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Acute effect of fatty acids on metabolism and mitochondrial coupling in skeletal muscle

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Abstract

Acute effects of free fatty acids (FFA) were investigated on: (1) glucose oxidation, and UCP-2 and -3 mRNA and protein levels in 1 h incubated rat soleus and extensor digitorium longus (EDL) muscles, (2) mitochondrial membrane potential in cultured skeletal muscle cells, (3) respiratory activity and transmembrane electrical potential in mitochondria isolated from rat skeletal muscle, and (4) oxygen consumption by anesthetized rats. Long-chain FFA increased both basal and insulin-stimulated glucose oxidation in incubated rat soleus and EDL muscles and reduced mitochondrial membrane potential in C2C12 myotubes and rat skeletal muscle cells. Caprylic, palmitic, oleic, and linoleic acid increased O_2 consumption and decreased electrical membrane potential in isolated mitochondria from rat skeletal muscles. FFA did not alter UCP-2 and -3 mRNA and protein levels in rat soleus and EDL muscles. Palmitic acid increased oxygen consumption by anesthetized rats. These results suggest that long-chain FFA acutely lead to mitochondrial uncoupling in skeletal muscle.

Keywords: Long-chain fatty acid; Skeletal muscle; Mitochondrial uncoupling; Glucose metabolism; Mitochondria

1. Introduction

Long-chain fatty acids (LCFA) have protonophoric action on the inner mitochondrial membrane [1]. This effect requires rapid translocation of both protonated and unprotonated forms of the fatty acid in the lipid bilayer [2]. The protonated form of LCFA can rapidly ($t_{1/2}<1$ s) cross the lipid bilayer [3]. However, the passage of unprotonated form through the lipid bilayer is difficult and very slow, suggesting that the mitochondrial uncoupling effect of LCFA involves membrane proteins [4]. In addition, LCFA can participate in the generation of mitochondrial unspecific permeability pore [5,6].

Various proteins have been described to mediate the transport of the anionic form of LCFA such as ADP/ATP carrier [4], glutamate/aspartate carrier [7], dicarboxylate carrier [8], and uncoupling proteins (UCP). UCP-1, present in brown adipose tissue, has a role in nonshivering thermogenesis in newborn mammals, cold-adapted or overfed rodents and hibernating mammals [9]. UCP-1 creates a parallel pathway to dissipate the proton gradient, thus uncoupling substrate oxidation from ATP synthesis. As a result of mitochondrial uncoupling, there are increased substrate oxidation, O_2 consumption, CO_2 generation, and heat production and decreased ATP synthesis [10]. Fatty acids operate as obligatory activators and purine nucleotides as inhibitors of UCP-1 [10].

New UCP isoforms (UCP-2 to -5 in mammals) have been identified in different tissues [11–14] but their functions are not fully known yet [15,16]. Skeletal muscles are the most important contributors (approximately 40%) of the body mass

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for the basal metabolic rate in humans and mammals [17]. Thus, UCP-2 and -3, the two isoforms present in skeletal muscles, may play a central role in the control of basal metabolic rate and body weight gain [17,18]. In addition, UCP-2 and -3 can be involved in the lipid metabolism [19], defense against excessive production of oxygen reactive species [20,21], protection against lipotoxicity in mitochondria [22], adaptive thermogenesis (e.g., fever) [16], and insulin sensitivity and secretion [23,24]. Transgenic mice overexpressing UCP-3 are hyperphagic but have low weight gain and high resting O_2 consumption and present low fasting glycemia and insulinemia when compared to controls [25]. UCP-2 and -3 dysfunction is found in various diseases with altered lipid metabolism, such as obesity, type 2 diabetes mellitus and heart failure [22,26].

UCP-2 and -3 activities are regulated by purine nucleotides [27] and depend on the redox state of endogenous Coenzyme Q (CoQ). Jarmuszkiewicz et al. [28] reported that muscle UCPs under respiratory state 4 are insensitive to GTP due to the high reduction level of CoQ. In contrast, muscle UCPs under respiratory state 3 becomes more sensitive to the GTP due to the more oxidized level of CoQ. Then, muscle UCPs seem to be regulated by purine nucleotides only when the ADP/ATP ratio is high, improving ATP synthesis efficiency. In contrast, at low ADP/ATP ratio, purine nucleotides do not inhibit muscle UCPs favoring thermogenesis and possibly regulating the production of reactive oxygen species during respiratory state 4 [28].

Evidence that LCFA are required for UCP-2 and -3 activities comes from experiments showing that (i) overexpression of UCP-2 and -3 in yeast increases proton leak in the presence of LCFA [7]; (ii) LCFA reduce proton gradient in reconstituted liposomes containing UCP-2 or -3 [29]; and (iii) addition of LCFA to isolated mitochondria of skeletal muscle leads to uncoupling [30]. However, there is no data about the effect of LCFA on mitochondrial uncoupling in intact skeletal muscle cells. In this study, we investigated the acute effect of various fatty acids on glucose metabolism and mitochondrial uncoupling in incubated rat skeletal muscles and cultivated skeletal muscle cells and compared with the results obtained with mitochondria isolated from rat skeletal muscle. The effect of the fatty acids was tested on: (1) glucose oxidation in rat soleus and extensor digitorium longus (EDL) muscles incubated for 1 h; (2) mitochondrial polarization in cultured skeletal muscle cells; (3) respiratory activity and transmembrane electrical potential in mitochondria isolated from rat skeletal muscle; (4) UCP-2 and -3 mRNA and protein levels in rat soleus and EDL muscles incubated for 1 h; and (5) consumption of O_2 by anesthetized rats.

2. Material and methods

2.1. Material

All reagents for buffers were obtained from Sigma Chemical Co. (St. Louis, USA). Regular insulin was purchased from Biobrás (Brazil). D-[U-^{14}C]Glucose was obtained from Amersham International Plc (Bucks, UK). DMEM, RPMI 1640 medium, bovine fetal serum, horse serum, penicillin, and SYBR GREEN were purchased from Invitrogen (Carlsbad, USA). Rhodamine 123 was obtained from Molecular Probes (Eugene, USA). BRAZOL reagent was purchased from LGC Biotechnology (São Paulo, Brazil). Anti-UCP-2 was obtained from Calbiochem (Darmstadt, Germany) and anti-UCP-3 from Chemicon International (Temecula, USA). Anti-rabbit IgG antibody conjugated to horseradish peroxidase and ECL Western Blotting System Kit were purchased from Amersham International Plc (Bucks, UK).

