucleotides, suggesting that an UCP-3 –independent uncoupling mechanism might exist [24, 25]. Recently, we demonstrated that LCFA acutely lead to mitochondrial uncoupling in 1h- incubated rat skeletal muscles [26].In this study, the UCP-3 inhibitor, GDP, had no effect on mitochondrial respiration, which supports the proposition for an UCP-3 independent acute effect of fatty acids. In addition to this effect, LCFA increased glucose oxidation in both extensor digitorium longus (EDL) and soleus muscles. The latter muscle is expected to exhibit low UCP activity, confirming the proposition of a possible UCP-3 –independent uncoupling mechanism. In line with this proposition, other studies have observed the same phenomenon in liver mitochondria from hypertriglyceridemic mice [27, 28]. UCP-3 has also been described as an important regulator of mitochondrial superoxide production and cell death in skeletal muscle [29, 30].

Treatment of L6 myotubes with mitochondrial uncoupling DNP (2,4-dinitrophenol) increased lactate production, decreased the oxidative phosphorylation and raised the contribution of glycolysis for ATP formation [22]. The information above led us to postulate that, during intense muscle contraction, LCFA might reduce mitochondrial ATP/ADP ratio by an UCP-3 independent mechanism, as indicated by the treatment with GDP, raising the contribution of glycolysis to maintain an adequate fuel supply for muscle contraction. To test this hypothesis, we examined in rats the effect of a single infusion of lipid emulsion on: a) intramuscular contents of lactate, glucose-6-phosphate (G6-P), citrate and glycogen; b) expression of UCP-3, and c) muscle force during 20 min of intense contraction induced by electrical stimulation. We also examined in cultured cells isolated from rat skeletal muscles the effect of lipid emulsion on: a) rat mitochondrial respiration; b) UCP-3 content, and c) lactate content.

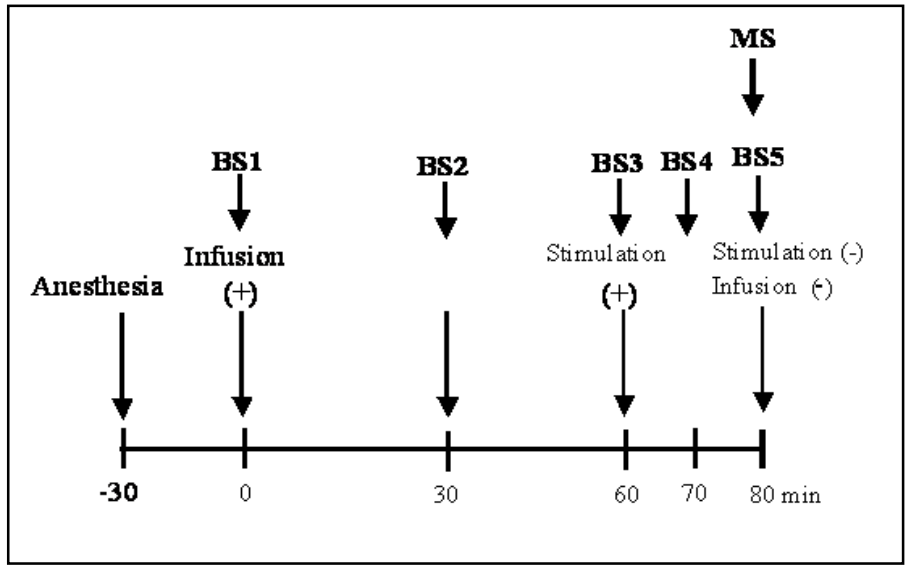


Fig. 1. Design of the experimental protocol used in this study. Blood (BS) and muscle samples (MS) are indicated by arrows. The symbols (+) and (-) denote the beginning and the end of either infusion or electrical stimulation, respectively. Blood samples were collected at 0, 30 and 60 min during rest infusion period and at 0, 10 and 20 min during muscle contraction period in both groups. Soleus (SO), red gastrocnemius (RG) and white gastrocnemius (WG) muscles were collected immediately after the end of the contraction period.

Materials and Methods

In vivo experiments

The *in vivo* experimental procedures were approved by the Ethical Committee of the Institute of Biomedical Sciences, University of São Paulo. Sixteen Male Wistar rats weighing 300 ± 6 g were maintained on a 12:12 h light-dark cycle at 23° C with free access to food and water.

The rats were divided into 2 groups of 8 animals each: 1) control; infused with saline and 2) experimental; infused with Lipovenus. The animals were anesthetized by intraperitoneal injection of pentobarbital sodium (5 mg/100 g body wt) and surgically prepared for either Krebs Ringer solution (KR) or lipid emulsion (Lipovenus) infusion. The skin and subcutaneous tissues were removed and jugular vein exposed through a midline ventral incision of approximately 0.5 cm length in the neck. A 12 cm cannula (Tygon I.D. 0.05 mm) was inserted into the jugular vein approximately 1.5 cm deep. After insertion, the cannula was attached to a perfusion pump (model 102, Corning) and KR or Lipovenus was pumped (8 µL/min) throughout the experiment. The KR solution contained (in mM): NaCl, 118; $NaHCO_3$, 24; KCl, 5; $MgSO_4$, 1; KH_2PO_4, 1; $CaCl_2$, 2.5; pH 7.3 was equilibrated with 95% O_2 - 5% CO_2 and the temperature was kept at 37° C. The Lipovenus solution (20%, Fresenius Kabi, Campinas, Brazil) is a soybean oil-based emulsion rich in essential polyunsaturated fatty acids; linoleic and α-linolenic acids representing about 60% of the total fatty acids [31]. The composition of Lipovenus emulsion was determined by thin layer chromatography [32] using as a standard mixture of lipids containing cholesterol (18.72%), free fatty acids (28.45%) and triacylglycerol (52.82%). The amount of Lipovenus after infusion was roughly calculated in 2% of the blood volume.

One leg was surgically prepared for electro-stimulation of the sciatic nerve and fixed on the perfusion platform as previously described [33]. The leg was further connected to an isometric force transducer (Narco Bio-System, Houston, USA). The resting length (L0) and the stimulation voltage (V0) were adjusted, so that maximum force level was reached during contraction. Muscle contractions were induced by intense electro-stimulation during 20 min. The protocol consisted of trains of 200 ms delivered every 1s. The impulse frequency and duration within the train were of 100 Hz and 0.1 ms, respectively [33].

