JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Acute Administration of Grape Seed Proanthocyanidin Extract Modulates Energetic Metabolism in Skeletal Muscle and BAT Mitochondria

D. Pajuelo, S. Díaz, H. Quesada, A. Fernández-Iglesias, M. Mulero, A. Arola-Arnal, M. J. Salvadó, C. Bladé, and L. Arola*

Nutrigenomics Group, Universitat Rovira i Virgili, Tarragona, Spain

ABSTRACT: Proanthocyanidin consumption might reduce the risk of developing several pathologies, such as inflammation, oxidative stress and cardiovascular diseases. The beneficial effects of proanthocyanidins are attributed to their antioxidant properties, although they also can modulate gene expression at the transcriptional level. Little is known about the effect of proanthocyanidins on mitochondrial function and energy metabolism. In this context, the objective of this study was to determine the effect of an acute administration of grape seed proanthocyanidin extract (GSPE) on mitochondrial function and energy metabolism. To examine this effect, male Wistar rats fasted for fourteen hours, and then they were orally administered lard oil containing GSPE or were administered lard oil only. Liver, muscle and brown adipose tissue (BAT) were used to study enzymatic activity and gene expression of proteins related to energetic metabolism. Moreover, the gastrocnemius muscle and BAT mitochondria were used to perform high-resolution respirometry. The results showed that, after 5 h, the GSPE administration significantly lowers plasma triglycerides, free fatty acids, glycerol and urea concentrations. In skeletal muscle, GSPE lowers FATP1 mRNA levels and increases mitochondrial oxygen consumption, using pyruvate as the substrate, suggesting a promotion of glycosidic metabolism. Furthermore, GSPE increased the genetic expression of key genes in energy metabolism such as peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PGC1α), and modulated the enzyme activity of proteins, which are involved in the citric acid cycle and electron transport chain (ETC) in BAT. In conclusion, GSPE affects mainly the skeletal muscle and BAT mitochondria, increasing their oxidative capacity rapidly after acute supplementation.

KEYWORDS: mitochondria, polyphenols, proanthocyanidins, skeletal muscle, brown adipose tissue, UCP 1, PGC1α, Sirt1, citrate synthase, COX, ATPase

INTRODUCTION

Mitochondria play a central role in energy homeostasis and are the principal energy sources of the cell that convert nutrients into energy through cellular respiration.[1] Compromised mitochondrial function has been linked to numerous diseases, including those of the metabolic and cardiovascular systems.[2]

Evidence from several sources indicates that the failure of the mitochondria to produce energy from food substrates may be a common pathway in the development and perpetuation of obesity.[3] Furthermore, mitochondrial dysfunction helps to explain a number of the common signs and symptoms of obesity, including low energy expenditure; chronic food intake, in excess of expenditure; and the presence of markers for low-grade systemic inflammation. In recent years, the interest in skeletal muscle mitochondrial function has risen due to findings of a mitochondrial dysfunction in a wide range of disease from type 2 diabetes mellitus to the process of aging.[4] In fact, the mitochondria of type 2 diabetes patients demonstrate reduced electron transport chain (ETC) capacities and reduced citrate synthase (CS) activity.[5] These conditions may result from the varying energetic roles and needs of the different tissues. The variation in the individual and regional predisposition to obesity and type 2 diabetes mellitus may result from the interaction of modern dietary caloric intake with ancient mitochondrial genetic polymorphisms. Therefore, mitochondria may provide a targetable means to influence health, linking an environmental component such as diet and genetic components.[1]

Proanthocyanidins are a class of polyphenolic compounds that are one of the most ubiquitously distributed in the human diet. Proanthocyanidins are considered bioactive compounds as they influence physiological and cellular processes and, therefore, can have beneficial effects on health.[6] It has been suggested that proanthocyanidins play an important role in the prevention of some pathologies, such as obesity,[7] diabetes,[8] cardiovascular diseases and certain forms of cancer.[9] Moreover, proanthocyanidins have antioxidant[10−12] and anti-inflammatory properties[13] and can act like hypolipidemic agents.[14,15] Several authors have studied the effect some types of flavonoids, such as resveratrol[2] and quercetin,[16,17] have on energy metabolism and mitochondrial function. These studies suggest that these natural compounds increase the aerobic capacity of muscle tissue, improving mitochondrial function. This finding implies that these specific flavonoids have metabolic consequences in energetic homeostasis, helping to protect against obesity and body weight maintenance,[18] both in humans and in rats. Despite the prior

Received: January 25, 2011
Accepted: February 25, 2011
Revised: February 25, 2011
Published: March 14, 2011

research findings, very little is known about the effect of dietary administration of proanthocyanidins on mitochondrial function and energy metabolism *in vivo*.

In this context, the objective of this study was to determine whether grape seed proanthocyanidin extract (GSPE) has, as a target, the mitochondria of key tissues involved in energy homeostasis and how GSPE affects mitochondrial function and energy metabolism. The mitochondrial function was assessed by high-resolution respirometry, and energy metabolism was analyzed by gene expression and the enzymatic activities of key enzymes involved in the citric acid cycle and the ETC.

MATERIALS AND METHODS

Proanthocyanidin Extract. GSPE was kindly provided by Les Dérives Résiniques et Terpéniques (Dax, France). This proanthocyanidin extract contained monomeric (21.3%), dimeric (17.4%), trimeric (16.3%), tetrameric (13.3%) and oligomeric (5−13 units) (31.7%) proanthocyanidins.

Animals and Experimental Design. Nine-week-old male Wistar (Crl:Wi) rats, weighing 300−350 g, were purchased from Charles River (Barcelona, Spain). They were housed, in pairs, in animal quarters, at 22 °C with a 12 h light/dark cycle (light from 8:00 a.m. to 20:00 p.m.). The rats were fed *ad libitum* a standard chow diet (Panlab 04, Barcelona, Spain) and were provided tap water. After a week of adaptation, the rats were randomly divided into two groups: control and GSPE group ($n = 6$ each). On the day of the experiment, the rats were deprived of food for 14 h. To GSPE group was administered an oral gavage dose of 250 mg/kg of body weight of GSPE in a suspension of lard oil (2.5 mL/kg of body weight). The same gavage dose of lard oil was administered to control group. The Animal Ethics Committee of our University approved all procedures.

Tissue Collection. Five hours after administration, the rats were anesthetized with a combination of ketamine and xylazine. After anesthetization, the rats were sacrificed by exsanguination from the abdominal aorta. The removed blood was collected; plasma was extracted by centrifugation and stored at −80 °C until analysis. A portion of interscapular brown adipose tissue (BAT), the gastrocnemius muscle and liver were immediately excised and frozen in liquid nitrogen to be stored at −80 °C until RNA extraction. Another portion of these tissues was placed on ice and was used for mitochondrial isolation, as described by Fuster et al.,[19] to perform the enzymatic assays.

