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Improvement of Mitochondrial Function in Muscle of Genetically Obese Rats after Chronic Supplementation with Proanthocyanidins

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ABSTRACT: The aim of this study was to determine the effect of chronic dietary supplementation of a grape seed proanthocyanidin extract (GSPE) at a dose of 35 mg/kg body weight on energy metabolism and mitochondrial function in the skeletal muscle of Zucker obese rats. Three groups of 10 animals each were used: lean *Fa/fa* lean group (LG) rats, a control *fa/fa* obese group (OG) of rats, and an obese supplemented *fa/fa* proanthocyanidins obese group (POG) of rats, which were supplemented with a dose of 35 mg GSPE/kg of body weight/day during the 68 days of experimentation. Skeletal muscle energy metabolism was evaluated by determining enzyme activities, key metabolic gene expression, and immunoblotting of oxidative phosphorylation complexes. Mitochondrial function was analyzed by high-resolution respirometry using both a glycosidic and a lipid substrate. In muscle, chronic GSPE administration decreased citrate synthase activity, the amount of oxidative phosphorylation complexes I and II, and Nrf1 gene expression, without any effects on the mitochondrial oxidative capacity. This situation was associated with lower reactive oxygen species (ROS) generation. Additionally, GSPE administration enhanced the ability to oxidize pyruvate, and it also increased the activity of enzymes involved in oxidative phosphorylation including cytochrome c oxidase. There is strong evidence to suggest that GSPE administration stimulates mitochondrial function in skeletal muscle specifically by increasing the capacity to oxidize pyruvate and contributes to reduced muscle ROS generation in obese Zucker rats.

KEYWORDS: proanthocyanidins, mitochondria, *fa/fa* rat, obesity, muscle, high-resolution respirometry

INTRODUCTION

Skeletal muscle accounts for >50% of the total body mass in an adult and, thus, metabolic alterations in this tissue have a major impact on the entire body.[1] In recent years, interest in skeletal muscle mitochondrial function has risen due to the discovery of mitochondrial dysfunction in a wide range of diseases; specifically type 2 diabetes mellitus, the aging process,[2] and obesity.[3] A reduction in mitochondrial mass and activity has been proposed to explain the mitochondrial dysfunction found in obese subjects with type 2 diabetes.[4]

Alterations in mitochondrial function may be central in explaining the metabolic changes and insulin resistance that characterize obesity and type 2 diabetes.[1] It has been described that the severity of insulin resistance in skeletal muscle in type 2 diabetes and obesity is associated with alterations in mitochondrial function[1] and decreased oxidative enzyme activity.[5−7]

Obesity is closely related to changes in mitochondrial gene expression, which impact its functionality.[8] The major metabolic abnormalities that have been identified in obesity are diminished fat oxidation and a greater dependence on glucose for ATP synthesis,[9] an excess of lipid accumulation in skeletal muscle fibres that is inversely associated with insulin sensitivity,[10] and a low basal ATP concentration, which has been related to smaller mitochondria with lower energy-generating capacity.[4] These differences not only favor the development and progression of obesity but also have serious implications in the treatment of obesity.[3]

Besides an intrinsic impairment in oxidative capacity, increased oxidative stress could produce insulin resistance[11] and impair glucose transport in muscles.[12] The superoxide anion ($^{-}O_2^{\bullet}$) is derived from several cellular sources, but the main contributor in skeletal muscle may be electron leakage from mitochondria.[13] At rest, approximately 0.1−0.2% of consumed oxygen is converted to reactive oxygen species (ROS).[14] Insulin-resistant animal models have higher levels of superoxide production,[15] and healthy individuals fed a high-fat diet have increased ROS generation in permeabilized muscle fibres without a change in mitochondrial respiration.[16]

Proanthocyanidins are the most abundant polyphenols in human diets.[17] The widespread presence of proanthocyanidins in plants makes them an important part of the human diet, as they are found in fruits, berries, beans, nuts, cocoa, and wine.[18] Epidemiological studies strongly suggest that proanthocyanidins protect against cardiovascular diseases.[19] Proanthocyanidins are known to be important antioxidant, anti-inflammatory agents[17] and reduce spongy cells, prevent aortic atherosclerosis, and improve endothelial function.[20]

In our laboratory, previous studies of acute high-dose GSPE administration to healthy Wistar rats has shown that mitochondria are the target of proanthocyanidins after 5 h of administration, mainly in skeletal muscle and brown adipose tissue (BAT), increasing the activity of key enzymes of energy metabolism in BAT, including citrate synthase and cytochrome *c* oxidase as well as increasing lipid and glycosidic oxidation in muscle.[21]

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With these data as background, we next wanted to study the effect of proanthocyanidins on mitochondrial function and therefore in the energy metabolism of Zucker rats, a genetic obesity model. We hypothesized that prolonged consumption of a moderate dose of proanthocyanidins would improve skeletal muscle mitochondrial function in the Zucker rat model. To test our hypothesis, we determined the enzyme activity of two enzymes involved in oxidative phosphorylation and the expression of key energy metabolic and mitochondrial biogenesis genes such as PGC1α, Sirt1, and Nrf1. We also assessed mitochondrial function using high-resolution respirometry.