Blood and muscle analysis

Blood (100 µL) samples were collected at 0, 30 and 60 min during rest infusion period and at 0, 10 and 20 min during muscle contraction period in both groups as described in detail in the Figure 1 legend. The samples were collected in tubes containing EDTA and immediately centrifuged at 4,000 rpm for 10 min at 4° C. Plasma was frozen in liquid nitrogen and stored at -80° C for determination of free fatty acids [34] and lactate [35]. Samples from soleus (SO), red gastrocnemius (RG) and white gastrocnemius (WG) muscles were collected immediately after the end of the contraction period. The samples were freeze-dried, dissected and divided in two aliquots. One aliquot (50 mg) was homogenized in percloric acid (0.6 N) using a dilution ratio of 1:6 (w/v) and the contents of lactate [35], G6-P [36] and citrate [37] were spectrophotometrically determined. Total glycogen content of the muscles was determined as described by Leighton et al. [38] and Hirabara et al. [39]. The another aliquot was used for total mRNA extraction.

Real time PCR

UCP-2 and -3 expression was evaluated by real-time PCR [40] using a ROTOR GENE 3000 equipment (Corbett Research, Mortlake, Australia). Total RNA was obtained from 50-100 µg of soleus, red gastrocnemius and white gastrocnemius muscles using Trizol reagent™ (Invitrogen Life Technologies, Rockville,

Genes	Primer sense	Primer anti-sense	Annealing temperature
G3PDH	5′-*ACCACAGTCCATGCCATCAC*-3′	5′-*TCCACCACCCTGTTGCTGTA*-3′	60°C
UCP 2	5′- *TCCTGAAAGCCAACCTCATGA*-3′	5′-*CAATGACGGTGGTGCAGAAG*-3′	56°C
UCP 3	5′-*AGCAGTTCTACACCCCCAAAGG* -3′	5′-*TTTACCACATCCACTGGGGAGG*-3′	59°C

Table 1. Primer sequences and temperatures used for UCP-3 and G3PDH mRNA analysis. G3PDH = glyceraldehyde 3-phosphate dehydrogenase; UCP = uncoupling protein.

MD, USA), as previously described [41]. Briefly, muscles were lysed using 1 mL Trizol reagent and then, after 5 min incubation at room temperature, 200 μL chloroform were added to the tubes and centrifuged at 12.000 x g. The aqueous phase was transferred to another tube and the RNA was pelleted by centrifugation (12,000 x g) with isopropyl alcohol. RNA pellet was washed with ethanol (75%) by centrifugation at 7,500 x g for 5 min and dried in air. RNA pellets were eluated in RNase-free water and treated with DNase I. Afterwards, RNA was stored at -80°C until to the reverse transcription reaction being performed. RNA was quantified by measuring absorbance at 260 nm. The purity of the RNA preparations was assessed by the 260/280 nm ratio and on a 1% agarose gel eletrophoresis stained with ethidium bromide at 5 μg/mL [42].

cDNA probes were synthesized using 4 μg of total RNA and a mixture of the following reagents: 146 ng of random primers, 200 U of reverse transcriptase (Invitrogen Life Technologies, Rockville, MD, USA), reaction buffer 5x (50 mM Tris-HCl, pH 8.0, 75 mM KCl, 3 mM $MgCl_2$), 5 mM DTT, 500 μM dNTP in a final volume of 20 μL. The reaction was incubated for 2 min, at 25°C, assembling the oligonucleotides and RNA hybridization, followed by heating at 42°C for 50 min. cDNA was stored at -20°C prior to the real time PCR assay. To perform the real time PCR reaction, 1 μL of cDNA was used in a final volume of 25 μL, containing 100 μM of dNTPs, reaction buffer 10x (10 mM Tris-HCl, 50 mM KCl, 2 mM $MgCl_2$), 1 U of Taq DNA polimerase (Invitrogen Life Technologies, Rockville, MD, USA), 0.1 μM of each primer (sense and antisense), and SYBR GREEN (25,000 x diluted) (Invitrogen, Carlsbad, CA, EUA) as fluorescent dye. The sequences of the primers were designed using information contained in the public database in the GeneBank of the National Center for Biotechnology Information (NCBI) (Table 1).

Quantification of gene expression was carried out as described by Liu and Saint [43], using glyceraldehyde 3-phosphate dehydrogenase (G3PDH) gene as inner control. Amplification efficiency was calculated as described by Ramakers *et. al.*[44].

Effect of Lipovenus acid on O_2 consumption by anesthetized rats

Oxygen consumption was measured through an indirect open circuit calorimeter (Columbus Instruments' Oxymax Deluxe System, Ohio, USA). This system monitors O_2 by volume at the inlet and outlet ports of a chamber through which a known flow of air is being forcibly ventilated. The flow rates were adjusted according to the animal weights to maintain the changes in the composition of the expired gases less than 0.05%. The flow meter was previously calibrated with gases of known concentrations. Rats were anesthetized with sodium pentobarbital (50 mg per kg body weight, intraperitoneal injection) and surgically prepared as described before. After 30 min, the O_2 consumption was measured under basal condition without Lipovenus. After then, Lipovenus was infused during 60 min at (8 μL/min) and the O_2 consumption measured again. In the control experiment, KR infusion did not change the basal O_2 consumption (data not shown).

In vitro experiments

A) Isolation of skeletal muscle mitochondria. Mitochondria were isolated from control animals (300g, b.w.). Briefly, hindlimb skeletal muscles were quickly removed and homogenized in ice-cold medium containing 100 mM sucrose, 100 mM KCl, 50 mM Tris-HCl, 1 mM K_2HPO_4, 0.1 mM EGTA, and 0.2% bovine serum albumin (BSA), pH 7.4, followed by differential centrifugation [45]. The final mitochondrial pellet was resuspended in cold buffer containing 0.2 M mannitol, 0.1 M sucrose, 10 mM Tris-HCl, 0.1 mM EDTA, at pH 7.4. The protein concentration was usually around 30-40 mg/mL, as determined by a modified Biuret method [46]. The presence of BSA (0.2%) in the buffers throughout the isolation procedure depleted the mitochondria of endogenous fatty acids [47].