Moreover, the remaining gastrocnemius muscle and a portion of the BAT were rapidly utilized for mitochondrial isolation according to Hoeks et al.[4] and Cannon and Nedergaard,[20] respectively. Freshly isolated mitochondria were used immediately for mitochondrial respirometry. The remaining mitochondria were added to a protease inhibitor cocktail (P8340, Sigma) and were stored at −80 °C for further analysis. The protein concentration in the mitochondrial pellet was measured using fluorescamine (Fluram , Sigma); bovine serum albumin (BSA) was used as the standard.[21]

Blood Plasma Assays. Commercial enzymatic colorimetric kits were used to determine plasma metabolites: glucose, triglycerides (TGs) and urea (QCA, Barcelona, Spain), ketone bodies (IVD, BEN Srl - Italy), NEFAS (Wako chemicals GmbH) and glycerol (R-Biopharm AG, Roche, Germany).

Mitochondrial Respirometry. Mitochondrial isolation from the gastrocnemius muscle was performed according to Hoeks et al.,[4] using a buffer containing 100 mM sucrose, 50 mM KCl, 20 mM K^+-TES, 1 mM EDTA and 0.2% (w/v) BSA with proteinase subtilisin A (Sigma) 1 mg/g tissue. The homogenates were centrifuged at 8500*g* for 10 min at 4 °C, and the resulting pellets were resuspended with the same buffer and centrifuged at 800*g* for 10 min at 4 °C. The resultant supernatants were centrifuged at 8500*g* for 10 min, and the final pellets were resuspended with approximately 150 μL of isolation buffer in a small glass homogenizer.

Freshly isolated gastrocnemius muscle mitochondria (0.2 mg of mitochondrial protein for pyruvate; 0.2 mg for glutamate, malate and succinate; 0.5 mg for carnitine−palmitoyl-CoA) were incubated in a medium containing 100 mM sucrose, 20 mM K^+-TES (pH 7.2), 50 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 4 mM KH_2PO_4, 3 mM malate and 0.1% BSA. The substrates used were 5 mM pyruvate, 2 mM carnitine plus 50 μM palmitoyl-CoA and 10 mM glutamate plus 10 mM succinate (state 2 of respiration). State 3 of respiration was initiated by addition of 450 μM ADP. State 4o of respiration was measured as the residual respiration following addition of 1 μg/mL of oligomycin. The Uncoupled state (Unc), which is the maximum oxygen flux rate, was achieved by titration with 0.5 μM additions aliquots of the chemical uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP).[20]

Mitochondrial isolation from BAT was performed according to Cannon and Nedergaard.[20] Portions of mitochondrial protein from freshly isolated BAT mitochondria weighing 0.2 mg were used for pyruvate. The portions were incubated in a medium consisting of 100 mM KCl, 20 mM K-TES, 4 mM KH_2PO_4, 2 mM MgCl, and 1 mM EDTA and reached an equilibrium pH of 7.2.[20] Next, 5 mM pyruvate was added as substrate (state 2 of respiration). Then 3.2 mM GDP was added to inhibit the activity of UCP1, avoiding the uncoupling state. Later 450 μM ADP was added to obtain state 3 of respiration. State 4o of respiration was measured as the residual respiration following addition of 1 μg/mL of oligomycin. The Unc state was achieved by titration utilizing 0.5 μM additions of FCCP.

In addition, 0.5 mg of mitochondrial protein from freshly isolated BAT mitochondria was incubated in a medium consisting of 8 mM *N*-tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid buffer, pH 7.4, 50 mM KCl, 6 mM $MgCl_2$, 1 mM EDTA, 23 mM sucrose, 3.3 mM malate and 14 mM KH_2PO_4.[22] Mitochondrial recoupling was carried out in the previously described incubation solution, containing an additional 1.65 mM adenosine triphosphate (ATP), 1.65 mM L-carnitine and 5 μM CoASH.[22] This incubation received an additional 12.5 μM palmitoyl-CoA (state 2 of respiration). The Unc state was achieved by titration using 0.5 μM additions of FCCP.[22]

All substrates were dissolved in Milli-Q water, except oligomycin and FCCP, which were dissolved in 96% ethanol. The functionality of oxidative phosphorylation of *ex vivo* mitochondria was determined measuring oxygen consumption in different mitochondrial states polarographically as described in ref 20 at 37 °C using a two-chamber Oxygraph (Oroboros Instruments, Innsbruck, Austria), expressed as nmol of O_2 per mg of mitochondrial protein per minute.

Enzymatic Assays. The mitochondria extract was isolated according to Fuster et al.[19] and was used to analyze CS, cytochrome *c* oxidase (COX) and ATP synthase (ATPase) total activities. Tissues were homogenized using a Teflon and glass homogenizer at 4 °C in a 1:3 (w:v) buffer containing 250 mM sucrose, 1 mM EDTA and 10 mM Tris-HCl, pH 7.4. After this step, the homogenates were centrifuged at 700*g* for 10 min at 4 °C; the supernatants were collected and centrifuged at 12000*g* for 10 min at 4 °C. The resulting pellet that contained the purified mitochondria was resuspended in 100 μL of a buffer composed of 70 mM sucrose, 220 mM mannitol, 2 mM HEPES and 1 mM EDTA, pH 7.4.

CS activity was measured as describe by Srere's procedure,[23] measuring changes in absorbance at 412 nm in presence of 5,5′-dithiobis-(2-nitrobenzoate) (DTNB) 0.1 mM (dissolved in Tris-HCl 1 M, pH 8.1). COX activity was measured as described in ref 19, by monitoring changes in absorbance at 550 nm in the presence of the reduced substrate 0.056 mM ferrocytochrome *c* (Sigma). ATPase activity measurement was performed according to Penefsky and Bruist,[24] by monitoring changes in absorbance at 339 nm in a solution containing