MATERIALS AND METHODS

Proanthocyanidin Extract. Grape seed proanthocyanidin extract (GSPE) was kindly provided by Les Dérives Résiniques et Terpéniques (Dax, France). This proanthocyanidin extract is composed by the following proanthocyanidin fractions: monomers (21.3%), dimers (17.4%), trimers (16.3%), tetramers (13.3%), and oligomers (5−13 units) (31.7%). The proanthocyanidin extract that we used in the present study has been analyzed and characterized previously.[22]

Animals and Experimental Design. Twenty female Zucker Fatty Rats (*fa/fa*) and 10 heterozygous lean littermates (*Fa/fa*) were purchased from Charles River (Barcelona, Spain). Both genotypes arrived at 5 weeks of age and were housed in genotypic pairs in the animal quarters at 22 °C with a 12 h light/dark cycle (light from 8:00 a.m. to 8:00 p.m.), and they were fed ad libitum with a standard chow diet (Panlab 04, Barcelona, Spain) and tap water.

After 1 week of adaptation, the animals were divided into three groups of 10. The first group, termed the lean group (LG), contained *Fa/fa* lean rats. The other two groups were composed of *fa/fa* rats that were randomly divided into either a control obese group (OG) or an GSPE-supplemented proanthocyanidins obese group (POG), in which the rats were treated with a 35 mg dose of GSPE/kg of body weight/day, which was administrated in 0.6 mL of condensed skimmed milk (La Lechera, Spain; composition per 100 g: 8.9 g protein, 0.4 g fat, 60.5 g carbohydrates, 281 kcal) and diluted 1:6 with tap water. The diluted milk with and without proanthocyanidins was administrated daily at 4:00 p.m. during the last 68 days to each group. All experimental procedures were performed according to the national and institutional guidelines for animal care and use that are in place at our university.

Tissue Collection. On the last day of the study, the rats were anaesthetized with sodium pentobarbital and were sacrificed by abdominal aorta exsanguination using syringes containing heparin as an anticoagulant. The plasma was obtained by centrifugation, and it was stored at −80 °C until analysis. Samples of gastrocnemius muscle were rapidly excised, immediately frozen in liquid nitrogen, and stored at −80 °C until RNA extraction. Other parts of this tissue were placed on ice and processed for mitochondrial isolation for enzymatic assays and respiratory analysis. The protein concentration in the mitochondrial pellet was measured using fluorescamine (Fluram, Sigma, St. Louis, MO) and bovine serum albumin (BSA, Sigma) as standard.[23]

Plasma Triglycerides Assay. A commercial enzymatic colorimetric kit was used to determine plasma triglycerides (TGs) (QCA, Barcelona, Spain) according to the manufacturer's protocol.

Oxidative Phosphorylation in Isolated Mitochondria. Mitochondrial isolation from the gastrocnemius muscle for respiratory analysis was performed according to Hoeks et al.[2]

Freshly isolated gastrocnemius muscle mitochondria (0.2 mg of mitochondrial protein for pyruvate; 0.2 mg for glutamate, malate, and succinate; 0.5 mg of mitochondrial protein for carnitine + palmitoyl-CoA) were incubated in a medium according to the method of Hoeks et al.[2] The substrates used were 5 mM pyruvate, 2 mM carnitine plus 50 μM palmitoyl-CoA, and 10 mM glutamate plus 10 mM malate plus 10 mM succinate (state 2 respiration). State 3 of respiration was initiated by the addition of 450 μM ADP. State 4o of respiration was measured as the residual respiration following the addition of 1 μg/mL oligomycin. The uncoupled state (Unc), which is the maximum oxygen flux rate, was achieved by titration with the addition of 0.5 μM aliquots of the chemical uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). All substrates were dissolved in Milli-Q water, except oligomycin and FCCP, which were dissolved in 96% ethanol, and were purchased from Sigma.

The functionality of oxidative phosphorylation of ex vivo mitochondria was analyzed by measuring the oxygen consumption in different mitochondrial states polarographically as described by Hoeks et al.[2] at 37 °C using a two-chamber Oxygraph (Oroboros Instruments, Innsbruck, Austria). Data were expressed as nanomoles of O_2 per millilgram of mitochondrial protein per minute.

Enzymatic Assays. Mitochondrial fractions were isolated according to Fuster et al.[24] and used in several enzymatic assays.

Citrate synthase (CS) activity was measured as described in Srere's method,[25] measuring changes in absorbance at 412 nm in the presence of 5,5′-dithiobis(2-nitrobenzoate) (DTNB) 0.1 mM (dissolved in Tris-HCl 1 M, pH 8.1).

Cytochrome c oxidase (COX) activity was measured as described in Fuster et al.,[24] by monitoring changes in absorbance at 550 nm in the presence of the reduced substrate 0.056 mM ferrocytochrome *c* (Sigma).