B) Determination of mitochondrial respiration. Mitochondrial respiration was measured using a Clark-type electrode (Hansatech Instruments, Ltd, using the software OXYGRAPH V1.10, England) equipped with magnetic stirring, in 1 ml of standard incubation medium (28°C) containing: 225 mM mannitol, 75 mM sucrose, 10 mM Tris-HCl, pH 7.4, 10 mM KH_2PO_4, 0.1 mM EDTA, 5 mM NAD^+-linked substrates (α-ketoglutarate, pyruvate, malate, and glutamate), 1 μg/mL oligomycin (ATP-synthase inhibitor) and 1 μM carboxyatractyloside (CAT, adenine nucleotides carrier inhibitor). Additions of Lipovenus (1%), 2 mM guanosine di-phosphate (GDP, UCP inhibitor) and 0.05% BSA were performed as indicated in the Figure 4. The respiratory control ratio (state-III/state-IV respiration rate) was over 4.0. Respiration rates are given in nmoles O_2/mg protein/min.

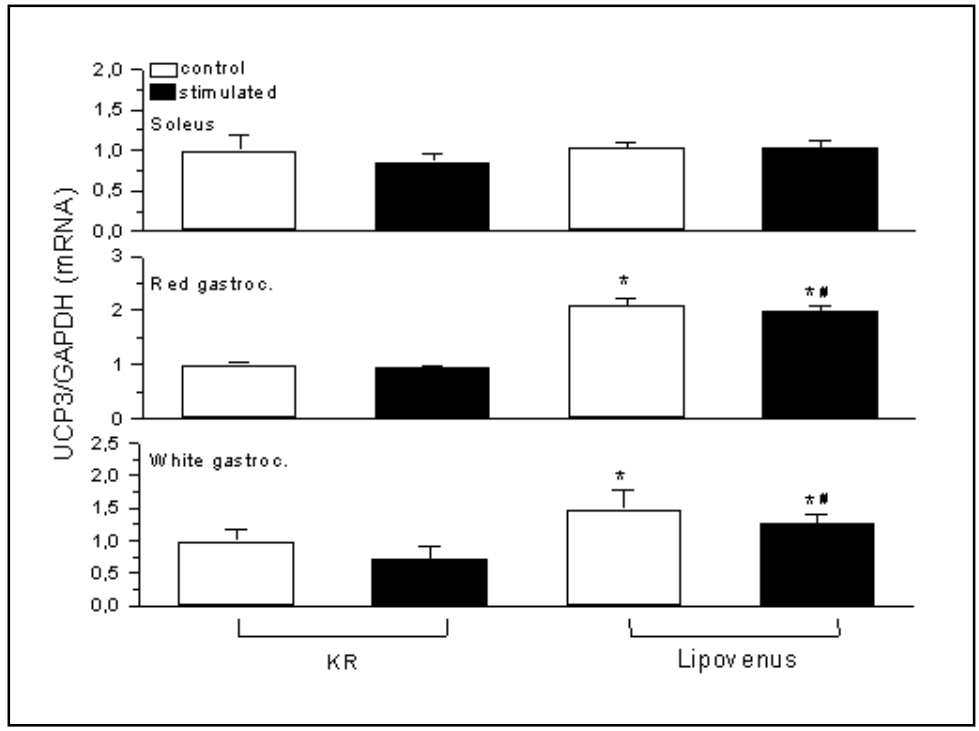


Fig. 2. Effect of intense electro-stimulation on UCP-3 mRNA level in SO, RG and WG muscles after Lipovenus or KR perfusion. The animals were infused during 60 min and the leg muscles were then electro-stimulated for 20 min. Gene expression was determined by comparative cycle threshold method, using G3PDH gene expression as inner control. The values are relative to control set as 1 and presented as mean ± SEM (n = 6). * $p < 0.05$ as compared with KR control and # $p < 0.05$ as compared with stimulated KR.

Rat skeletal muscle cell culture

For culture, skeletal muscle cells where isolated from control male Wistar rats (~150 g, b.w.). The rats were killed by cervical dislocation and the hindlimbs were quickly removed and used to prepare muscle cell culture as previously described by Lynge et al. [48]. The cells were cultured in DMEM with 10% horse serum.

Effect of Lipovenus on UCP-3 protein levels of rat skeletal muscle cells in culture

After being cultured, muscle cells were incubated during 80 min in KR equilibrated with 95% O_2 - 5% CO_2 mixture at 37° C, containing Lipovenus (2%). Afterwards, the cells were scraped off and frozen in liquid N_2 and homogenized in 0.5 mL extraction buffer (100 mM Trizma, pH 7.5; 10 mM EDTA; 100 mM NaF; 10 mM sodium pyrophosphate; 10 mM sodium orthovanadate; 2 mM phenylmethanesulfonyl fluoride; 0.01 mg/mL aprotinin; at 4 °C) for 30 s. Triton X 100 was added at 1% and the samples incubated for 30 min at 4°C. Samples were centrifuged at 13,000×*g* for 20 min, at 4°C. Aliquots of supernatants were used for the measurement of total protein content. Equal amounts of proteins of each sample (10 µg) were separated by using 15% SDS-gel polyacrylamide [49]. Western blotting was carried out following the method described by Towbin et al. [50]. The proteins of the gel were transferred to a nitrocellulose membrane at 120 V for 1 h. Non-specific bounds were blocked by incubating the membranes with 5% defatted milk in basal solution (10 mM Trizma, pH 7.5, 150 mM NaCl, 0.05% Tween 20), at room temperature, for 2 h. Membranes were washed 3 times for 10 min each in basal solution and then incubated with anti-UCP-2 (1:1,000 dilution) or anti-UCP-3 (1:500 dilution) antibodies in basal solution containing 3% defatted milk, at room temperature, for 3 h. Membranes were washed again (3 times for 10 min each) and incubated with anti-IgG antibody (1:10,000 dilution) linked to horseradish peroxidase in basal solution containing 1% defatted milk, at room temperature, for 1 h. Following the washings again, membranes were incubated with substrate for peroxidase and chemiluminescence enhancer (ECL Western Blotting System Kit) for 1 min and immediately exposed to X-ray film. Films were then revealed in the conventional manner.

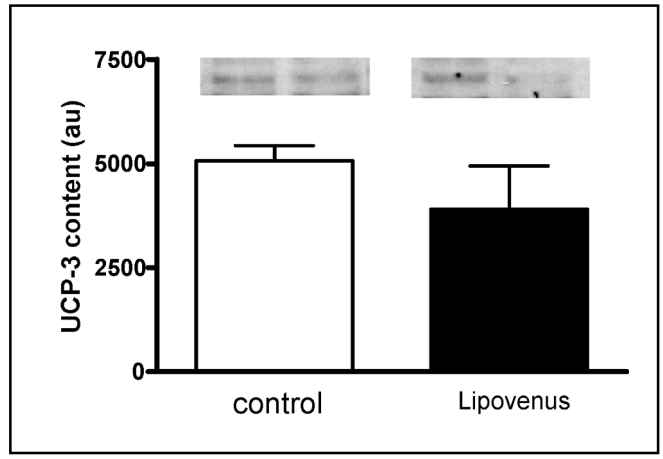


Fig. 3. Representative Western blot of UCP-3 in rat skeletal muscle cells in culture incubated for 80 min with or without Lipovenus. UCP-3 content was determined as decribed in methods. Data are presented as mean ± S.E.M. of 2 experiments ($P > 0.05$).