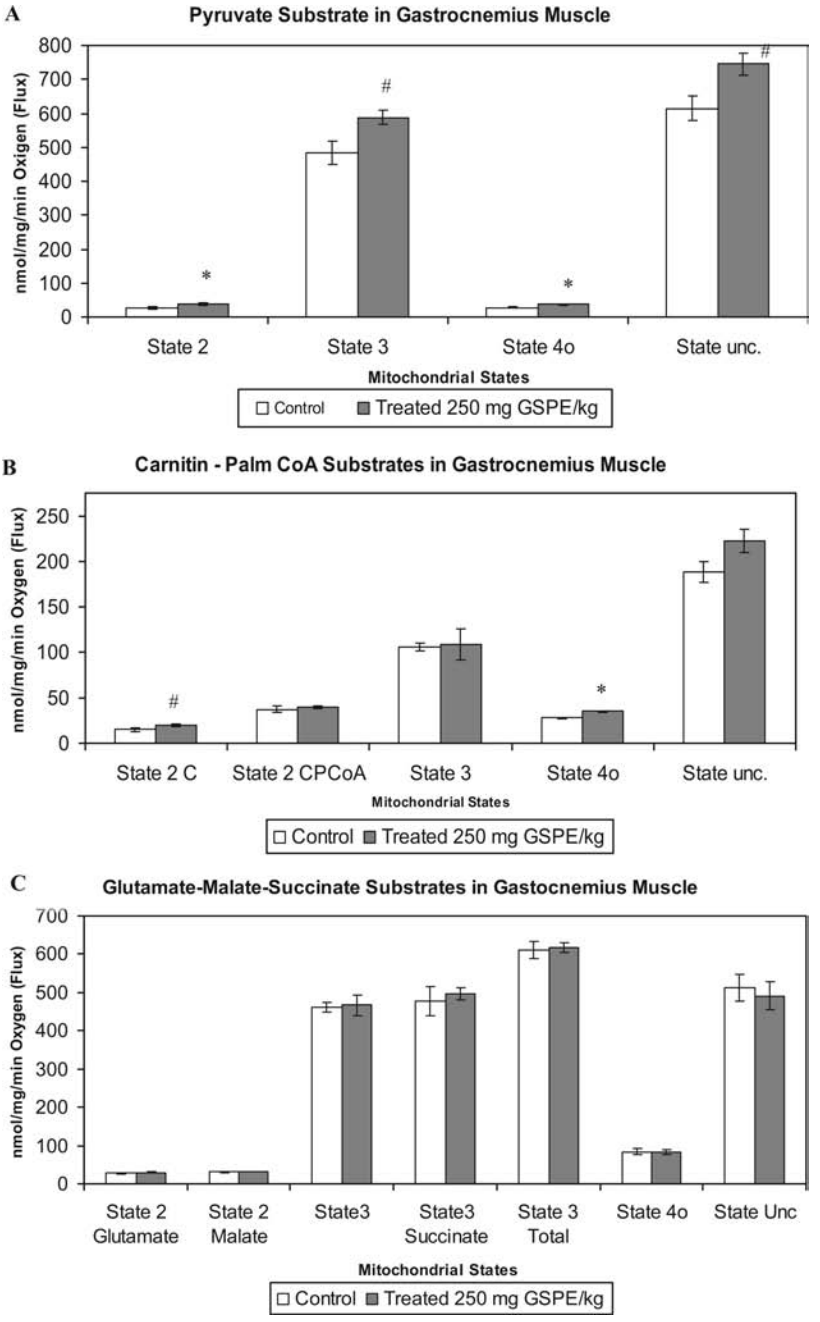

Figure 1. Oxygen flux of oxidative phosphorylation of gastrocnemius muscle mitochondria respiring on incubation medium plus (A) pyruvate, (B) palmitoyl-CoA + carnitine and (C) glutamate + malate + succinate as a substrate in different mitochondrial states. White bars represent control rats while gray bars represent the rats treated with 250 mg of GSPE/kg body weight. Values are expressed as means ± SEM ($n = 6$ per group). * indicates a significant difference versus control group (Student's t test, $p < 0.05$). # indicates a significant difference versus control group (Student's t test, $p < 0.1$).

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, 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 three minutes.

Gene Expression. Liver, BAT and muscle total RNA was obtained using the RNeasy Lipid Tissue Kit (QIAGEN), following the manufacturer's protocol. Synthesis of cDNA was obtained from 2 μg of total RNA using the TaqMan Reverse transcription reagent kit (High Capacity cDNA Reverse Transcription kit, Applied Biosystems). Gene expression levels were first quantified by a custom low-density array card (TLDA) (Applied Biosystems TaqMan) using 100 μL (6 ng/μL) of each cDNA sample and adding the same volume of 2X Taqman Universal PCR Master Mix (Applied Biosystems). After gentle mixing, the mixture was transferred into a loading port on a TLDA. Each low-density array card has eight separated loading ports that feed into 48-gene sets. Each 2 μL well contains specific user-defined probes, capable of detecting a single gene. Each set of 48 genes also contains three housekeeping genes, RPLP2, Ppia and 18S. However, we used Ppia (as a housekeeping gene) to evaluate the results of this study.