2.2. Animals

The Ethical Committee of the Institute of Biomedical Sciences, University of São Paulo, approved the study. Male Wistar rats were obtained from the Department of Physiology and Biophysics, Institute of Biomedical Sciences, University of São Paulo. Animals were housed at 12:12 h light–dark cycle, 23±1 °C, and allowed access to food and water ad libitum until the day of the experiment. Rats weighing 200±20 g were utilized in all experiments, except for skeletal muscle incubation, in which rats with 150±10 g were utilized because of the ideal body weight for incubation procedure.

2.3. Effect of fatty acids on D-[U-^{14}C]glucose oxidation in incubated rat skeletal muscle

Soleus and EDL muscles were isolated and incubated as previously described [31,32]. The rats were killed by cervical dislocation and the muscles rapidly and carefully isolated, split longitudinally in two equal portions, weighed (25–35 mg) and pre-incubated, at 37 °C, in Krebs–Ringer bicarbonate buffer containing 5.6 mM glucose, pH 7.4, pre-gassed for 30 min with 95% O_2/5% CO_2, with agitation at 120 rpm for 30 min. Subsequently, the muscles were transferred to the same buffer with 0.3 μCi/mL D-[U-^{14}C]glucose and incubated under similar conditions for 1 h, in the absence or presence of 10 mU/mL insulin. Phenylethylamine, diluted 1:1 v/v in methanol, was added into a separate compartment for $^{14}CO_2$ adsorption. Oxidation of D-[U-^{14}C]glucose was determined as previously described [33]. The fatty acids were previously dissolved in ethanol (20 mM fatty acid solution). As a control, some muscles were incubated in the presence of 0.5% vehicle. Concentrations ranging from 50 to 200 μM of palmitic acid were previously tested. The maximal effect on glucose oxidation was observed with 100 μM of palmitic acid (data not shown). Thus, further experiments were performed with 100 μM of different fatty acids: caprylic (8:0), lauric (12:0), myristic (14:0), palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2), and eicosapentaenoic (20:5) acid.

2.4. Effect of fatty acids on free coenzyme A content in incubated rat skeletal muscle

Soleus and EDL muscles were isolated and incubated for 1 h as described above in the absence or presence of 100 μM of caprylic, palmitic or linoleic acid. After the incubation period, muscles were frozen in liquid N_2 and free coenzyme A content determined as previously described [34,35]. The muscles were homogenized in 0.5 M perchrolic acid (1:6 w/v) and centrifuged at 3000 × *g* for 10 min, at 4 °C. The supernatant was neutralized with 2 N KOH. The content of free coenzyme A was measured at 412 nm in a buffer containing 100 mM Tris–HCl, pH 7.5, and 0.125 mM 5,5′-dithiobis-2-nitrobenzoic acid (DNTB).

2.5. Effect of fatty acids on mitochondrial polarization in cultivated skeletal muscle cells

2.5.1. Culture of C2C12 myotubes

Culture of C2C12 myotubes was performed as previously described [36]. Mice C2C12 myoblasts were maintained in RPMI medium, at pH 7.5, containing 10% bovine fetal serum, 2 mM glutamine, 15 mM HEPES, and 500 U/mL penicillin, with 95% O_2/5% CO_2, at 37 °C. For differentiation, myoblasts were seeded in 24-well plates (70,000 cells per well) and cultivated in RPMI medium containing 1% bovine fetal serum for 3–4 days.

The effects of caprylic, palmitic, and linoleic acid on mitochondrial polarization in situ in C2C12 myotubes were analyzed by rhodamine 123 technique as previously described [37,38]. Cells were incubated in Krebs–Ringer bicarbonate buffer, pH 7.4, containing 5.6 mM glucose, in the absence or in the presence of 100 μM caprylic, palmitic, or linoleic acid (20 mM fatty acid

ethanolic solution), at 37 °C, and 5% CO_2 for 1 h. As a control, C2C12 myotubes were incubated in the presence of 0.5% vehicle. Rhodamine 123, at 10 μg/mL, was added 15 min prior to the end of the incubation. The cells were washed twice for 1 min with PBS at 37 °C and analyzed by fluorescence microscopy at 590 nm. Five fields of each well were photographed. Fluorescence intensity was determined by the KS300 program (Eching, Munich, Germany).

2.5.2. Primary culture of rat skeletal muscle

Primary culture of cells from rat skeletal muscles was performed as previously described [39]. Briefly, soleus, gastrocnemius, and quadriceps muscles were dissected from rats and cut into small pieces for digestion with 2% type II collagenase, 0.25% trypsin and 0.1% DNAse for isolation of myoblasts. The cells were placed into plates of 24 wells (60,000 cells per well) for growing in DMEM containing 1% penicillin, 10% fetal bovine serum and 10% horse serum. After 2–3 days of the culture period, myoblasts were differentiated into skeletal muscle cells in DMEM containing 1% penicillin and 10% horse serum for 4–5 days.

The effect of caprylic, palmitic and linoleic acid (100 μM) on mitochondrial polarization was determined as described above. Moreover, the effect of palmitic acid was also evaluated in the presence of insulin (10 mU/mL).

Antimycin A (inhibitor of the complex III of electron transporter chain) and carbonylcyanide-n-chlorophenylhydrazone (CCCP, classical mitochondrial uncoupler), both at 10 μM (2 mM ethanolic solution), were used as positive and negative controls of mitochondrial coupling, respectively. The cells were incubated for 30 min in the absence or presence of antimycin A or CCCP and the mitochondrial polarization evaluated as described above.

2.6. Effect of fatty acids on respiratory activity and transmembrane electrical potential in mitochondria isolated from skeletal muscle

2.6.1. Isolation of skeletal muscle mitochondria

Muscle mitochondria were isolated from rat hindlimb skeletal muscles by homogenization 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% BSA, pH 7.4, followed by differential centrifugation [40]. The final mitochondrial pellet was resuspended in ice cold storage 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 [41]. The presence of fatty acid-free BSA (0.2%) in the buffers throughout the isolation procedure completely depleted the mitochondria of endogenous fatty acids.