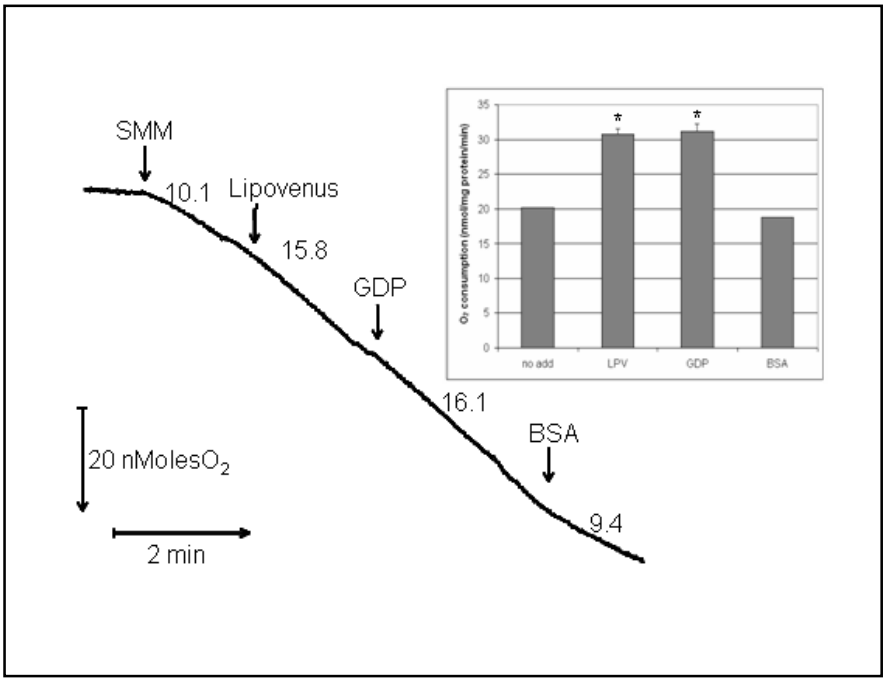


Fig. 4. Effect of Lipovenus on resting respiration during 2 min in isolated skeletal muscle mitochondria (SMM). The addition of Lipovenus (2%), GDP (2 mM) or BSA (0.05 %) are indicated. Respiratory rates in nmoles O_2/mg protein/min. The results are representative of 3 independent experiments. Insert graphic is showing the statistic analysis. * $P < 0.05$ compared to SMM.

Lactate content in cultured rat skeletal muscle cells

The cells were incubated at 37° C in KR solution equilibrated with 95% O_2 - 5% CO_2. Lipovenus (2%) or CCCP (10 µm) were added to the medium and the cells were electrically stimulated for 20 min [33]. Immediately after stimulation, the medium was collected and stored at -80° C for lactate determination [35].

Protein determination

The total protein content of the muscles and muscle cells was measured by the method of Bradford [51] using BSA as standard.

Statistical analysis

The results are presented as means ± SEM. Significance differences into KR and Lipovenus groups were assessed by using paired Student t test. Significances among the groups were tested by using one-way analysis of variance (ANOVA) followed by Tukey *post hoc* test. $P < 0.05$ was considered as significant (Statistics for Windows 4.3).

Results

Muscle UCP-3 and UCP-2 mRNA levels

The KR infusion had no effect on UCP-3 mRNA levels. However, mRNA levels of UCP-3 were significantly increased in RG and WG muscles after Lipovenus infusion in both control and stimulated legs. In SO muscle, UCP-3 mRNA levels were not changed by Lipovenus infusion. UCP-3 mRNA level in stimulated leg from RG and WG muscles after Lipovenus infusion was significantly increased compared to stimulated muscles after KR infusion (Figure 2). UCP2 mRNA levels were not changed by neither KR nor Lipovenus infusion (data not shown).

Effect of Lipovenus on UCP-3 protein content in rat skeletal muscle cells in culture

Lipovenus had no significant effect on UCP-3 protein content in 80 min incubated rat skeletal muscle cells in culture (Figure 3).

Mitochondrial respiration

Resting respiration (non-phosphorylating) of skeletal muscle mitochondria was increased by Lipovenus treatment. The addition of the GDP, as UCP inhibitor, had no effect on mitochondrial O_2 consumption. However, the addition of BSA, a fatty acid chelator, substantially reestablished mitochondrial respiration to the previous level of the Lipovenus treatment (Figure 4).

Effect of Lipovenus on O_2 consumption by anesthetized rats

Lipovenus administration induced a significant increase of basal O_2 consumption when values before and after Lipovenus administration were compared (Figure 5). However, the RER (respiratory exchange ratio) values did not show differences when both conditions were compared (data not shown).

Fig. 5. Muscle force was recorded during 20 min of intense electrical stimulation and the absolute values were obtained through a standard curve with known weights (●) represents the Lipovenus trial (n=8) and (■) represents the KR trial (n=8).