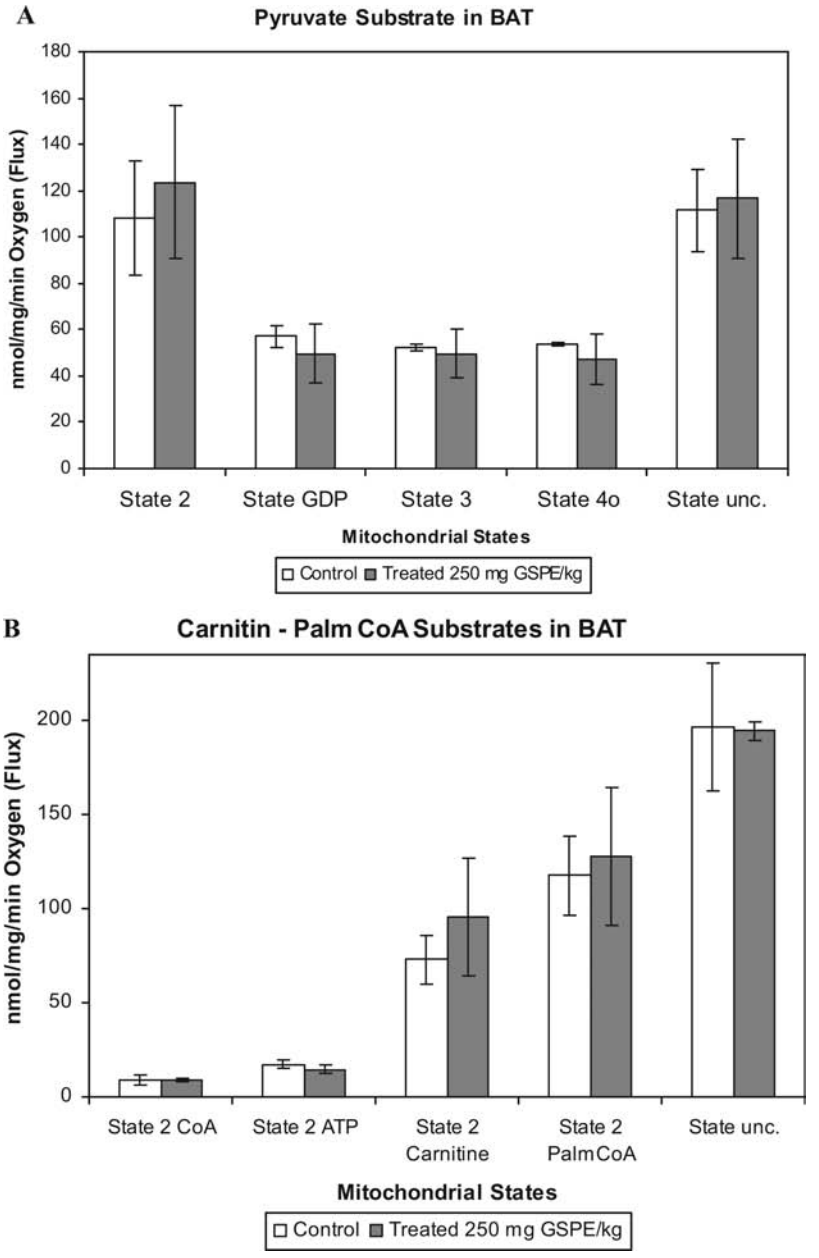

Figure 2. Oxygen flux of oxidative phosphorylation of BAT mitochondria respiring on incubation medium plus (A) pyruvate, (B) palmitoyl-CoA + carnitine as a substrate in different mitochondrial states. White bars represent control rats while gray bars represent the rats treated with 250 mg of GSPE/kg body weight. Values are expressed as means ± SEM ($n = 6$ per group). * indicates a significant difference versus control group (Student's t test, $p < 0.05$). # indicates a significant difference versus control group (Student's t test, $p < 0.1$).

The array was centrifuged twice for a minute at 1200 rpm to disperse the samples from the loading port into each well. The card was then sealed and PCR amplification was performed using an Applied Biosystems Prism 7900HT sequence detection system. Thermal cycling conditions were as follows: 2 min at 50 °C, 10 min at 94.5 °C, 30 s at 97 °C and 1 min at 59.7 °C repeated for 40 cycles.[25]

Genes having relative quantification differing significantly from control samples were checked with quantitative RT-PCR amplification of 20 ng of the corresponding cDNA samples using TaqMan Universal 2X PCR Master Mix (Applied Biosystems). Specific TaqManR Assay-On-Demand probes (Applied Biosystems) were used to study the level of expression for different genes. Cyclophilin A (Ppia) was used as an endogenous control. Real-time quantitative PCR reactions were performed using the ABI Prism 7300 SDS Real-Time PCR system (Applied Biosystems).[25]

Statistical Analysis. Results are reported as the mean ± SEM of six animals. Group means were compared with an independent-samples Student's t test ($P \leq 0.05$) using SPSS version 17.0 for Windows (SPSS, Inc., Chicago, IL, USA).

RESULTS

GSPE Administration Enhanced Pyruvate Oxidation in Muscle Mitochondria Whereas BAT Mitochondria Were Not Affected. *Skeletal Muscle (Gastrocnemius).* The results obtained using pyruvate as a substrate indicate that GSPE significantly increased state 2 (respiration stimulated by pyruvate) and state 4o (oligomycin dependent respiration) of mitochondrial respiration

while it tended to increase state 3 (ADP-stimulated respiration), and the Unc state (uncoupled state, respiration stimulated by FCCP) (Figure 1A). These results suggest that the GSPE supplementation enhance pyruvate oxidation in muscle mitochondria.

The uncoupling control ratios (UCR, state 3 divided by the Unc state respiration) and respiratory control ratios (RCR, state 3 divided by state 4 respiration) were derived from results of these data. The average value of UCR was similar between the control and the GSPE group (0.79 ± 0.02 and 0.81 ± 0.01, respectively). A similar trend for values of RCR emerged, which had no significant differences between control and treated groups (18.01 ± 0.92 and 17.11 ± 0.99, respectively). RCR on pyruvate yielded high RCR values, indicative of high quality mitochondria.[26]

The results obtained using carnitine−palmitoyl-CoA as substrates indicated that GSPE administration tended to increase the 2C state (respiration stimulated by carnitine) and significantly increased the 4o state (Figure 1B). Neither the 2CPCoA state, state 3 nor the Unc state was significantly affected by GSPE administration (Figure 1B). These results indicate that GSPE administration led to an increase of basal proton leak in nonphosphorylation (oligomycin dependent state 4) conditions.

Both the UCR and the RCR values did not show significant differences between the control and the treatment groups: 0.56 ± 0.02 and 0.63 ± 0.07, respectively, for UCR and 3.55 ± 0.10 and 3.05 ± 0.51, respectively, for RCR. Levels of UCR and RCR are very similar to those obtained using the protocol with pyruvate as the substrate.

The results obtained using glutamate−malate−succinate as substrates showed that there were no significant differences between the two groups in any of the states of mitochondrial respiration (Figure 1C). Similarly, UCR and RCR values were not significantly different in the control compared to the treatment group: 1.34 ± 0.12 and 1.22 ± 0.06, respectively, for UCR and 7.78 ± 0.62 and 7.33 ± 0.71, respectively, for RCR.

Brown Adipose Tissue. The results of mitochondrial respiration shown in Figures 2A and 2B indicated that all mitochondrial states were not significantly modified by GSPE administration using either pyruvate or carnitine−palmitoyl-CoA as substrates.

GSPE Supplementation Improves the Activity of the Key Mitochondrial Enzymes in BAT Whereas in Liver and Muscle Tissues Activity Was Not Affected. To determine whether the mitochondria and mitochondrial function are targets of GSPE, we evaluated the enzyme activities of CS, COX and ATPase, which are key enzymes present in the citric acid cycle and the oxidative phosphorylation pathway. These activities are measured in the three major tissues involved in energy homeostasis: liver, skeletal muscle (gastrocnemius) and BAT.

The results of total CS activity (Table 2) showed that GSPE tended to increase CS activity in BAT, whereas in liver and muscle tissue it was not affected by GSPE administration. These results suggest that GSPE could improve the citric acid cycle in BAT.

Besides, the results of total COX activity (Table 3) showed that GSPE significantly increased this activity in BAT. Neither muscle nor liver tissue revealed COX activity was affected by GSPE administration. These results suggest that GSPE could improve the flux of the ETC.

Finally, the results of total ATPase activity (Table 4) showed that this activity was not affected by GSPE administration in liver, muscle and BAT.

GSPE Supplementation Modulated the Expression of Some Energetic Metabolism Related Genes Primarily in BAT. In order to determine the expression of key genes that control the energetic metabolism in liver, muscle and BAT after 5 h of GSPE administration, a low density array card (TLDA) was used.

Genes tested coded for key proteins involved in thermogenesis (UCP1), fatty acid oxidation (Cpt1a, Cpt2), and other genes that are believed to defend against ROS production (UCP2, UCP3) as well as proteins that control cellular energy balance (AMPK, PGC1α) and the NAD^+-dependent deacetylase Sirt1. Additionally, in skeletal muscle pyruvate dehydrogenase genes (Pdhb) and fatty acid transport protein 1 (FATP1) were determined as marker genes of muscle catabolism.