2.6.2. Standard incubation procedure

The experiments were carried out at 28 °C with continuous magnetic stirring. Skeletal muscle mitochondria (0.5 mg/mL) were added in a standard reaction medium containing 225 mM mannitol, 75 mM sucrose, 10 mM Tris–HCl, pH 7.4, 10 mM KH_2PO_4 and 0.1 mM EDTA. For measurements of O_2 consumption, mitochondria were incubated with 5 mM NAD^+-linked substrates (α-ketoglutarate, pyruvate, malate, and glutamate), 1 μg/mL oligomycin (ATP-synthase inhibitor), 1 μM carboxyatractyloside (CAT, ATP/ADP carrier inhibitor), and 1 μM cyclosporine A (inhibitor of the formation of mitochondrial permeability transition pore). Additions of 2 mM GDP (UCP inhibitor) and 0.05% BSA (free fatty acid bind) were performed as indicated in the figures. For measurements of mitochondrial electrical potential, mitochondria were incubated with 15 mM succinate, 2 μM rotenone (inhibitor of complex I) and 80 nM ATP (required for activating succinate dehydrogenase). Concentrations ranging from 0.5 to 10 μM of caprylic, palmitic, oleic and linoleic acid were previously tested. The maximal effect on oxygen consumption was observed with 0.5 μM for caprylic and palmitic acid, 4 μM for oleic acid and 1 μM for linoleic acid (data not shown). Thus, further experiments were performed using these concentrations.

2.6.3. Determination of mitochondria respiration rates and phosphorylation efficiency

Oxygen consumption was measured using a Clark-type electrode (Hansatech Instruments, Ltd., using software OXYGRAPH V1.10, England). The respiratory control ratio (state-III/state-IV respiration rate) was over 4.0, as measured using NAD^+-linked substrates. Respiration rates are given in nAtoms oxygen/mg protein per min.

2.6.4. Measurement of mitochondrial transmembrane electrical potential (ΔΨ)

Changes in the safranine fluorescence was employed to obtain a continuous record of the mitochondrial membrane potential [42]. Fluorescence changes of safranine O were recorded on a F-4500 Hitachi spectrofluorometer (Hitachi Ltd., Tokyo, Japan) operating at excitation and emission wavelengths of 495 and 586 nm, respectively, with slit widths of 3 nm. The assay was performed in standard reaction medium (1 mL) in the presence of 5 μM safranine as previously described [42]. Palmitic (0.5 μM) or linoleic (1 μM) acid was added in the absence or presence of 2 mM GDP (UCP inhibitor). To change CoQ state to its more oxidized form [28], 100 μM malonate (inhibitor of succinate dehydrogenase activity) was added. CCCP was added at 1 μM to evaluate the integrity of the mitochondria at the end of experiment.

2.7. Effect of insulin and caprylic and palmitic acid on UCP-2 and -3 protein levels in incubated rat skeletal muscles

Soleus and EDL muscles were incubated for 1 h as described above in the absence or presence of 10 mU/mL insulin or 100 μM of caprylic or palmitic acid. After the incubation period, muscles were 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. After homogenization, 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, as described by Bradford [43]. Equal amounts of proteins of each sample (75 μg) were separated by using 15% SDS-gel polyacrylamide [44]. Western blotting was carried out following the method described by Towbin et al. [45]. 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.

2.8. Effect of insulin and caprylic and palmitic acid on UCP-2 and -3 mRNA levels in incubated rat skeletal muscles

Soleus and EDL muscles were incubated for 1 h as described above in the absence or presence of 10 mU/mL insulin or 100 μM of caprylic or palmitic acid. After the incubation period, muscles were frozen in liquid N_2 and RNA extracted as previously described [46] by using BRAZOL reagent. Total RNA (4 μg) was reverse transcribed to cDNA synthesis. UCP-2 and -3 expression was evaluated by real-time PCR [47] in ROTOR GENE 3000 equipment (Corbett Research, Mortlake, Australia), using SYBR GREEN as fluorescent dye. The sequence of the utilized primers were: 5′TCCTGAAAGCCAACCTCATGA3′ and 5′CAATGACGGTGGTGCAGAAG3′ for sense and antisense primers of the UCP-2 gene, respectively; 5′AGCAGTTCTACACCCCCAAAGG3′ and 5′ TTTACCACATCCACTGGGGAGG3′ for sense and antisense primers of the UCP-3 gene, respectively; and 5′ACCACAGTCCATGAAATCAC3′ and 5′ TCCACCACCCTGTTGCTGTA3′ for sense and antisense of the G3PDH gene, respectively. Quantification of gene expression was determined by comparative cycle threshold method, using G3PDH gene expression as inner control [48].

2.9. Effect of palmitic 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). Palmitic acid (2.05 mg per kg body weight, ethanolic solution of 20 mM) and ethanol in controls (0.4 mL per kg body weight) were administered through the penian vein. After the fatty acid administration, the animals were immediately placed into the calorimeter chamber. After a 10-min stabilization period of the gases in the interior of the chamber, O_2 consumption was determined every 1 min during a 15 min period. Other group of animals was submitted to serum free fatty acid determination. Blood samples were collected before and after 10, 20, and 30 min of the administration of palmitic acid or ethanol. Serum free fatty acids were determined by the acyl-CoA synthase/acyl-CoA oxidase method [49], using the kit NEFA-C obtained from Wako Chemicals GmbH (Neuss, Germany).

2.10. Statistical analysis

The data are presented as mean±S.E.M. and analyzed by one-way ANOVA and Tukey test ($P<0.05$). The areas under the curves of O_2 consumption were determined and compared between the two groups by using the Student's t test.

3. Results

3.1. Effect of fatty acids on D-[U-^{14}C]glucose oxidation by incubated rat skeletal muscles

In incubated soleus muscle, caprylic acid decreased insulin-stimulated D-[U-^{14}C]glucose oxidation by 29% ($P<0.001$), whereas palmitic, stearic, oleic, linoleic, and eicosapentaenoic acid increased by ≥59% in the absence ($P<0.05$) and by ≥32% in the presence ($P<0.05$) of 10 mU/mL insulin (Fig. 1A). Lauric and myristic acid did not exhibit significant effect on glucose oxidation (Fig. 1A). In EDL muscle, palmitic and linoleic acid increased D-[U-^{14}C]glucose oxidation by ≥56% ($P<0.05$) in the absence and by ≥26% ($P<0.05$) in the presence of insulin (Fig. 1B). There was no change in intracellular ATP content in soleus muscle incubated in the presence of palmitic acid (data not shown).