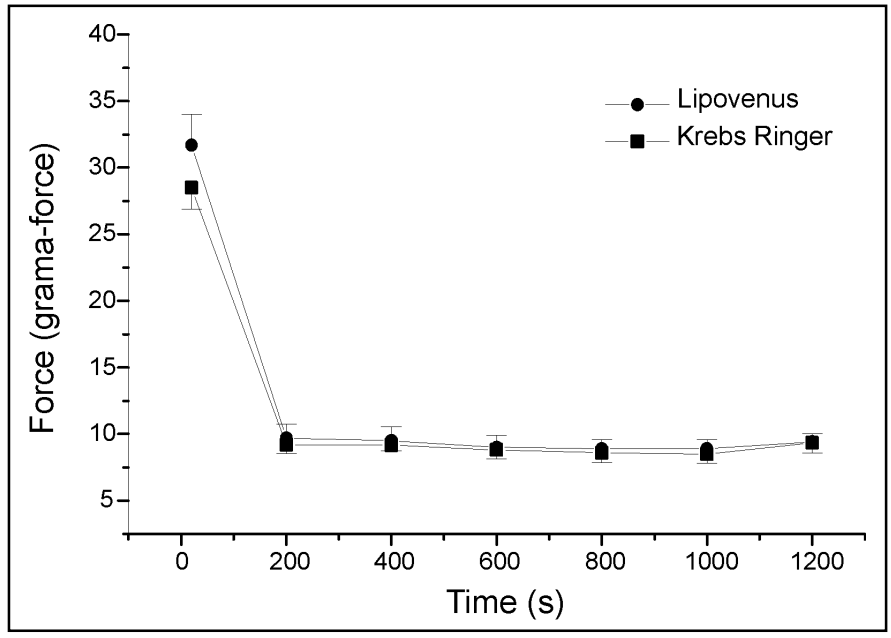
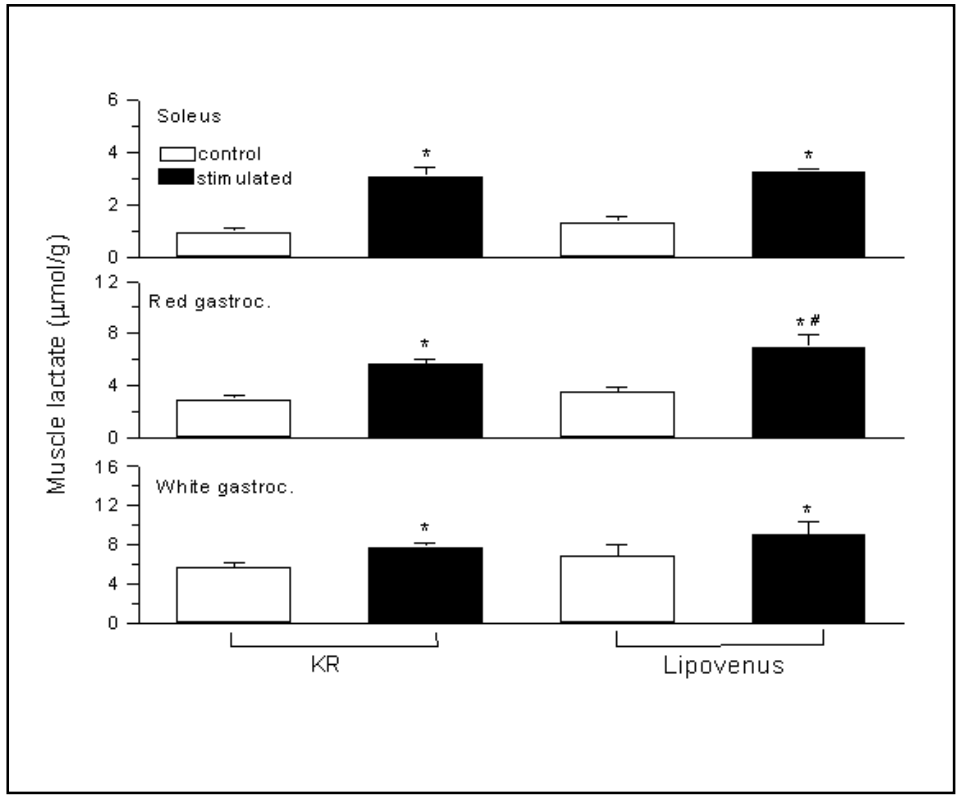


Fig. 6. Effect of intense stimulation by 20 min on muscle lactate content after Lipovenus or Krebs Ringer solution (KR) infusion. The values are presented as mean ± SEM (n = 7). * $p < 0.05$ as compared with control (KR or Lipovenus) and # $p < 0.05$ compared with stimulated KR.



Muscle contraction performance

Muscle force during 20 min of intense contractions showed a rapid decline in the first 200 s and remained unchanged afterwards. The force produced was not statistically significant when KR and Lipovenus treatments were compared (Figure 6).

Muscle metabolite content

A) Lactate. Lactate content was increased in the SO, RG and WG muscles by electro-stimulation in rats that received KR or Lipovenus infusion (Figure 7). However, lactate content of the RG muscle was significant increased by Lipovenus infusion after

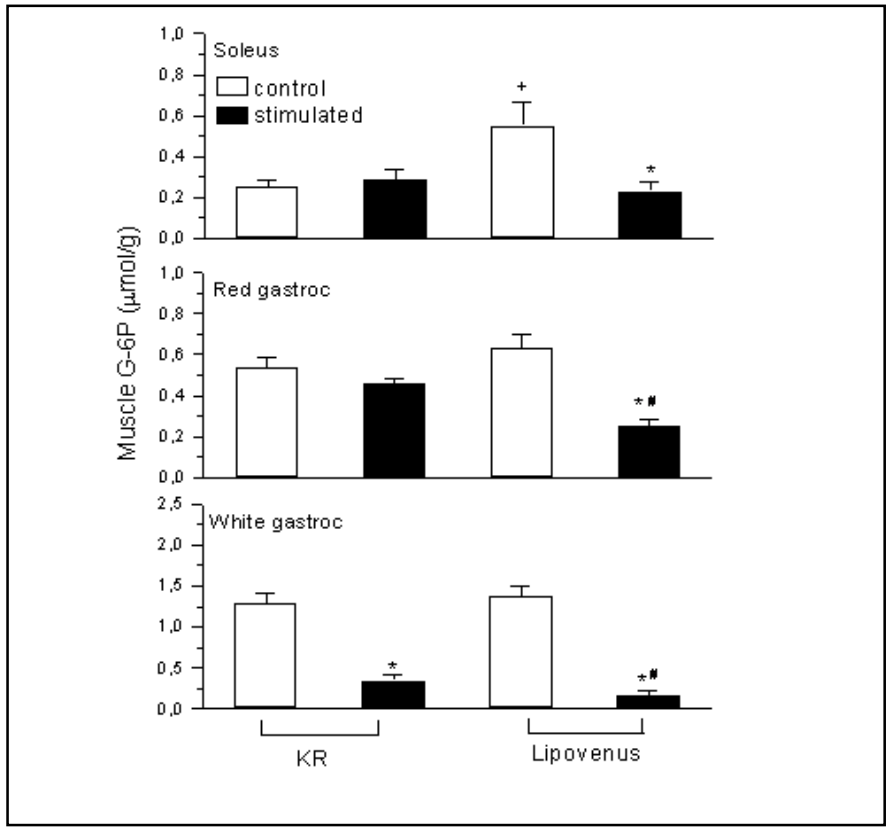


Fig. 7. Effect of 20 min intense stimulation on muscle G-6P content after Lipovenus or Krebs Ringer solution (KR) infusion. The values are presented as mean ± SEM (n = 8). * $p < 0.05$ compared with control (not-stimulated), # $p < 0.05$ compared with stimulated KR, and + $p < 0.05$ compared with not-stimulated KR.