ATPase activity measurements were performed according to the method of Penefsky and Bruist,[26] by monitoring changes in absorbance at 339 nm in a solution containing Tris-base 1 M, pH 7.5, 4 mM $MgSO_4$, 2 mM phosphoenolpyruvate (PEP), 62 kU/L lactate dehydrogenase (LDH), 0.048 mM NADH, 2 mM ATP, and 160 kU/L pyruvate kinase. This reaction began by the addition of the sample mitochondria extract to the solution; linear activity rates were obtained for at least 3 min.

Gene Expression. Total RNA from gastrocnemius muscle was obtained using an RNeasy Lipid Tissue Kit (Qiagen) following the manufacturer's protocol. cDNA was synthesized from 2 μg of total RNA using the TaqMan Reverse transcription kit. A total of 20 ng of cDNA was subjected to quantitative RT-PCR amplification using the TaqMan Universal 2X PCR Master Mix. Specific TaqManR Assay-On-Demand probes were used to study the level of expression for the different genes: UCP2 (Rn 00571166_m1), UCP3 (Rn 00565874_m1), PGC1α (Rn 00580241_m1), Sirt1 (Rn 01428093_m1), Nrf1 (Rn 01455958_m1), COX5a (Rn 00821806_m1), IDH3g (Rn 01757321_g1), Acadm (Rn 00566390_m1), and Casp3 (Rn 00563902_m1). Peptidylprolyl isomerase A (Ppia) (Rn 00690933_m1) was used as an endogenous control. Real-time quantitative PCR reactions were performed using the ABI Prism 7 300 SDS Real-Time PCR system. All reagents were purchased from Applied Biosystems, San Diego, CA.

Lipid Peroxidation Assay. A microplate assay kit for malondialdehyde (MDA) and 4-hydroxyalkenals (4-HAE) (Oxford Biomedical Research, Oxford, MI) was used. This assay is based on the reaction of the chromogenic reagent *N*-methyl-2-phenylindole (R1) with MDA and 4-hydroxyalkenals.

Immunoblotting. The relative quantification of representative subunits of each mitochondrial oxidative phosphorylation complex was measured in the mitochondrial fraction from gastrocnemius muscle. Samples were treated according to the protocol of the manufacturer (Mitosciences, Eugene, OR). The relative quantification was measured by 12% acrylamide/bis SDS-PAGE and immunoblotting detection as described by Paulson and Laemmli.[27] The proteins were transferred to a poly(vinylidene fluoride) membrane, which was blocked for 1 h with 5% milk in phosphate-buffered saline (PBS) Tween-20 0.1% on a room temperature shaker. After the blocking, we used a cocktail of five monoclonal antibodies (MS604; Mitosciences) in PBS with 0.1% milk overnight at 4 °C on a shaker. VDAC was used as a loading control, which is a mitochondrial protein that is generally used for this purpose,

and we assumed that VDAC is not affected by GSPE administration. The α-VDAC primary antibody (Sigma) was diluted 1:1000 in PBS Tween-20 0.1% and 0.1% milk overnight at 4 °C on a shaker. In all cases, the secondary antibody was applied with the appropriate anti-mouse IgG peroxidise-conjugated polyclonal antibody (Bio-Rad, Hercules, CA) and used at a concentration of 1:1 000 in 0.1% milk in PBS Tween-20 for 1 h at room temperature in a shaker. ImageJ software was used to perform the relative quantification.

Statistical Analysis. The results are reported as the mean ± SEM of 10 animals per group. The group means were compared with independent-sample Student's *t* test ($p \leq 0.05$) using SPSS for Windows v. 17.0 software (SPSS Inc., Chicago, IL). Only five rats per group were used for the analysis of high-resolution respirometry.

RESULTS

Zucker rats are a well-defined obesity model in which these animals develop hyperglycemia, hypertriglyceridemia, and hyperinsulinemia very early in life.[28,29] To confirm these abnormalities, we determined the levels of plasma triglycerides in LG and OG (75.38 ± 2.40 vs 612.68 ± 99.83 mg/dL, respectively; $p < 0.005$), and we found a great disparity in triglyceride levels, being much higher in OG than in LG, indicating that *fa/fa* rats were hypertriglyceridemic. A well-known role of GSPE is the ability to reduce plasma triglyceride levels in vivo in normal adult rats [30] and even in high-fat diet obese rats.[20] The chronic administration of a 35 mg/kg body weight/day GSPE dose in Zucker *fa/fa* rats slightly, but not significantly, decreased plasma triglyceride differences between OG and POG, respectively (612.68 ± 99.83 vs 582.26 ± 48.57 mg/dL). Moreover, chronic consumption of proanthocyanidins produced a moderate but significant 15.1% increase in food intake and consequently a slight, but significant, 7.4% increase in body weight.