3.2. Effect of fatty acids on free coenzyme A content in incubated rat skeletal muscle

Caprylic, palmitic and linoleic acid caused a marked decrease in the free coenzyme A content as compared to control group The results were expressed as nmol per g muscle fresh weight decreased due to incubation with fatty acids and presented as mean±S.E.M. of eight determinations from two experiments. The decrease in soleus muscle was of 44.0±7.6, 14.5±4.0 and 14.5±1.6 by caprylic, palmitic and linoleic acid, respectively, whereas, in EDL muscle, the decrease was of 32.8±7.9, 12.3±2.2 and 13.1±1.6, respectively.

3.3. Effect of fatty acids on mitochondrial polarization in cultivated skeletal muscle cells

We evaluated the effect of two classical drugs, well characterized, that alter the mitochondrial coupling: antimycin A (inhibitor of complex III of electron transport chain) and CCCP (mitochondrial uncoupler). As expected, antimycin A

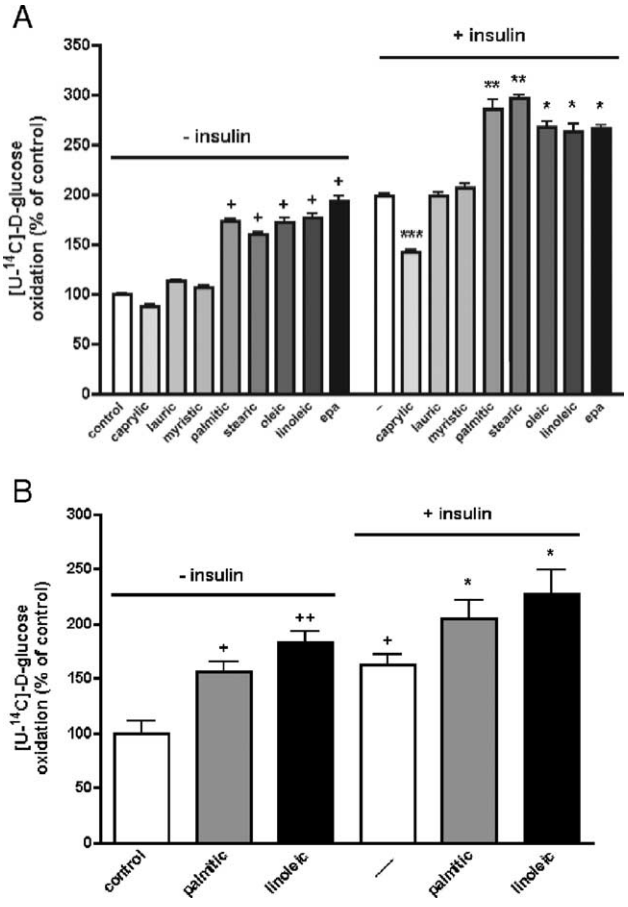

Fig. 1. Effect of fatty acids on D-[U-^{14}C]glucose oxidation in 1h incubated rat soleus (A) and extensor digitorium longus (EDL) (B) muscles. Muscles were incubated for 1 h in the absence or presence of 10 mU/mL insulin and/or 100 μM of different fatty acids. Phenylethylamine (0.4 mL solution 1:1 in methanol, v/v) was added into a separate compartment inside the flask to allow $^{14}CO_2$ adsorption. Values are presented as mean±S.E.M. of 4 experiments ($n=12$) for soleus muscle and 2 experiments ($n=6$) for EDL muscle. $^{+}P<0.05$ and $^{++}P<0.001$ as compared to the control group; $^{*}P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ as compared to the insulin group (in the absence of fatty acid). EPA, eicosapentaenoic acid.

increased by 20% ($P<0.001$) and CCCP decreased by 29% ($P<0.001$) the mitochondrial polarization, demonstrating that the utilized method is appropriate for analyzing the mitochondrial polarization in cultured cells (Fig. 3).

Palmitic and linoleic acid decreased mitochondrial polarization in C2C12 myotubes ($P<0.05$; Fig. 2) and in primary culture of rat skeletal muscle cells ($P<0.001$; Fig. 3), whereas caprylic acid had no significant effect. Palmitic acid also reduced mitochondrial polarization in primary culture of rat skeletal muscle cells in the presence of insulin (10 mU/mL) (9.19±0.19%; $P<0.01$).

3.4. Effect of fatty acids on O_2 consumption by mitochondria isolated from rat skeletal muscle

Resting respiration of mitochondria from skeletal muscle was significantly increased ($P<0.05$) in the presence of fatty acids (Fig. 4). Caprylic acid exhibited the less pronounced