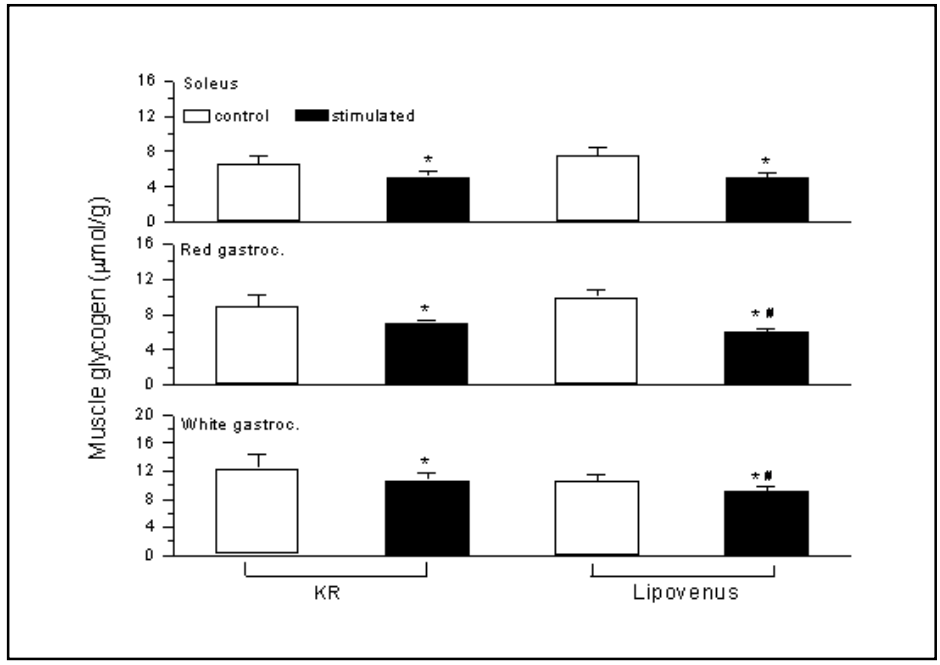


Fig. 8. Effect of 20 min intense electro-stimulation on muscle glycogen content after Lipovenus or Krebs Ringer solution (KR) infusion. The values are presented as mean ± SEM (n = 7). * $p < 0.05$ compared with control (not-stimulated) and # $p < 0.05$ compared with stimulated KR.

stimulation as compared with KR infusion (Figure 7, RG painel).

B) Glucose-6-phosphate (G6-P). G6-P content of the SO muscle was significantly reduced after electro-stimulation compared to control (not-stimulated) in the Lipovenus group. In contrast, G6-P content of the SO muscle in control after Lipovenus infusion was significantly increased compared with control after KR infusion (Figure 8, SO panel). In the RG muscle, G6-P content was significantly reduced by electro-stimulation

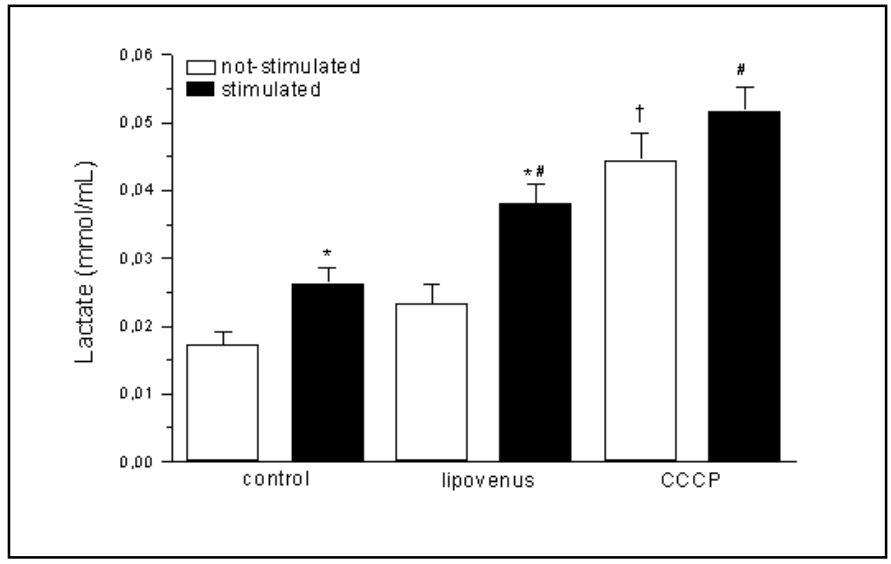


Fig. 9. Effect of electro-stimulation, electro-stimulation and Lipovenus (2%) and electro-stimulation and mitochondrial uncoupling CCCP (carbonyl-cyanide-n-chlorophenylhydrazone, 10 µM) on lactate levels in culture medium of rat skeletal muscle cells after 20 min contractions. The experiments were performed in KR solution containing glucose (5 mM) and O_2 (95%) – CO_2 (5%). The values are presented as mean ± SEM (n = 7). * $p < 0.05$ compared with not-stimulated cells, # $p < 0.05$ compared to stimulated cells from control experiment and † $p < 0.05$ compared to not-stimulated cells from control and Lipovenus.

compared to not-stimulated after Lipovenus treatment. There was no effect in KR infusion (Figure 8, RG panel). G6-P content of the RG stimulated muscle after Lipovenus infusion was significantly reduced compared with stimulated muscle after KR infusion (Figure 8, RG panel). In the WG muscle, G6-P content was decreased by electro-stimulation after infusion of both KR and Lipovenus (* $p < 0.05$). In addition, G6-P content of stimulated WG muscle after Lipovenus infusion was significantly reduced compared with stimulated WG muscle after KR infusion (Figure 8, WG panel).

C) Glycogen. SO, RG and WG muscles exhibited reduced glycogen content in stimulated groups compared to control not-stimulated after KR or Lipovenus infusion, respectively (Figure 9). This effect was significantly lower in stimulated muscles after Lipovenus infusion as compared with stimulated muscle after KR infusion (Figure 9, RG and WG panels).