The results of gene expression in the gastrocnemius muscle revealed no significant differences between most of the genes studied (Table 5). The GSPE treatment only significantly decreased the FATP1 gene expression.

In BAT the gene expression did not follow the same profile as the skeletal muscle. In this case, the results showed that PGC1α gene

Table 1. Results of Plasma Parameters in the Two Experimental Groups[a]

parameters	control group	250 mg/kg GSPE group[b]
glucose (mg/dL)	75.71 ± 6.81	87.66 ± 2.34
ketone-body (mg/dL)	39.05 ± 6.96	32.68 ± 7.47
lactate (mg/dL)	10.21 ± 1.51	10.92 ± 1.59
triglycerides (mg/dL)	106.85 ± 17.66	56.73 ± 5.08*
glycerol (mg/dL)	7.32 ± 1.32	4.26 ± 0.60#
free fatty acids (mg/dL)	31.72 ± 5.82	19.61 ± 2.15#
urea (mg/dL)	31.94 ± 1.99	24.11 ± 1.38*

[a] Values are means ± SEM. [b] * indicates a significant difference versus control group (Student's *t* test, $p < 0.05$). # indicates a significant difference versus control group (Student's *t* test, $p < 0.1$).

Table 2. Total Citrate Synthase Activity (nkat/g of Tissue)[a]

tissue	control group	250 mg/kg GSPE group[b]
liver	1.01 ± 0.27	0.71 ± 0.13
muscle	0.79 ± 0.39	0.61 ± 0.09
brown adipose tissue (BAT)	32.17 ± 2.68	40.36 ± 3.36#

[a] Values are means ± SEM. [b] # indicates a significant difference versus control group (Student's *t* test, $p < 0.1$).

Table 3. Total COX Activity (nkat/g of Tissue) [a]

tissue	control group	250 mg/kg GSPE group[b]
liver	17.49 ± 3.09	12.13 ± 1.73
muscle	3.98 ± 0.47	3.03 ± 0.66
brown adipose tissue (BAT)	26.56 ± 2.72	36.52 ± 3.37*

[a] Values are means ± SEM. [b] * indicates a significant difference versus control group (Student's *t* test, $p < 0.05$).

Table 4. Total ATPase Activity (nkat/g of Tissue)[a]

tissue	control group	250 mg/kg GSPE group
liver	6.07 ± 0.75	4.67 ± 0.92
muscle	1.45 ± 0.14	1.46 ± 0.27
brown adipose tissue (BAT)	6.29 ± 0.81	7.83 ± 0.82

[a] Values are means ± SEM.

Table 5. mRNA Levels of Energetic Metabolism Related Genes in Gastrocnemius Muscle of Control Rats and Treated Rats with GSPE 250 mg/kg Body Weight [a]

	rel expression	
genes	control group	250 mg/kg GSPE group[b]
Ucp2	1.02 ± 0.12	0.81 ± 0.18
Ucp3	1.08 ± 0.29	0.96 ± 0.17
PGC1α	1.16 ± 0.40	1.02 ± 0.16
Sirt1	1.02 ± 0.14	0.96 ± 0.06
Cpt1α	1.02 ± 0.14	0.87 ± 0.09
Cpt2	1.18 ± 0.42	0.78 ± 0.03
Prkaa2	1.09 ± 0.27	0.94 ± 0.20
Pdhb	1.06 ± 0.11	0.88 ± 0.13
FATP1	1.05 ± 0.12	0.69 ± 0.10*

[a] Abbreviations: GSPE, grape seed proanthocyanidin extract; Ucp2, uncoupling protein 2; Ucp3, uncoupling protein 3; PGC1α, Ppargc1α (peroxisome proliferator-activated receptor gamma, coactivator 1 alpha); Sirt1, sirtuin 1 (silent mating type information regulation 2 homologue 1); Cpt1a, carnitine palmitoyltransferase 1a; Cpt2, carnitine palmitoyltransferase 2; Prkaa2, protein kinase, AMP-activated, alpha 2 catalytic subunit; Pdhb, pyruvate dehydrogenase complex, beta subunit; FATP1, fatty acid transport protein 1. Values are the mean ± SEM and are expressed as fold change, using PPIA expression as the endogenous control. [b] * indicates a significant difference ($P \leq 0.05$) versus control group.

Table 6. mRNA Levels of Energetic Metabolism Related Genes in BAT of Control Rats and Treated Rats with GSPE 250 mg/kg Body Weight[a]

	rel expression	
genes	control group	250 mg/kg GSPE group[b]
Ucp1	1.09 ± 0.18	1.45 ± 0.26
Ucp2	1.02 ± 0.13	0.94 ± 0.05
Ucp3	1.02 ± 0.14	1.71 ± 0.63
PGC1α	1.01 ± 0.05	1.64 ± 0.24*
Sirt1	1.01 ± 0.06	0.81 ± 0.02*
Cpt1α	1.01 ± 0.11	1.07 ± 0.23
Cpt2	1.01 ± 0.09	0.96 ± 0.11
Prkaa2	1.03 ± 0.17	1.23 ± 0.19

[a] Abbreviations: Ucp1, uncoupling protein 1. Values are the mean ± SEM and are expressed as fold change, using PPIA expression as the endogenous control. Abbreviations and symbols as in Table 5. [b] * indicates a significant difference versus control group (Student's t test, $p < 0.05$).

expression increased significantly after the GSPE administration (Table 6). However, only UCP1, UCP3 and Prkaa2 tended to increase their expression after GSPE supplementation (Table 6). In contrast, Sirt1 was the only gene that decreased expression significantly after the GSPE administration (Table 6). The remaining genes were not altered by acute GSPE treatment (Table 6).

In the case of liver tissue, there is also a significant decrease in Sirt1 gene expression caused by acute GSPE treatment (Table 7). The others genes studied were not affected after acute treatment with GSPE, although notably mRNA levels of UCP2 and AMPK tended to decrease compared to control group (Table 7).

GSPE Administration Tended To Improve Plasma Lipid Profile. GSPE supplementation reduced significantly triglyceride

Table 7. mRNA Levels of Energetic Metabolism Related Genes in Liver of Control Rats and Treated Rats with GSPE 250 mg/kg Body Weight[a]

	rel expression	
genes	control group	250 mg/kg GSPE group[b]
Ucp2	1.01 ± 0.08	0.82 ± 0.07
PGC1α	1.03 ± 0.17	1.14 ± 0.40
Sirt1	1.00 ± 0.03	0.77 ± 0.02*
Cpt1α	1.00 ± 0.03	0.90 ± 0.07
Cpt2	1.01 ± 0.09	1.10 ± 0.14
Prkaa2	1.02 ± 0.13	0.77 ± 0.03

[a] Values are the mean ± SEM and are expressed as fold change, using PPIA expression as the endogenous control. Abbreviations and symbols as in Table 5. [b] * indicates a significant difference versus control group (Student's t test, $p < 0.05$).