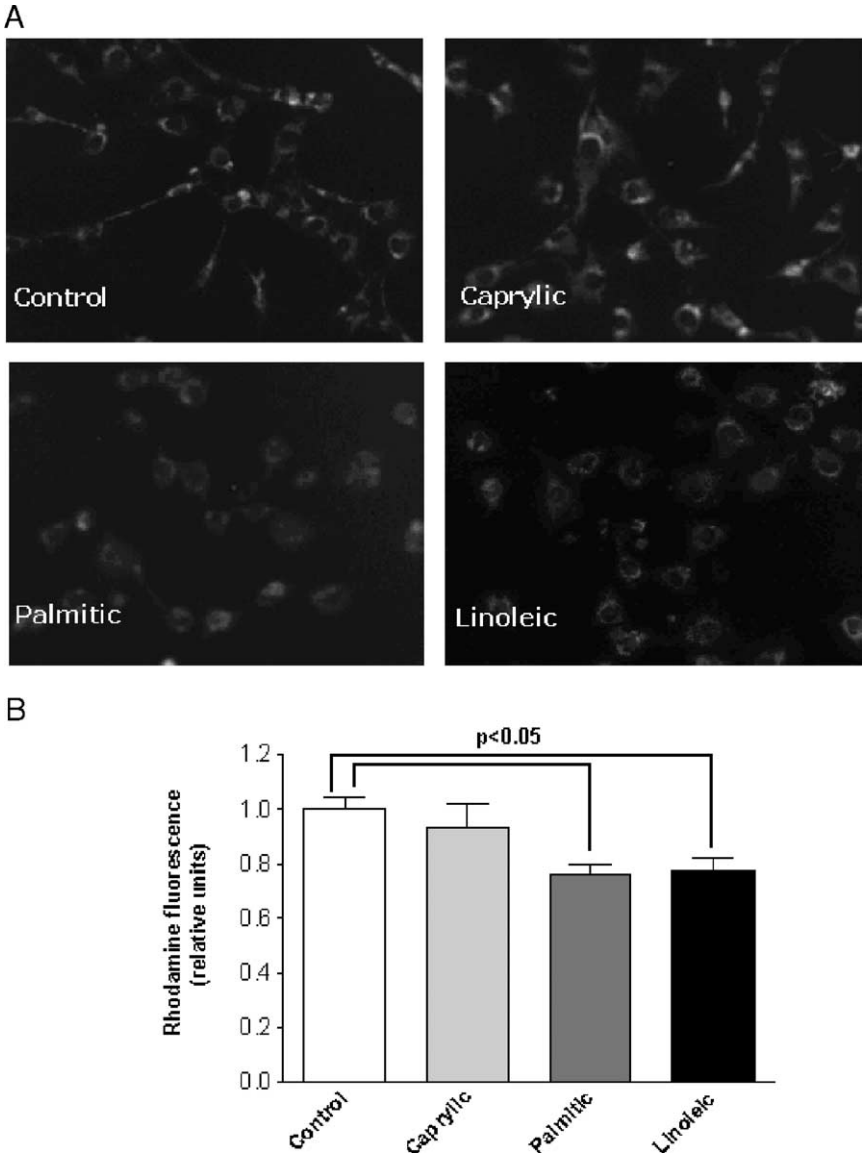

Fig. 2. Effect of caprylic, palmitic and linoleic acid on mitochondrial polarization of C2C12 myotubes. Muscle cells were incubated in Krebs–Ringer bicarbonate buffer, containing 5.6 mM glucose, in the absence or presence of 100 μM of fatty acids (ethanolic solution at 20 mM), at 37 °C, and 5% CO_2 for 1 h. Control cells were incubated in the presence of 0.5% ethanol. Rhodamine 123 (10 μg per mL) was added at 15 min before the end of the incubation period. The cells were washed twice for 1 min each with PBS at 37 °C and immediately evaluated in a fluorescence microscope at 590 nm. (A) Representative figure of one experiment (100×). (B) Mean±S.E.M. of 9 determinations of 3 experiments.

effect, rising mitochondrial oxygen consumption by 81%, whereas palmitic, oleic, and linoleic acid increased oxygen consumption by 146%, 129%, and 120%, respectively. The UCP inhibitor (GDP) had no effect on the increase of mitochondrial oxygen consumption induced by palmitic, caprylic, and oleic acid. However, GDP (2 mM) partially reduced oxygen consumption in the presence of linoleic acid. BSA (0.01%) fully abolished the increase in oxygen consumption induced by the fatty acids. The involvement of ATP-synthase, ATP/ADP carrier or mitochondrial permeability transition pore in the fatty acid-induced mitochondrial uncoupling was ruled out by using 1 μg/mL oligomycin, 2 mM carboxyatractylate and 1 μM cyclosporin A, respectively.

In the experiments depicted in Fig. 5, we studied the effect of GDP when coenzyme Q (CoQ) was maintained predominantly in an oxidized state by the presence of 100 μM malonate, an inhibitor of succinate dehydrogenase activity. As shown in previous work [28], this protocol promotes significant oxidation of CoQ, a decrease of about 10% in the rate of O_2 consumption and no significant reduction of $\Delta\psi$. The UCP activity was observed when the more oxidized form of coenzyme Q was obtained by inhibition of s succinate dehydrogenase (Fig. 5A2 and B2). Under this condition, GDP prevented the drop in mitochondrial membrane potential induced by palmitic (Fig. 5A2) and linoleic (Fig. 5B2) acid. In absence of malonate (Fig. 5A1 and B1), GDP was not able to prevent the drop in mitochondrial membrane potential, indicating that the dependence of the more oxidized form of coenzyme Q on the inhibition of UCP by GDP is in agreement with data obtained by Jarmuszkiewicz et al. [28].