D) Citrate. There was no change in muscle citrate content after Lipovenus or KR infusion in both control and stimulated groups ($p > 0.05$, data not shown).

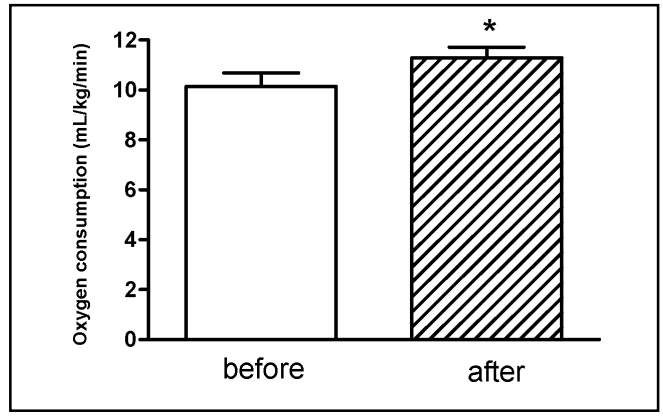


Fig. 10. Effect of Lipovenus on O_2 consumption in anesthetized rats. The rats were anesthetized with sodium pentobarbital and surgically prepared as described in Methods. After a stabilization period of 10 min, O_2 consumption was determined minute by minute during 15 min. Lipovenus or Krebs Ringer solution was infused during 60 min (8µL/min). Immediately after the administration, O_2 consumption was determined again minute by minute during 15 min. The values are presented as mean ± S.E.M (n=5 rats).

Lactate levels in the medium of cultured rat muscle cells

Lactate. Lactate concentration was significantly increased in the culture medium of stimulated cells in the control and Lipovenus groups compared with the respective not-stimulated cells. There was no difference of lactate concentration in the medium of the cells treated with CCCP when not-stimulated and stimulated cells were compared. Lactate concentration in the medium of stimulated cells treated with Lipovenus and CCCP was significantly higher compared to stimulated cells in the control group. Lactate content in the medium of not-stimulated cells treated with CCCP was significantly increased compared to not-stimulated cells of both control and Lipovenus (Figure 10).

Plasma levels of fatty acids and lactate

A) Fatty acids. Lipovenus perfusion for 60 min markedly increased plasma levels of saturated and unsaturated fatty acids as compared with control KR infusion, except for palmitoleic acid and eicosapentaenoic acid (EPA) (Table 2).

Plasma Fatty Acid Composition			
		Infusion	
	Control	Krebs Ringer	Lipovenus
Saturated (ng/μL)	0 min	60 min	60 min
lauric	13.5±1.4	16.3±1.1	61.45±23.4*
myristic	36.52±2.8	38.2±2.8	111.56±17.8*
palmitic	199.58±19.1	215.7±11.1	616.22±37.1*
estearic	165.14±30.09	150.2±0.3	582.93±120.2*
Unsaturated (ng/μL)			
palmitoleic	20.88±8.3	36.3±4.7	43.88±23.02
oleic	75.47±5.1	87.2±5.1	252.87±40.05*
linoleic	194.50±15.1	216.0±6.0	637.80±61.02*
γ-linolenic	18.48±5.5	14.2±0.1	68.66±15.5*
arachidonic	73.19±27.9	117.3±6.8	161.85±68.5*
eicosapentaenoic acid (EPA)	6.12±1.3	8.1±0.1	20.02±11.7
docosahexaenoic acid (DHA)	16.44±1.4	18.01±0.6	42.81±2.8*

Table 2. Plasma fatty acid composition after 60 min of KRS or Lipovenus infusion in rats. The values are presented as mean ± SEM (n = 7). * $p < 0.05$ as compared with control (0 min).

B) Lactate. Intense muscle contractions increased plasma lactate concentration in the KR and Lipovenus groups during muscle contraction compared with rest condition ($p< 0.05$). When compared with KR, Lipovenus infusion did not change the plasma lactate levels at rest and during intense muscle contraction ($P > 0.05$, data not shown).

Discussion

Although the physiological role of UCP-3 is still under debate, this protein has been reported to exert an important role in net energy production by uncoupling oxidative phosphorylation, resulting in reduced oxidative ATP production [20]. However, the physiological role of UCP-3 is still under debate. Under elevated mitochondrial respiratory ratio, as expected during intense muscle contractions, UCP-3 activity is very low due to the GDP inhibiting effect [26]. The hypothesis put forward in the present study was that, during intense muscle contraction, LCFA might reduce mitochondrial ATP/ADP ratio by an UCP-3 independent mechanism, raising the contribution of glycolysis for ATP production. Our findings showed that fatty acid infusion for 1h led to an increase of UCP-3 mRNA levels in RG and WG muscles (Figure 2), but had no effect on UCP-2 mRNA levels (data not shown). UCP-3 is upregulated in situations where fatty acid availability exceeds fat oxidation, including fasting, high-fat diet, exercise and T3 treatment [18]. Thus, the lack of Lipovenus effect on UCP-3 mRNA levels in soleus muscle may be related to the high capacity of this mitochondria rich muscle to oxidize fatty acids. In contrast, RG and WG muscles exhibit lower oxidative capacity compared to soleus muscle, which could contribute for an intracellular accumulation of fatty acids leading to upregulation of UCP-3 mRNA expression. Other studies have also shown that fatty acids induce UCP-3 expression *in vivo* [52, 53]. However, despite the fact that Lipovenus induced an increase of UCP-3 mRNA levels, the UCP-3 protein content was not changed (Figure 3). The absolute amount of this protein in skeletal muscle is much lower compared with the amount of UCP1 in brown adipose tissue [54], a well established mitochondrial uncoupling protein. This suggests that the role of UCP-3 in skeletal muscle is not exclusively to induce proton leak to mitochondrial matrix [18]. Our results showed that Lipovenus administration

increased resting respiration in isolated muscle mitochondria (Figure 4). The experiments were carried out in the presence of oligomycin to inhibit ATP synthesis and carboxyatractyloside (CAT) to rule out the possibility that the adenine nucleotides (ADN) carrier could contribute to the increased resting respiration of mitochondria by catalyzing a CAT-sensitive futile cycling of FFA [20]. The addition of GDP (an UCP inhibitor) did not reduce mitochondrial respiration, suggesting that uncoupling respiration induced by Lipovenus was not mediated by UCP. The inhibiting effect of BSA on mitochondrial respiration indicates that Lipovenus may induce *per se* mitochondrial uncoupling. Fatty acids are supposed to act as an H^+ shuttle lowering proton content in the inner mitochondrial space [27, 24].