(47%) and urea (25%) levels compared to the control group (Table 1), whereas the GSPE administration tended to reduce free fatty acids and glycerol levels (Table 1) compared to the control group. Additionally, glucose, lactate and ketone bodies concentrations (Table 1) were unaffected by GSPE supplementation. Thus, the GSPE administration demonstrates improved lipid metabolism after lard oil load.

DISCUSSION

Mitochondria convert nutrients into energy through cellular respiration. Compromised mitochondrial function has been linked to numerous diseases, including those of the metabolic and cardiovascular systems.[2]

Previous studies from our group have demonstrated that one of the main effects produced by GSPE administration in high doses was to decrease postprandial hypertriglyceridemia,[14,15] offsetting thereby one of the main risk factors of cardiovascular disease. In this study, we focused on whether homeostatic adjustment of energy metabolism may explain why GSPE supplementation tends to maintain circulating triglyceride levels despite being fed a diet high in fat; our group has observed this behavior repeatedly in previous *in vivo* experiments. These experiments have focused on the study of mitochondrial function in skeletal muscle and BAT. In addition, due to lack of knowledge about the effect of proanthocyanidins in mitochondrial function, we wanted to evaluate this effect by studying the gene expression and enzyme activity of key genes and enzymes related to the mitochondrial function.

Pursuant, we have evaluated the mitochondrial function by measuring the levels of oxygen consumption in different states of mitochondrial oxidative phosphorylation in *ex vivo* fresh isolated mitochondria from the gastrocnemius muscle and BAT. Mitochondrial respiration was studied in three different situations: (a) with pyruvate as substrate, which allowed us to evaluate the functionality of the transport system of pyruvate and the pyruvate dehydrogenase complex, (b) with carnitine and palmitoyl-CoA as substrates that allowed us to assess the ability to transport fatty acids into the mitochondria via the carnitine palmitoyl transferase (CPT) and the beta oxidation of fatty acids within mitochondria, and (c) with glutamate, malate and succinate as substrates, which are also the three substrates that allow completing the citric acid cycle, evaluation of the electron transport and the entry of electrons complex I and complex II of the ETC.

The high-resolution respirometry performed in mitochondria isolated from the gastrocnemius muscle, using pyruvate, showed higher levels of mitochondrial respiration induced by treatment with GSPE in mitochondrial respiratory states 2 and 4o. The 4o state was achieved by addition of oligomycin. The 4o state gave us information about oxygen consumption that occurred in conditions not conducive to phosphorylation. So we were able to evaluate the basal uncoupling of mitochondria. Oligomycin is an inhibitor of complex V blocking the phosphorylation of ADP to ATP. Under these conditions, the proton motive force is only diminished by the basal proton leak.[27] Thus, oxygen consumption is proportional to the intensity of the proton leak and the capacity of the respiratory chain to generate and maintain the membrane potential.[28]

Moreover, GSPE administration tended to increase the mitochondrial states 3 and Unc. Stimulation of state 3 of respiration by the addition of ADP provides information on the mitochondrial capacity to oxidize NADH and FADH2 from the citric acid cycle or β-oxidation in the ETC, resulting in the oxidative phosphorylation of ADP to ATP by ATPase.[29] Under physiological conditions, state 3 of respiration is limited by or under the control of the phosphorylation system activity, including ATPase and adenine nuclease translocase (ANT).[29]

The Unc state (uncoupled) is achieved with the addition of FCCP,[29] a chemical uncoupler that creates pores in the mitochondrial inner membrane, causing the entry of protons into the mitochondrial matrix while avoiding the phosphorylation system (complex V). The respiration levels of the Unc state provide evidence of the maximum capacity of ETC and dehydrogenase enzymes that are involved depending on the substrate provided. This may, therefore, represent the maximum uncontrolled mitochondrial oxidative capacity.[29] As expected, we consistently observed that the uncoupling induced by FCCP in the Unc state goes further than the oxygen consumption levels of state 3 of mitochondrial respiration, indicating that the phosphorylation system capacity (determined as a function of the synthesis of ATP and the activity of ANT1) controls the maximum flow through the ETC. This was observed both with pyruvate and with palmitoyl-CoA as substrates in the transport system,[30] although it was not observed using the substrates glutamate, malate and succinate. This agrees with another study,[26] which suggests that this combination of substrates produced an additive effect of convergent electrons into complexes I and II of the ETC.

These results suggest that GSPE supplementation improve the skeletal muscle mitochondrial function (with pyruvate as the substrate) as GSPE produced an increase of all mitochondrial states.

In contrast, we analyzed whether GSPE modified the activity of enzymes involved in the citric acid cycle. We observed that it is unlikely, there were no significant differences between the control and the GSPE groups in the protocol (glutamate−malate−succinate), which complete the citric acid cycle; this is indicative of GSPE not altering the activity and functionality of the citric acid cycle.

Contrariwise, using palmitoyl-CoA as a substrate we saw an increase in respiration primarily in the 4o state. These results suggest that the GSPE supplementation increases the basal proton leak in skeletal muscle mitochondria using a carnitine−palmitoyl-CoA.

The literature has reported uncoupling effects of flavonoids on artificial membranes of vesicles[31] and in cells *in vitro*;[32] here we show that GSPE also produced a significant *ex vivo* uncoupling in mitochondria isolated from gastrocnemius muscle of Wistar rats after 5 h of acute GSPE administration, using both pyruvate and carnitine−palmitoyl-CoA as substrates. A slight uncoupling of oxidative phosphorylation in muscle is related to a protective effect against oxidative stress and decreased production of reactive oxygen species (ROS).[33,34]

Due to these results, we analyzed several genes involved in energy metabolism and the production of ROS. We found that FATP1, involved in fatty acid uptake in muscle, was the only gene expression that decreased significantly. Overexpression of FATP1 leads to increased fatty acid incorporation.[35] Therefore, the decrease in FATP1 expression is related to lower storage capacity of fatty acids in muscle, thereby protecting intramyocellular lipid accumulation in muscle tissue, and could be the basis of mitochondrial dysfunction: a part of energy production, mitochondria are the major source of intracellular ROS.[36]

The accumulation of fatty acids in the vicinity of the mitochondria causes vulnerability to lipid peroxidation induced by ROS.[36] Consequently, this lipid peroxide toxicity cooperation could have effects on mtDNA, RNA and proteins of the mitochondrial machinery, leading to mitochondrial dysfunction.[36] Furthermore, the accumulation of intramyocellular lipids is the main cause of poor insulin signaling in type 2 diabetes and obesity.[36,37] Similarly, it was found EGCG inhibits, in a dose-dependent manner, the accumulation of lipids in premature adipocytes.[38] Moreover, muscle gene expression of UCP2 and UCP3 is not altered by GSPE administration, suggesting that the increase of basal proton leak observed in the mitochondria could occur through an alternative mechanism of which UCP's proteins were not involved.