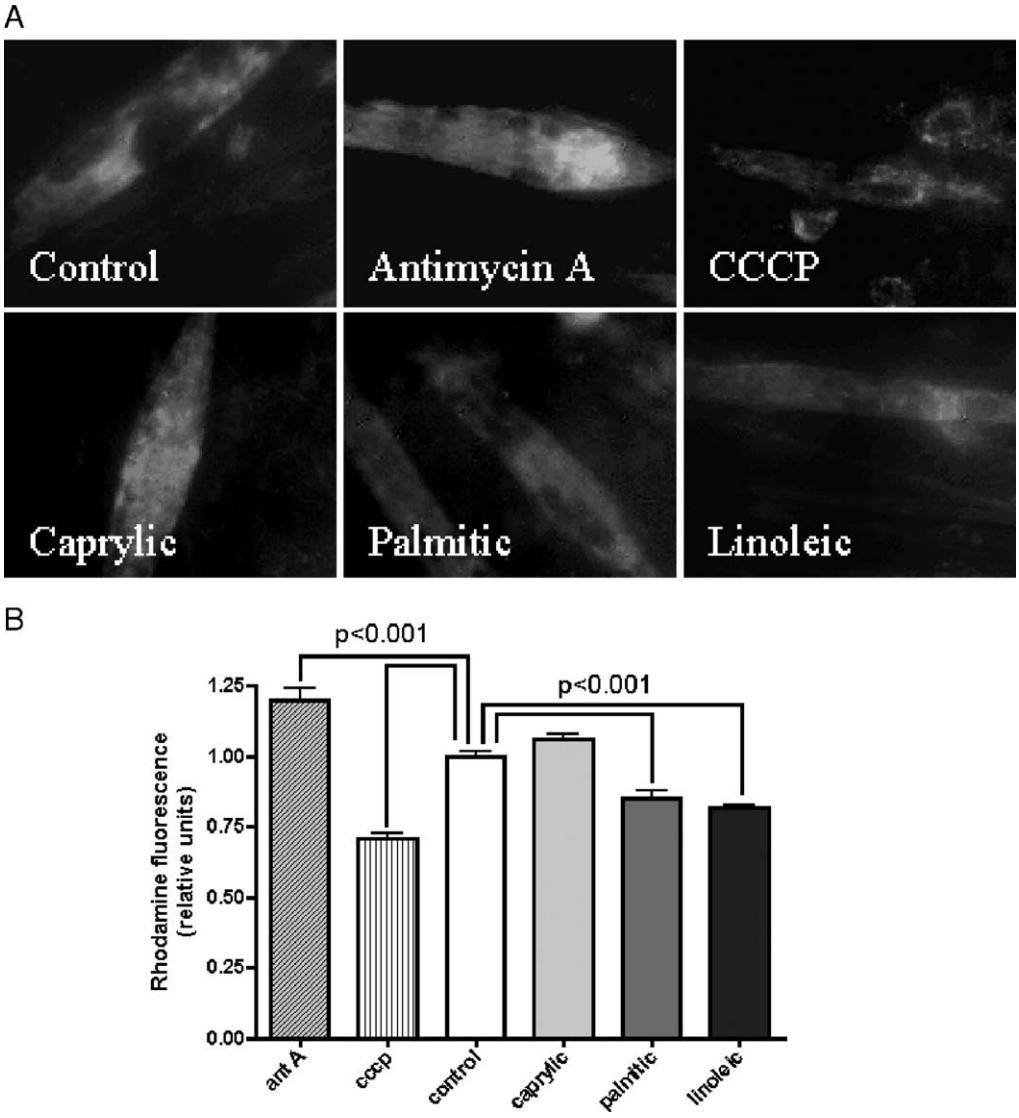

Fig. 3. Effect of antimycin A (Ant A), carbonylcyanide-n-chlorophenylhydrazone (CCCP) and caprylic, palmitic and linoleic acid on mitochondrial polarization of primary culture cells from rat skeletal muscle. The muscle cells were incubated in Krebs–Ringer bicarbonate buffer, containing 5.6 mM glucose, in the absence or presence of 10 μM Ant A, 10 μM CCCP or 100 μM caprylic, palmitic or linoleic acid (ethanolic solution at 20 mM fatty acid), at 37 °C, and 5% CO_2 for 30 min (Ant A and CCCP) or 1 h (fatty acids). Rhodamine 123 (10 μg per mL) was added 15 min before the end of the incubation period. The cells were washed twice for 1 min each with PBS at 37 °C and immediately evaluated in a fluorescence microscope at 590 nm. (A) Representative figure of one experiment (400×). (B) Mean ± S.E.M. of 3 experiments (n = 9).

3.5. Effect of insulin and caprylic and palmitic acid on UCP-2 and -3 mRNA and protein levels in incubated rat skeletal muscle

Insulin and caprylic and palmitic acid had no significant effect on UCP-2 and -3 mRNA (data not shown) and protein (Fig. 6) levels in 1 h incubated rat soleus and EDL muscles.

3.6. Effect of palmitic acid on O_2 consumption by anesthetized rats

Administration of palmitic acid caused a rapid increase in the plasma free fatty acid concentrations. The values (in mM) were: 0.34 ± 0.01 vs. 0.34 ± 0.02 before the treatment; 0.40 ± 0.05 vs. 0.78 ± 0.07 after 10 min (P < 0.01); 0.41 ± 0.07 vs. 0.88 ± 0.03 after 20 min (P < 0.001); and 0.40 ± 0.09 vs. 0.78 ± 0.09 after 30 min (P < 0.01) of the treatment with ethanol (control group) and palmitic acid, respectively. The results are presented as mean ± S.E.M. of 4 determinations from 2 experiments.

There was no difference in the O_2 consumption when the groups were compared in each minute individually (data not shown). However, when the area under the curve was determined, a significant increase in O_2 consumption was observed in rats that received palmitic acid when compared to animals treated with the vehicle (P < 0.01; Fig. 7).

4. Discussion

Mitochondrial uncoupling leads to an increase in metabolite oxidation, O_2 consumption, and heat production, and a decrease

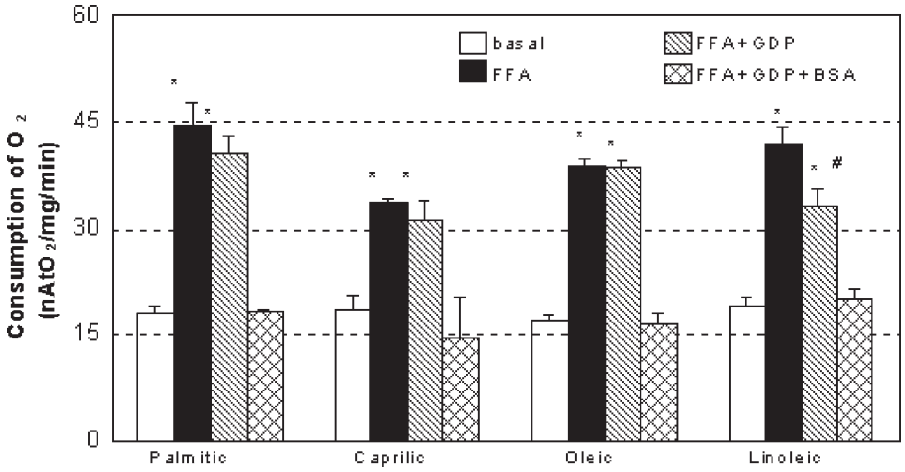

Fig. 4. Effect of free fatty acids (FFA, 0.5 μM caprylic and palmitic acid, 4 μM oleic acid, or 1 μM linoleic acid), GDP (2 mM) and BSA (0.05%) on mitochondrial O_2 consumption of isolated muscle mitochondria. Values were obtained from 3 different experiments. *$P<0.05$ as compared with basal; #$P<0.05$ as compared to FFA group.