GSPE Administration Enhances the Mitochondrial Respiration Using Pyruvate as a Substrate. The mitochondrial respiration results obtained using pyruvate as a substrate indicate that GSPE administration significantly increases state 3 (ADP-stimulated respiration), state 4o (oligomycin dependent respiration), and the Unc state (uncoupled state, respiration stimulated by FCCP); however, state 2 remained unchanged (Figure 1A). From these results, we calculated the uncoupling control ratio (UCR), which is defined as state 3 divided by the Unc state, and the respiratory control ratio (RCR), which is defined as state 3 divided by state 4o. The UCR values were higher in OG than in POG mitochondria (0.79 ± 0.012 vs 0.73 ± 0.018*, respectively; $p = 0.018$). Although no significant difference between OG and POG was found in the RCR values (28.6 ± 3.1 vs 23.9 ± 0.9, respectively; $p = 0.226$), respiratory control ratios of pyruvate yielded high RCR values, which indicate high-quality mitochondria.[31] These results suggest that chronic GSPE administration improves the capacity to oxidize pyruvate as a substrate in muscle mitochondria (Figure 1A).

The mitochondrial respiration levels using carnitine in combination with palmitoyl-CoA as substrate were significantly increased in two mitochondrial states: state 2 CPCoA (respiration stimulated by carnitine and palmitoyl-CoA addition) and state 4o (Figure 1B). No differences were found between OG and POG for both the UCR (0.80 ± 0.06 vs 0.83 ± 0.02, respectively; $p = 0.621$) and RCR (2.76 ± 0.58 vs 1.80 ± 0.29, respectively; $p = 0.162$) values.

The use of glycosidic and lipidic substrates exhibited higher levels of mitochondrial respiration in state 4o, indicating that

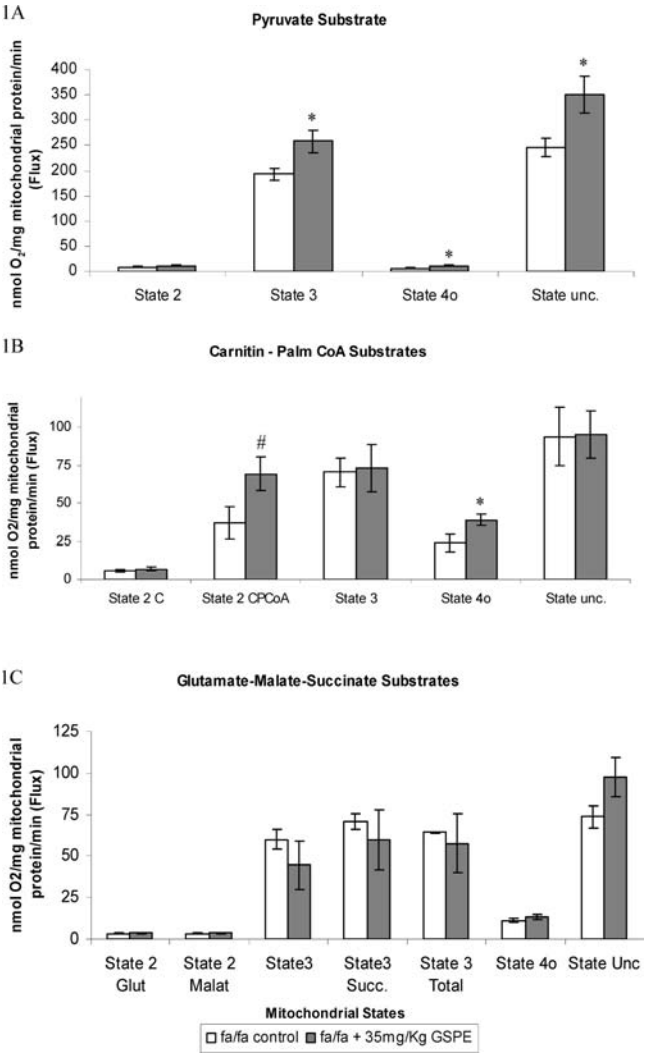

Figure 1. Oxygen flux of oxidative phosphorylation of gastrocnemius muscle mitochondria respiring on incubation medium plus (A) pyruvate, (B) palmitoyl-CoA + carnitine, and (C) glutamate + malate + succinate as substrates in different mitochondrial states. White bars represent *fa/fa* control rats, whereas gray bars represent the *fa/fa* rats treated with 35 mg GSPE/kg body weight. Values are expressed as the mean ± SEM ($n = 5$ per group). ∗ indicates a significant difference versus *fa/fa* control group (Student's *t* test, $p < 0.05$). # indicates a significant difference versus *fa/fa* control group (Student's *t* test, $p < 0.1$).

chronic GSPE administration induces a slight increase in basal uncoupling in skeletal muscle mitochondria.

The use of glutamate, malate, and succinate as substrates allowed us to evaluate the full citric acid cycle function. There were no significant differences between OG and POG in any of the mitochondrial states (Figure 1C). Similarly, for OG and POG, the UCR (0.80 ± 0.05 vs 0.58 ± 0.17, respectively; $p = 0.279$) and RCR (5.90 ± 0.32 vs 4.17 ± 1.15, respectively; $p = 0.214$) values were unaltered. These results indicate that chronic GSPE administration does not affect the citric acid cycle functionality.