We recently demonstrated that LCFA acutely induce mitochondrial uncoupling in rat skeletal muscle by an UCP-3 independent mechanism [26]. This effect of fatty acids has also been reported to occur in membrane preparations. LCFA may induce proton translocation across lipid vesicles, which acidification is greatly enhanced by fatty acids added to the medium [24, 25]. Moreover, the GDP effect on UCP-3 activity is highly dependent on mitochondrial redox state. Evidence was obtained that when reduced state of CoQ was decreased (i.e, oxidized), GDP was able to inhibit UCP-3 activity, abolishing the uncoupling effect induced by fatty acids [26]. These results are in agreement with a previous work by Jarmuszkiewicz et al. [55], suggesting a redox mechanism in the regulation of UCPs by purine nucleotides in skeletal muscle. This mechanism probably improves ATP synthesis efficiency when ATP demand is high. Under such condition, mitochondria are closer to respiratory state 3 and CoQ is expected to be in a more oxidized state favoring UCP inhibition by GDP. The high metabolic demand imposed by electrical stimulation in this study might be associated with elevated mitochondrial respiration rate, when UCP-3 is supposely to be inhibited by GDP [26, 55]. Alberici et al [28], examining the effect of elevated plasma lipid levels in apolipoprotein CIII overexpressing mice, demonstrated that liver mitochondria presented higher resting respiratory activity. The lack of GDP effect suggested that the increase of respiration rate was not due to UCP-3 activity. These authors found that activity and amount of mitoKATP channel are augmented in hypertriglyceridemic mice, providing a mechanistic explanation for the observed UCP independent uncoupling mechanism. Mitochondrial uncoupling has also been proposed to be induced by H^+ transport associated to K^+, Na^+ or Ca^{++} [28, 20, 56]. Therefore, mitochondrial uncoupling can also occur by other mechanisms in addition to UCP-3.

The reason for the mitochondrial uncoupling to occur is still unknown, once at elevated energy demand high ATP production rate is required. However, the force level during intense contractions remained unchanged in Lipovenus perfused muscles (Figure 5). Hesselink et al. [57] were also unable to show significant difference in the torque from knee-extensor muscles in humans receiving either low-fat or high-fat diets during 20 maximal voluntary contractions. The lack of effect on performance is intriguing, since elevated fatty acid availability has been reported to induce mitochondrial uncoupling resulting in a decreased energy gradient (ΔP) [20].

Kiens et al [58] showed that at moderate exercise intensities cellular LCFA content decreases probably due to enhanced fatty acid oxidation. In contrast, during intense exercise, when fat oxidation is low, the intracellular LCFA content was the same to that observed at rest condition. However, mitochondrial uncoupling is absent during rest. Although the mechanism is still unknown, during intense muscular activity, glycolysis is greatly stimulated, resulting in high rates of pyruvate/acetyl-CoA formation [59]. The elevated acetyl-CoA formation may reduce the cytosolic free CoA content, leading to a decrease of the acyl-CoA/fatty acid ratio. The reduced availability of cytosolic CoA could decrease fatty-acyl-CoA formation and oxidation, raising the intracellular fatty acid content and consequently favoring the mitochondrial uncoupling.

The increase in intracellular lactate content in the RG muscle after Lipovenus infusion and eletro-stimulation suggests that high glycolysis activity may have offset the low oxidative mitochondrial ATP synthesis imposed by mitochondrial uncoupling (Figure 6). The augment of plasma free fatty acid levels and the low capacity of the RG muscle to oxidize fatty acids during intense contractions may have favored mitochondrial uncoupling [17,18]. In contrast, the lack of effect on lactate content in the WG muscle may be a consequence of a much lower oxidative capacity of this muscle (i.e. high glycolytic capacity). Although there is a negative relationship between oxidative capacity and mitochondrial uncoupling, type II muscle is expected to exhibit very low mitochondria density [17]. A rapid lactate diffusion to the extracellular space may also contributed for the relatively low lactate content in the WG muscle during intense contractions. Therefore, the elevated fatty acid availability for this tissue has little effect on mitochondrial

respiration. The low amount of UCP-3 in the SO muscle combined with its high capacity to oxidize fatty acids may have contributed for a lower lactate production. The results of intramuscular G-6P content also support the proposition of a shift from oxidative to anaerobic glycolytic ATP production. The RG and WG muscles from the Lipovenus group exhibited a lower G-6P content after contraction compared with the KR group (Figure 7), suggesting that glucose uptake by skeletal muscle was not negatively modulated by G6-P [1-4]. Accordingly, a more significant reduction of intracellular glycogen store in RG and WG muscles after Lipovenus infusion suggests that electro-stimulation recruited predominantly fast-twitch glycolytic fibers favoring anaerobic glycolysis (Figure 8). The lack of Lipovenus effect on citrate content (data not shown) is in agreement with the proposition for an elevated glycolytic flux, since this metabolite is an inhibitor of the glycolytic pathway [1, 2]. Recently, Bashan.et al. [22] demonstrated an increase of lactate production in L6 myotubes treated with the mitochondrial uncoupling 2,4-dinitrophenol (DNP), suggesting that DNP-induced mitochondrial uncoupling decreased mitochondrial ATP synthesis leading to a higher dependence of glycolysis for ATP formation. Control experiments in rat skeletal muscle cells in culture also evidenced that an elevated FFA availability during intense muscle contraction might contribute for an increase in glycolytic flux, since lactate concentration in the medium of cultured cells was higher in the presence of Lipovenus (Figure 9). The similar effect on lactate production induced by the chemical mitochondrial uncoupling CCCP suggests that Lipovenus might have decreased the mitochondrial DP, consequently increasing glycolytic flux. This is an indication that FA were also able to induce mitochondrial uncoupling in isolated muscle cells in culture. It is noteworthy that Lipovenus infusion induced an increase of O_2 consumption in anesthetized rats (Figure 10). Similar observation was obtained by Schiffelers et al. [60]. However, this is not a definitive proof that fatty acid load induces mitochondrial uncoupling *in vivo* and further studies are required to clarify this issue.

In summary, our results suggest that an elevated intracellular FA content in skeletal muscle during intense muscle contractions induces mitochondrial uncoupling contributing to an increase in the anaerobic glycolysis. The relevance of this study, however, is limited to conditions where fatty acid supply exceeds fatty acid oxidation such as fasting, caffeine administration and high-fat feeding during intense muscle contractions.

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