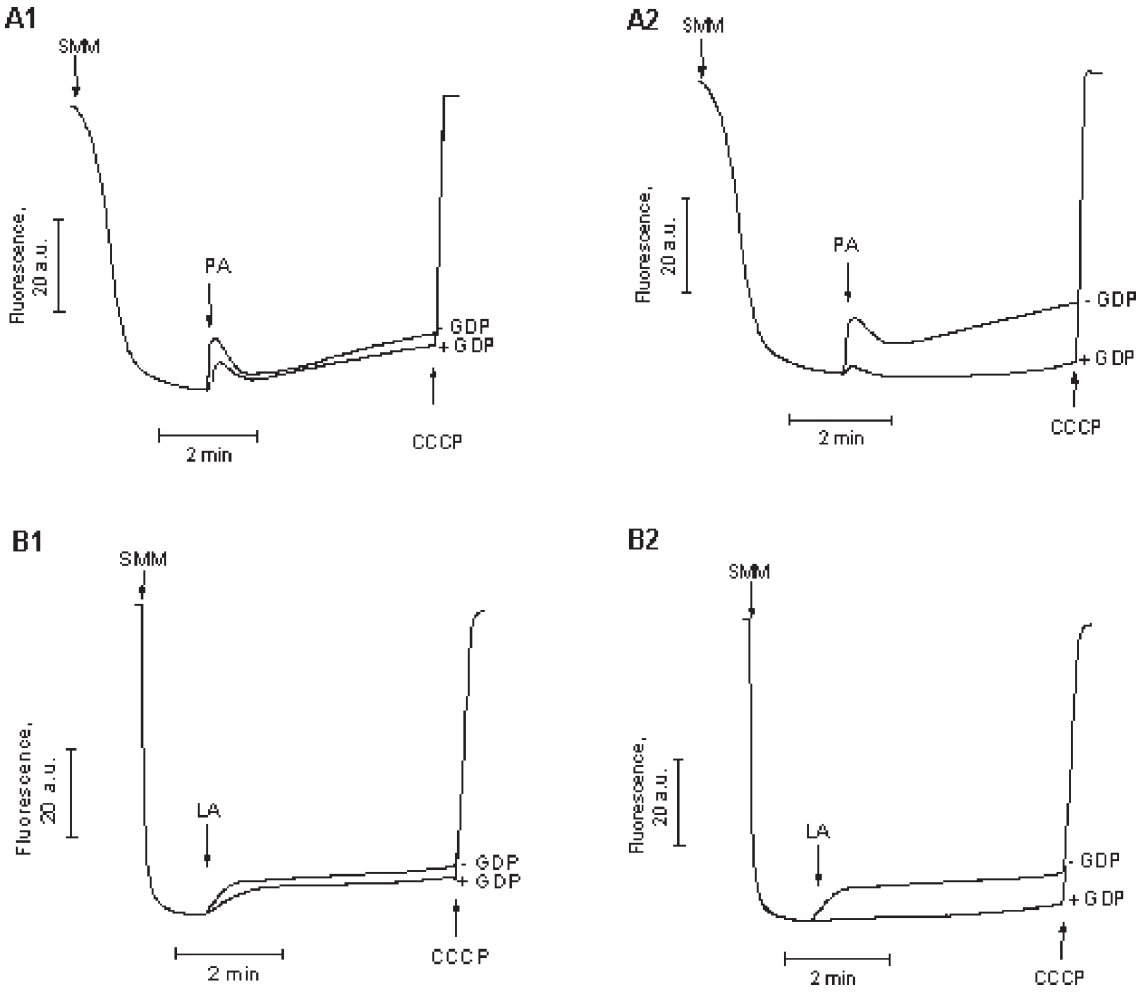

Fig. 5. Effect of GDP on the drop of mitochondrial electrical potential induced by palmitic (Panels A) and linoleic (Panels B) acid. Mitochondria were incubated in the absence (A1 and B1) or presence (A2 and B2) of 100 μM malonate (CoQ oxidized), and in the absence (−GDP) or presence (+GDP) of 2 mM GDP. The arrows indicate additions of 0.5 μM palmitic acid (PA), 1 μM linoleic acid (LA) and 1 μM carbonylcyanide-n-chlorophenylhydrazone (CCCP). Traces are representatives of 3 independent experiments of both.

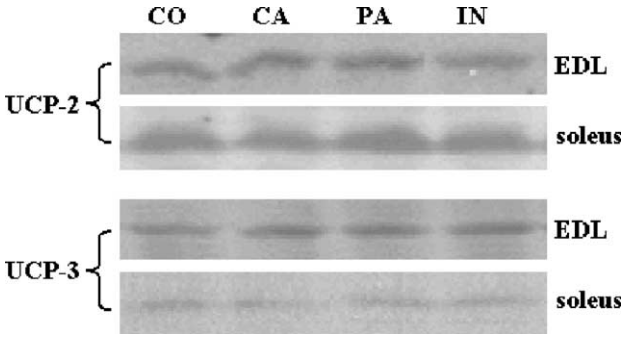

Fig. 6. Effect of insulin (IN) and caprylic (CA) and palmitic (PA) acid on UCP-2 and -3 protein levels in incubated rat skeletal muscle. Muscles were incubated for 1 h in the absence (control group –CO) or presence of 10 mU/mL insulin or 100 μM of caprylic or palmitic acid. At end of incubation, UCP-2 and -3 protein levels were determined by Western blotting as described under Materials and methods section. Data are presented as mean±S.E.M. of 2 experiments (n=3).

in ATP synthesis [10]. Evidence was obtained herein that acute treatment with LCFA causes mitochondrial uncoupling effect on incubated rat skeletal muscles, cultured rat skeletal muscle cells and C2C12 myotubes, and isolated rat skeletal muscle mitochondria.

Soleus muscle presents predominantly oxidative fibers (84% type I and 16% type IIa) and low UCP-3 expression as compared to glycolytic fibers. EDL muscle presents predominantly glycolytic fibers (3% type I, 57% type IIa and 40% type IIb) and high expression of UCP-3 as compared to oxidative fibers [50,51]. Consequently, it would be expected a more pronounced effect of fatty acids on EDL muscle than in soleus muscle. However, the increase in glucose oxidation induced by LCFA was similar in both muscles. This result may be related to the large amount of mitochondria in soleus muscle that may compensate for the low expression of UCP-3 in this tissue. In both muscles, the effect was additive to that of insulin, suggesting a different mechanism. Insulin increases glucose oxidation by activating pyruvate dehydrogenase (PDH) and the Krebs cycle [52,53]. We investigated here if the increased glucose oxidation induced by LCFA is related to their mitochondrial uncoupling effect.