Chronic GSPE Administration Decreased Total CS Activity While Significantly Increasing COX Activity. We determined the enzymatic activity of CS, COX, and ATPase in mitochondria isolated from gastrocnemius muscle in the OG and POG groups

Table 1. Total Enzyme Activity (nkat/g Tissue) [a]

	fa/fa	
total activity/ratio activity	*fa/fa* control	*fa/fa* + GSPE[b]
citrate synthase (CS) (nkat/g)	8.84 ± 0.64	5.17 ± 0.79 *
COX/CS[c]	1.88 ± 0.23	4.93 ± 1.49 ◆
ATPase/CS [d]	0.20 ± 0.02	0.38 ± 0.15

[a] Values are the mean ± SEM. [b] * indicates a significant difference ($p < 0.05$) and ◆ indicates a significant difference ($p < 0.10$) between groups. [c] Calculated ratio between COX activity and citrate synthase activity to represent the COX activity by mitochondrial content (arbitrary units). [d] Calculated ratio between ATPase activity and citrate synthase activity to represent the ATPase activity by mitochondrial content (arbitrary units).

to compare the effect of chronic GSPE administration on several marker enzymes of mitochondrial density (CS)[32,33] and oxidative phosphorylation functionality (COX and ATPase).

The total gastrocnemius muscle CS activity results reveal that chronic GSPE administration causes a decrease in CS activity. These results suggest a lower mitochondrial density in the muscle (Table 1).

To calculate the ratio of activities within COX/CS, we also determined the total COX activity in the gastrocnemius muscle and divided it by the CS activity to express COX activity by the mitochondrial content, as described by Garcia-Ramirez et al.[32] The results show a significant increase in the COX/CS ratio, indicating that GSPE administration produced an increase in muscle COX activity (Table 1).

Similarly, we analyzed the total ATPase activity in gastrocnemius muscle and divided it by CS to express ATPase activity by mitochondrial content. The results show that the GSPE supplementation caused no significant differences (Table 1).

Chronic Administration of GSPE Significantly Decreased Gene Expression of the Transcription Factor Nrf1 in *fa/fa* Rat Muscle. The gene expression data from gastrocnemius muscle (Table 2) demonstrated that chronic GSPE administration significantly decreased the expression of the Nrf1 transcription factor, whereas the genetic expression of other genes including PGC1α, COX5α, IDH3γ, and Acadm, tended to decrease slightly, but were nonetheless significant. Other genes such as UCP2 and UCP3, which are related to the protection against ROS, and other genes such as Casp3, Bax, and Bcl2, which are involved in the apoptotic process, were unchanged in response to GSPE administration in skeletal muscle.

GSPE Administration Decreases the Production of Intramuscular Lipid Peroxides. We also evaluated the amount of intramuscular lipid peroxide (MDA + 4-HAE) to study the antioxidant effect of GSPE on intramuscular ROS production. The results revealed a significantly lower content of MDA and 4-HAE in the gastrocnemius muscle in Zucker rats supplemented with proanthocyanidins (Figure 2).

The Number of Oxidative Phosphorylation Complexes I and II Was Diminished by Chronic GSPE Administration in Muscle Mitochondria of Obese Rats. The OXPHOS complex results from *fa/fa* rat muscle (Figure 3) demonstrated that GSPE administration caused a remarkable decrease in the concentration of some complexes but not others. In particular, complex I (NADH dehydrogenase) and complex II (succinate-ubiquinone oxidoreductase) were significantly reduced due to GSPE administration, whereas complex III (ubiquinol-cytochrome oxidoreductase), complex IV (cytochrome *c* oxidase), and complex V (ATP synthase) tended to slightly increase their amount in the mitochondrial inner membrane, although the difference was insignificant.

Table 2. mRNA Levels of Energetic Metabolism Related Genes in the Gastrocnemius Muscle of *fa/fa* Rats with and without GSPE Administration[a]

	fa/fa	
gene	*fa/fa* control	*fa/fa* + GSPE
Ucp2	1.01 ± 0.05	1.03 ± 0.11
Ucp3	1.01 ± 0.02	1.09 ± 0.07
PGC1α	1.02 ± 0.07	0.85 ± 0.09
Sirt1	1.02 ± 0.06	0.96 ± 0.10
Nrf1	1.02 ± 0.07	0.76 ± 0.04 *
Cox5α	1.03 ± 0.09	0.90 ± 0.11
Idh3γ	1.03 ± 0.10	0.85 ± 0.08
Acadm	1.04 ± 0.10	0.81 ± 0.08
Casp3	1.00 ± 0.02	0.94 ± 0.10
Bax	1.07 ± 0.06	1.04 ± 0.10
Bcl2	1.01 ± 0.06	1.01 ± 0.09

[a] Abbreviations: GSPE, grape seed proanthocyanidin extract; Ucp3, uncoupling protein 3, PGC1α, peroxisome proliferator-activated receptor γ, coactivator 1 α; Sirt1, silent mating type information regulation 2 homologue 1; Nfr1, nuclear respiratory factor 1; Cox5α, cytochrome *c* oxidase, subunit Va; Idh3γ, isocitrate dehydrogenase 3 NAD, γ; Acadm, acyl-coenzyme A dehydrogenase C-4 to C-12 straight chain; Casp3, caspase 3; Bax, Bcl2-associated X protein; Bcl2, B-cell lymphoma 2. Each value represents the mean ± SEM, and they are expressed as fold change, using PPIA expression as the endogenous control. * indicates a significant difference ($p \leq 0.05$) between groups.