Additionally, we analyzed several enzymatic activities in muscle tissue (CS, COX and ATPase), and we found that GSPE administration did not change the activities.

In contrast, we also performed high-resolution respirometry on BAT using pyruvate and carnitine−palmitoyl-CoA as substrates. We observed that GSPE administration did not affect mitochondrial function in BAT using either of the substrates.

As in the muscle tissue, we analyzed the gene expression of key genes involved in energy metabolism and we observed that GSPE administration increased the gene expression of PGC1α, a gene that plays an important role in controlling mitochondrial function and biogenesis.[39] The overexpression of PGC1α results in a robust increase in mitochondrial number, cellular respiration and intracellular ATP concentrations in a variety of cell types.[40,41] Moreover, we also studied the Sirt1 gene expression as it has been shown to function together with PGC1α to promote adaptation to caloric restriction (CR) by regulating the genetic program for gluconeogenesis and glucolysis in the liver.[42] Surprisingly, we found that GSPE decreased the Sirt1 gene expression in BAT. These results suggest that the GSPE affected the gene expression of PGC1α and Sirt1 by different mechanisms.

Although the majority of studies use the CS enzyme activity as a marker of mitochondrial density in chronic administration studies,[27,43] we have measured it as a marker of the activity of the citric acid cycle as CS is one of the enzymes that regulate the citric acid cycle. In addition, we determined the enzymatic activity of COX and ATPase to evaluate the possible effect of GSPE, into oxidative phosphorylation, in BAT. The results showed a significant increase in COX activity, and a tendency to increase CS activity. Taking into account these results and the increase of PGC1α gene expression, these all suggest that GSPE supplementation improves the activity and mitochondrial function in BAT.

We also determined the expression of the same genes and the enzymatic activities in the liver that we studied in muscle and BAT. The results showed that GSPE administration only reduced the Sirt1 gene expression, while the enzymatic activities were not affected by GSPE supplementation.

Finally, the results that we observed were that GSPE administration did not alter the levels of glucose, insulin and lactate. In contrast, triglycerides, glycerol and free fatty acids, which are important parameters that provide information about lipid metabolism, are decreased after GSPE administration. This decrease in the lipid plasma parameters has already appeared in published studies performed by our group.[14,15]

In conclusion, our results show that GSPE affects mitochondria and mitochondrial function after 5 h of treatment, specifically at the level of mitochondrial activity rather than the gene expression level. GSPE improves mitochondrial function in skeletal muscle with pyruvate as a substrate, suggesting an improvement in the activity of enzymes involved in oxidation and metabolism of pyruvate, and a shift in priority to the glycosidic metabolism rather than lipid metabolism. The decrease in the FATP1 gene expression in muscle suggests that GSPE protects against intramyocellular lipid accumulation. In addition, it appears that the GSPE caused a slight uncoupling of mitochondria, at least in skeletal muscle; given the chemical structure and nature of GSPE it promotes antioxidant effects against oxidative stress and decreases ROS.

AUTHOR INFORMATION

Corresponding Author

*Department of Biochemistry and Biotechnology, Universitat Rovira i Virgili, Marcel.lí Domingo, s/n, Campus Sescelades, 43007 Tarragona, Spain. E-mail: lluis.arola@urv.cat. Tel: +34977558449. Fax: +34977558232.

Funding Sources

This study was supported by Grant No. AGL 2008-00387/ALI from the Spanish Ministerio de Educación y Ciencia.

ACKNOWLEDGMENT

We gratefully acknowledge Santiago Moreno and Niurka Llopiz for their technical assistance.

ABBREVIATIONS USED

GSPE, grape seed proanthocyanidin extract; ATP, adenosine triphosphate; ETC, electron transport chain; BAT, brown adipose tissue; Unc, uncoupling; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; ANT, adenosine nucleotide translocase; ROS, reactive oxygen species; Ucp1, uncoupling protein 1; Ucp2, uncoupling protein 2; Ucp3, uncoupling protein 3; PGC1α, Ppargc1α (peroxisome proliferator-activated receptor gamma, coactivator 1 alpha); Sirt1, sirtuin 1 (silent mating type information regulation 2 homologue 1); Cpt1a, carnitine palmitoyltransferase 1a; Cpt2, carnitine palmitoyltransferase 2; Prkaa2, protein kinase, AMP-activated, alpha 2 catalytic subunit

REFERENCES

(1) Wallace, D. C. A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine. *Annu. Rev. Genet.* **2005**, *39*, 359–407.

(2) Lagouge, M.; Argmann, C.; Gerhart-Hines, Z.; Meziane, H.; Lerin, C.; Daussin, F.; Messadeq, N.; Milne, J.; Lambert, P.; Elliott, P.; Geny, B.; Laakso, M.; Puigserver, P.; Auwerx, J. Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1alpha. *Cell* **2006**, *127*, 1109–1122.

(3) Rogge, M. M. The role of impaired mitochondrial lipid oxidation in obesity. *Biol. Res. Nurs.* **2009**, *10*, 356–373.

(4) Hoeks, J.; Briede, J. J.; de Vogel, J.; Schaart, G.; Nabben, M.; Moonen-Kornips, E.; Hesselink, M. K.; Schrauwen, P. Mitochondrial function, content and ROS production in rat skeletal muscle: effect of high-fat feeding. *FEBS Lett.* **2008**, *582*, 510–516.

(5) Kelley, D. E.; He, J.; Menshikova, E. V.; Ritov, V. B. Dysfunction of mitochondria in human skeletal muscle in type 2 diabetes. *Diabetes* **2002**, *51*, 2944–2950.

(6) Blade, C.; Arola, L.; Salvado, M. J. Hypolipidemic effects of proanthocyanidins and their underlying biochemical and molecular mechanisms. *Mol. Nutr. Food Res.* **2010**, *54*, 37–59.

(7) Decorde, K.; Teissedre, P. L.; Sutra, T.; Ventura, E.; Cristol, J. P.; Rouanet, J. M. Chardonnay grape seed procyanidin extract supplementation prevents high-fat diet-induced obesity in hamsters by improving adipokine imbalance and oxidative stress markers. *Mol. Nutr. Food Res.* **2009**, *53*, 659–666.