Randle et al. [54] proposed that increased fatty acid oxidation results in elevated generation of acetyl-CoA that leads to inhibition of PDH and consequently reduced glucose oxidation. Prolonged exposure to fatty acids inhibits PDH activity by increasing the expression of PDH kinase [55]. Pyruvate generated from glucose is converted into CO_2 through pyruvate dehydrogenase and the Krebs cycle entering via pyruvate carboxylase reaction [56]. In a previous study, the effect of palmitic acid on [1-^{14}C]- and [2-^{14}C]-pyruvate oxidation in rat soleus muscle incubated for 1 h was determined in order to estimate the contribution of both decarboxylation sites (pyruvate dehydrogenase and Krebs cycle). [2-^{14}C]-Pyruvate is decarboxylated through the TCA cycle only in opposition to [1-^{14}C]-pyruvate, which produces $^{14}CO_2$ in both sites. Palmitic acid decreased [1-^{14}C]-pyruvate oxidation and enhanced [2-^{14}C]-pyruvate oxidation [57]. Thus, an increase in acetyl-CoA content from palmitate oxidation leads up to inhibition of pyruvate dehydrogenase activity, decreasing the conversion of pyruvate to acetyl-CoA, but also enhances pyruvate carboxylase activity, raising the conversion of pyruvate to oxaloacetate that entries into the Krebs cycle being then oxidized [57].

In order to examine the effect of fatty acids on mitochondrial polarization in cultured skeletal muscle cells, we used rhodamine 123. This compound is a mitochondrion-specific fluorescent cationic probe with high affinity to mitochondrial inner membrane due to its high electrical polarity [37,38]. Palmitic and linoleic acid decreased mitochondrial polarization in C2C12 myotubes and rat skeletal muscle cells in culture. Moreover, palmitic acid also reduced mitochondrial polarization in cultivated rat skeletal muscle cells in the presence of insulin. These results suggest that LCFA acutely lead to mitochondrial uncoupling in skeletal muscle cells.

We also evaluated the effect of fatty acids on respiratory activity and transmembrane electrical potential in mitochondria isolated from rat skeletal muscles. Under conditions of resting respiration (nonphosphorylating state), caprylic, palmitic, oleic and linoleic acid increased oxygen consumption. GDP did not markedly alter the effects of the fatty acids, except for linoleic acid, where GDP was able to inhibit by 20% linoleic acid-stimulated O_2 consumption. Tonkonogi et al. [30] demonstrated that incremental additions of oleic acid ranging from nanomolar to micromolar concentrations increase respiration in mitochondria isolated from human skeletal muscles. Similarly, this effect was unaffected by GDP, suggesting an UCP-independent uncoupling mechanism or a GDP-insensitive UCP-dependent uncoupling mechanism in this condition. In order to investigate this later possibility, we determined the effect of GDP when the reduced state of coenzyme Q was decreased, i.e., in the more oxidized form. Under this condition, GDP was able to abolish the drop of mitochondrial potential induced by palmitic or linoleic acid, suggesting an UCP-dependent uncoupling

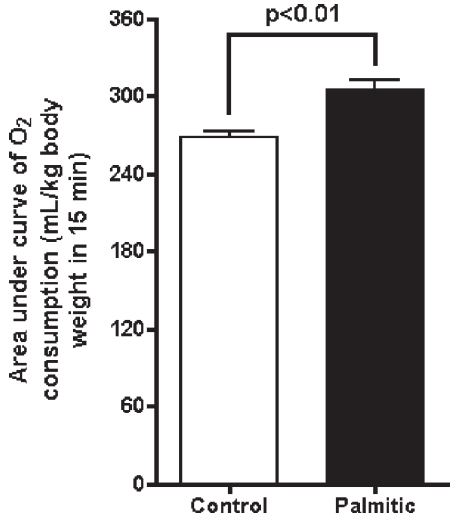

Fig. 7. Effect of palmitic acid on O_2 consumption in anesthetized rats. The rats were anesthetized with sodium pentobarbital and palmitic acid or ethanol as control was intravenously administered. Immediately after the administration, the animals were put into the chamber of the calorimeter. After a stabilization period of 10 min, O_2 consumption was determined minute by minute during the next 15 min. Areas under the curves were determined and the values analyzed by Student's t test. The values are presented as mean±S.E.M. of 5 rats.

mechanism. These results are in agreement with a previous work by Jarmuszkiewicz et al. [28], suggesting a redox mechanism in the regulation of UCPs by purine nucleotides in skeletal muscle. We hypothesize that the physiological function of this mechanism is to improve 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. In conditions of low metabolic demand, mitochondria are closer to respiratory state 4 and CoQ is in a more reduced form favoring mitochondrial uncoupling and thermogenesis.

Interestingly, caprylic acid, a medium chain fatty acid, caused similar effects to those of LCFA on isolated skeletal muscle mitochondria; however, it had no effect on intact skeletal muscle cells (incubated rat skeletal muscles and cultivated C2C12 myotubes and rat skeletal muscle cells). Therefore, the mitochondrial uncoupling effect of caprylic acid is supposedly lost by a cytoplasmatic processing of the molecule, possibly by generation of capryl-CoA derivatives, leading to its rapid oxidation. Moreover, caprylic acid does not require carnitine shuttle to entry into the mitochondria, speeding its metabolization. In accordance to this proposition, we found a greater reduction of free coenzyme A content in rat skeletal muscles incubated in the presence of caprylic acid as compared to palmitic and linoleic acid.

Expression of UCP-3 gene in skeletal muscle is up-regulated in various situations of increased fatty acid availability, such as fasting, food restriction, high-fat diet and a single exercise session, whereas it is down-regulated in situations of decreased fatty acid availability, such as exercise training and weight reduction [58]. The acute effect of fatty acids, however, was unknown. In this study, we found that caprylic and palmitic acid acutely did not have a significant effect on UCP-2 and -3 mRNA and protein levels in 1 h incubated soleus and EDL muscles but raised mitochondrial uncoupling activity.

The results herein reported support the proposition that LCFA acutely lead to mitochondrial uncoupling in skeletal muscle. Since skeletal muscle represents 40% of the body weight of a lean subject being the major determinant factor of the basal metabolic rate [17], one should expect an increase in the basal metabolic rate by increasing FFA supply. In order to investigate the effect of palmitic acid on O_2 consumption in vivo, palmitic acid was infused in anesthetized rats. This treatment caused a rapid increase in the serum FFA concentrations associated with an elevation in O_2 consumption. This result is indicative that LCFA acutely increase the basal metabolic rate that may be related to the mitochondrial uncoupling effect of these metabolites in vivo.

In summary, our findings led us to postulate that LCFA acutely induce mitochondrial uncoupling in skeletal muscle. This may be an important mechanism for the control of glucose oxidation and basal metabolic rate by LCFA.

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