DISCUSSION

Several groups have studied the effects of polyphenols, including resveratrol[34] and flavonoids such as quercetin,[35,36] on muscle mitochondria and have suggested that they produce an increase in mitochondrial function, which leads to increased energy expenditure, improved aerobic capacity, and increased sensorimotor muscle function. However, little is known about the role of proanthocyanidins on mitochondrial function and energy metabolism in skeletal muscle. Thus, in this study, we used genetically obese rats to determine if grape seed proanthocyanidins correct the dysfunction in muscular energy metabolism after 68 days of GSPE administration at moderate doses.

Previous studies have demonstrated the versatility of proanthocyanidins in the correction and prevention of metabolic diseases including cardiovascular disease by reducing circulating lipids[20] and reducing atherosclerotic risk modulating the small heterodimer partner (SHP), a key regulator of lipid homeostasis in liver.[30] The beneficial effect of proanthocyanidins as an antioxidant agent[37,38] and as an anti-inflammatory[39] was also reported.

GSPE consumption decreased plasma triglycerides under different conditions including the acute dose[30] and the chronic dose in cafeteria diet-induced obesity.[20] Chronic administration of GSPE slightly, but not significantly, decreased plasma triglycerides in genetically obese Zucker rats, similar to a study performed with a red wine polyphenols extract at a dose of 20 mg/kg of body weight/day.[29]

Proanthocyanidins are well tolerated in rats and devoid of marked toxicity.[40] As a matter of fact, no significant adverse

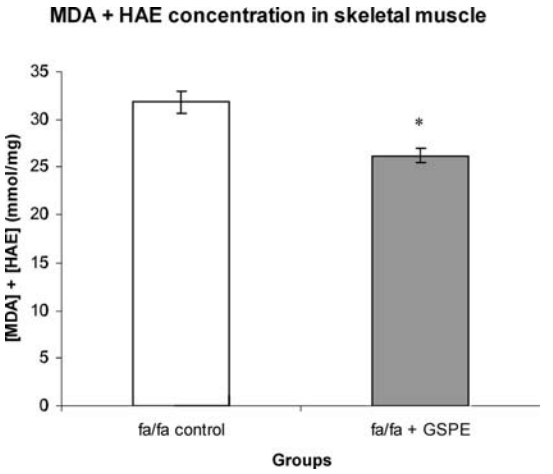

Figure 2. Concentration of gastrocnemius muscle homogenate polyunsaturated fatty acid peroxides: malondialdehyde (MDA) and 4-hydroxyalkenals (HAE) of *fa/fa* rats with and without GSPE administration. Each value represents the mean ± SEM ($n = 10$). * indicates a significant difference ($p \leq 0.05$) between groups.

effects have been evidenced in rats; even a dosage of 2% (w/w) (equal to 1501 mg/kg body weight/day in females) for 90 days did not induce noticeable signs of toxicity.[41] Nevertheless, mitochondrial toxicity has been suggested for proanthocyanidins in malignant tumor cells [42] through collapse of the mitochondrial membrane potential.

CS activity is a common marker of mitochondrial density.[32,33] GSPE administration caused a decrease in CS activity, suggesting that GSPE causes a decrease in the gastrocnemius muscle mitochondrial density in the POG group. One possible explanation could be that GSPE administration at a dose of 35 mg/kg may have pro-apoptotic activity, as occurs with high doses of resveratrol[43] and EGCG.[44,45] We determined Casp3, Bax, and Bcl2 as marker genes of the mitochondrial apoptosis process; however, our gene expression data show no significant differences and cannot confirm the suggested increase in apoptosis.

COX activity significantly increased in rats supplemented with GSPE. This fact, taking into account that small variations in the activity could cause important changes, suggests the possibility that proanthocyanidins may enhance the activity of oxidative phosphorylation in POG skeletal muscle.

Results obtained by other groups using mitochondria isolated from *fa/fa* rat gastrocnemius muscle[46] and soleus muscle[47] showed that there was no difference in the levels of oxygen consumption, suggesting that the *fa/fa* obesity rat model is not associated with mitochondrial dysfunction in skeletal muscle.

In the present study, mitochondrial respiration was studied in three different situations: (a) with pyruvate as a substrate, which allowed us to assess the functionality of the pyruvate transport system and the pyruvate dehydrogenase complex; (b) with carnitine and palmitoyl-CoA as a substrate, which allowed us to evaluate the ability to transport fatty acids into the mitochondria via the carnitine palmitoyl transferase (CPT) and the β-oxidation of fatty acids within mitochondria; and (c) with glutamate, malate, and succinate as substrates, which are also the three substrates that complete the citric acid cycle and allowed us to evaluate the electron transport and the entry of electrons into complexes I and II of the ETC.

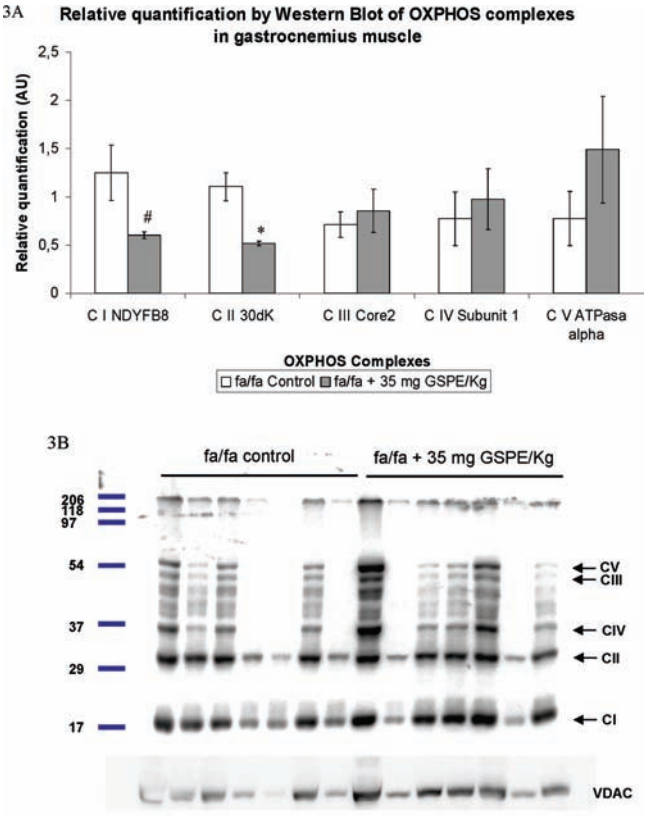

Figure 3. (A) Relative Western blot quantification of the oxidative phosphorylation complexes. White bars represent *fa/fa* control rats, whereas gray bars represent *fa/fa* rats treated with 35 mg GSPE/kg body weight. Values are expressed as the mean ± SEM ($n = 5$ per group). * indicates a significant difference versus the *fa/fa* control group (Student's t test, $p < 0.05$). # indicates a significant difference versus the *fa/fa* control group (Student's t test, $p < 0.1$). (B) Example of a Western blot image analysis that relatively quantified the amount of OXPHOS complexes.

The high-resolution respirometry performed in mitochondria isolated from gastrocnemius muscle showed a marked increase in state 3, state 4o, and Unc state respiration levels using pyruvate as a substrate (see Figure 4). State 3 respiration stimulated by the addition of ADP provides information on the mitochondrial capacity to oxidize NADH and $FADH_2$ from the citric acid cycle and β-oxidation in the ETC, resulting in oxidative phosphorylation of ADP to synthesize ATP by ATPase.[48] Under physiological conditions, state 3 respiration is limited by or under the control of the phosphorylation system activity (including ATPase and ANT).[48] Oligomycin state 4o provides insight into the oxygen consumption that is occurring in nonphosphorylative conditions, which evaluated basal mitochondrial uncoupling. Oxygen consumption is thus proportional to the intensity of the proton leak and the capacity of the respiratory chain to generate and maintain the membrane potential.[49] The Unc state is achieved with the addition of FCCP,[48] a chemical uncoupler that creates pores in the mitochondrial inner membrane, causing the entry of protons into the mitochondrial matrix and avoidance of the phosphorylation system (complex V). The Unc state respiration levels indicate the ETC maximum capacity and the dehydrogenase enzymes (such as pyruvate dehydrogenase) are involved depending on the substrate provided. Therefore, this

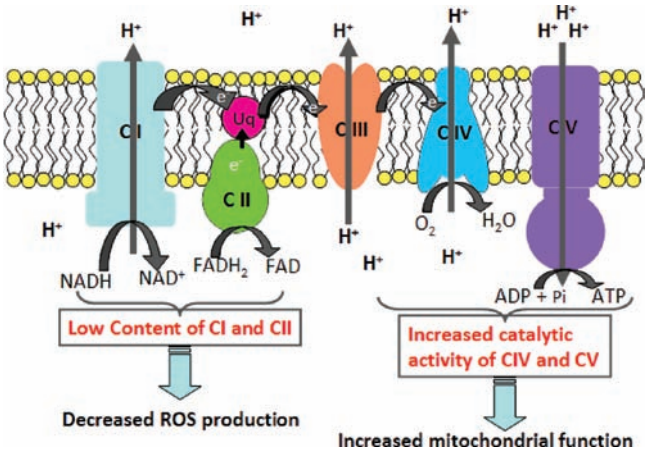

Figure 4. Schematic figure summarizing the main findings in this study. ETC complexes CI, CII, and CIII are ROS producers, and the low content of CI and CII caused by the administration of GSPE is directly linked to reduced lipid peroxidation in the gastrocnemius muscle homogenate. In addition, under physiological conditions, state 3 respiration is limited by or under the control of the activity of the phosphorylation system (including ATPase and ANT). The fact that GSPE supplementation increased the enzyme activity of the complex C IV (COX) and CV (ATPase) promotes improved mitochondrial function.

Unc state may refer to the maximum uncontrolled mitochondrial oxidative capacity.[48]

As expected, we consistently observed that the FCCP-induced uncoupling Unc state goes farther than the oxygen consumption levels of state 3 mitochondrial respiration, indicating that the phosphorylation system capacity (mainly determined by the ATP synthesis and the activity of ANT1) controls the maximum flow through the ETC. This finding was observed both with pyruvate and palmitoyl-CoA as substrate.[46]

The high-resolution respirometry results, using a combination of carnitine and palmitoyl-CoA as substrates, revealed that proanthocyanidins caused higher state 2 respiration levels, suggesting an improvement in fatty acid transport into the mitochondria and/or an increased capacity for fatty acid oxidation by β-oxidation.[20] Moreover, in parallel studies, we observed a significant increase in both the activity and the expression of CPT-1 in rats acutely treated with a dose of 250 mg GSPE/kg body weight (data not shown), suggesting that GSPE administration improves fatty acid transport into the mitochondria, with the consequent facilitation of β-oxidation. These results are similar to those of Lagouge et al.,[34] who studied the effect of resveratrol treatment in mice with increased aerobic capacity and endurance during exercise.

Notably, by using pyruvate and carnitine plus palmitoyl-CoA, we obtained higher levels of state 4o respiration in POG. In other words, chronic GSPE administration resulted in significantly higher basal gastrocnemius muscle mitochondria uncoupling. Additionally, the gene expression levels of UCP2 and UCP3 were not altered by GSPE supplementation. Although the literature has reported uncoupling effects of flavonoids on artificial membranes in vesicles[50] and in cells in vitro,[51] the present data show that GSPE also produced a slight but significant ex vivo uncoupling in mitochondria isolated from the gastrocnemius muscle of *fa*/*fa* rats using both pyruvate and carnitine plus palmitoyl-CoA as substrate.

The higher basal uncoupling together with a greater oxidative mitochondrial capacity may partially explain the observed decrease in plasma triglyceride levels. On the other hand, the consumption of proanthocyanidins induces a small increase in food intake accompanied by a slight increase in body weight, which does not correlate well with the observed increase of mitochondrial activity, but that can be explained by the decrease in mitochondrial density observed.

To test whether chronic GSPE supplementation had an impact on the OXPHOS complexes, we used Western blotting to evaluate the relative amount of each of the OXPHOS complexes. Interestingly, chronic GSPE administration resulted in a drastic decrease in OXPHOS complexes I and II. This decrease in the amount of complexes I and II did not affect the normal functioning of mitochondria, which, far from diminishing their functionality, increased their capacity to oxidize pyruvate and carnitine-palmitoyl-CoA. Moreover, this decrease in the amount of complexes I and II correlated with the reduced gene expression of the Nrf1 transcription factor, which is a nuclear factor that activates the expression of OXPHOS components, mitochondrial carrier, and ribosomal proteins.[52]

The mitochondrial ETC leads to ROS production, and complexes I and II are two sources of ROS generation.[53,54] The fact that POG exhibits fewer complexes I and II may be indicative of lower ROS production. Thus, we set out to evaluate the production of intramuscular ROS by determining the concentration of lipid peroxides (MDA + 4-HAE) in muscle. Our results showed that intramuscular lipid peroxidation was lower in GSPE-supplemented *fa*/*fa* rats, but did not compromise mitochondrial function.

In conclusion, our results show that chronic GSPE administration resulted in a significant improvement in skeletal muscle mitochondrial function, both in the ability to oxidize pyruvate and in the oxidation of fatty acids, such as palmitoyl-CoA. In addition, proanthocyanidins caused a slight mitochondrial uncoupling related to their known chemical properties and antioxidant effects. Chronic GSPE supplementation causes a sharp decrease in the amount of complexes I and II of the ETC, a fact that may be associated with a lower production of ROS in muscle. Importantly, this decrease in complexes I and II has not affected the normal muscle mitochondria function.

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ABBREVIATIONS USED

GSPE, grape seed proanthocyanidin extract; LG, lean group; OG, obese group; POG, proanthocyanidins obese group; OXPHOS, oxidative phosphorylation; ETC, electron transport chain; MDA,

malondialdehyde; 4-HAE, 4-hydroxyalkenals; Ucp3, uncoupling protein 3; PGC1α, peroxisome proliferator-activated receptor γ, coactivator 1 α); Sirt1, sirtuin (silent mating type information regulation 2 homologue 1; Nrf1, nuclear respiratory factor 1; Cox5a, cytochrome *c* oxidase, subunit Va; Idh3γ, isocitrate dehydrogenase 3 (NAD)γ; Acadm, acyl-coenzyme A dehydrogenase, C-4—C-12 straight chain; Casp3, caspase 3; Bax, Bcl2-associated X protein; Bcl2, B-cell lymphoma 2; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; PVDF, poly(vinylidene fluoride); PBS, phosphate-buffered saline.

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