(8) Lu, M.; Xu, L.; Li, B.; Zhang, W.; Zhang, C.; Feng, H.; Cui, X.; Gao, H. Protective effects of grape seed proanthocyanidin extracts on cerebral cortex of streptozotocin-induced diabetic rats through modulating AGEs/RAGE/NF-kappaB pathway. *J. Nutr. Sci. Vitaminol. (Tokyo)* **2010**, *56*, 87–97.

(9) Santos-Buelga, C.; Scalbert, A. Proanthocyanidins and tannin-like compounds. Nature, occurrence, dietary intake and effects on nutrition and health. *J. Sci. Food Agric.* **2000**, *80*, 1094–1117.

(10) Yousef, M. I.; Saad, A. A.; El-Shennawy, L. K. Protective effect of grape seed proanthocyanidin extract against oxidative stress induced by cisplatin in rats. *Food Chem. Toxicol.* **2009**, *47*, 1176–1183.

(11) Puiggros, F.; Llopiz, N.; Ardevol, A.; Blade, C.; Arola, L.; Salvado, M. J. Grape seed procyanidins prevent oxidative injury by modulating the expression of antioxidant enzyme systems. *J. Agric. Food Chem.* **2005**, *53*, 6080–6086.

(12) Llopiz, N.; Puiggros, F.; Cespedes, E.; Arola, L.; Ardevol, A.; Blade, C.; Salvado, M. J. Antigenotoxic effect of grape seed procyanidin extract in Fao cells submitted to oxidative stress. *J. Agric. Food Chem.* **2004**, *52*, 1083–1087.

(13) Terra, X.; Montagut, G.; Bustos, M.; Llopiz, N.; Ardevol, A.; Blade, C.; Fernandez-Larrea, J.; Pujadas, G.; Salvado, J.; Arola, L.; Blay, M. Grape-seed procyanidins prevent low-grade inflammation by modulating cytokine expression in rats fed a high-fat diet. *J. Nutr. Biochem.* **2009**, *20*, 210–218.

(14) Quesada, H.; del Bas, J. M.; Pajuelo, D.; Diaz, S.; Fernandez-Larrea, J.; Pinent, M.; Arola, L.; Salvado, M. J.; Blade, C. Grape seed proanthocyanidins correct dyslipidemia associated with a high-fat diet in rats and repress genes controlling lipogenesis and VLDL assembling in liver. *Int. J. Obes. (London)* **2009**, *33*, 1007–1012.

(15) Del Bas, J. M.; Fernandez-Larrea, J.; Blay, M.; Ardevol, A.; Salvado, M. J.; Arola, L.; Blade, C. Grape seed procyanidins improve atherosclerotic risk index and induce liver CYP7A1 and SHP expression in healthy rats. *FASEB J.* **2005**, *19*, 479–481.

(16) Nieman, D. C.; Williams, A. S.; Shanely, R. A.; Jin, F.; McAnulty, S. R.; Triplett, N. T.; Austin, M. D.; Henson, D. A. Quercetin's influence on exercise performance and muscle mitochondrial biogenesis. *Med. Sci. Sports Exercise* **2010**, *42*, 338–345.

(17) Davis, J. M.; Murphy, E. A.; Carmichael, M. D.; Davis, B. Quercetin increases brain and muscle mitochondrial biogenesis and exercise tolerance. *Am. J. Physiol.* **2009**, *296*, R1071–R1077.

(18) Westerterp-Plantenga, M. S.; Lejeune, M. P.; Kovacs, E. M. Body weight loss and weight maintenance in relation to habitual caffeine intake and green tea supplementation. *Obes. Res.* **2005**, *13*, 1195–1204.

(19) Fuster, A.; Oliver, P.; Sanchez, J.; Pico, C.; Palou, A. UCP1 and oxidative capacity of adipose tissue in adult ferrets (Mustela putorius furo). *Comp. Biochem. Physiol., Part A: Mol. Integr. Physiol.* **2009**, *153* (2), 106–112.

(20) Cannon, B.; Nedergaard, J. Studies of thermogenesis and mitochondrial function in adipose tissues. *Methods Mol. Biol.* **2008**, *456*, 109–121.

(21) Udenfriend, S.; Stein, S.; Bohlen, P.; Dairman, W.; Leimgruber, W.; Weigele, M. Fluorescamine: a reagent for assay of amino acids, peptides, proteins, and primary amines in the picomole range. *Science* **1972**, *178*, 871–872.

(22) Cannon, B. Control of fatty-acid oxidation in brown-adipose-tissue mitochondria. *Eur. J. Biochem.* **1971**, *23*, 125–135.

(23) Srere, P. A. Citrate Synthase. *Methods Enzymol.* **1969**, *13*, 3–11.

(24) Bergmeyer, H. U.; Bergmeyer, J.; Grabl, M. *Enzymes 2: Esterases, Glycosidases, Lyases, Ligases*, 3rd ed.; Methods of Enzymatic Analysis; Wiley-VCH: New York, 1984; Vol. IV, pp 324−331.

(25) Steg, A.; Wang, W.; Blanquicett, C.; Grunda, J. M.; Eltoum, I. A.; Wang, K.; Buchsbaum, D. J.; Vickers, S. M.; Russo, S.; Diasio, R. B.; Frost, A. R.; LoBuglio, A. F.; Grizzle, W. E.; Johnson, M. R. Multiple gene expression analyses in paraffin-embedded tissues by TaqMan low-density array: Application to hedgehog and Wnt pathway analysis in ovarian endometrioid adenocarcinoma. *J. Mol. Diagn.* **2006**, *8*, 76–83.

(26) Rasmussen, H. N.; Rasmussen, U. F. Small scale preparation of skeletal muscle mitochondria, criteria of integrity, and assays with reference to tissue function. *Mol. Cell. Biochem.* **1997**, *174*, 55–60.

(27) Flamment, M.; Arvier, M.; Gallois, Y.; Simard, G.; Malthiery, Y.; Ritz, P.; Ducluzeau, P. H. Fatty liver and insulin resistance in obese Zucker rats: no role for mitochondrial dysfunction. *Biochimie* **2008**, *90*, 1407–1413.

(28) Hafner, R. P.; Brand, M. D. Effect of protonmotive force on the relative proton stoichiometries of the mitochondrial proton pumps. *Biochem. J.* **1991**, *275* (Part 1), 75–80.

(29) Phielix, E.; Schrauwen-Hinderling, V. B.; Mensink, M.; Lenaers, E.; Meex, R.; Hoeks, J.; Kooi, M. E.; Moonen-Kornips, E.; Sels, J. P.; Hesselink, M. K.; Schrauwen, P. Lower intrinsic ADP-stimulated mitochondrial respiration underlies in vivo mitochondrial dysfunction in muscle of male type 2 diabetic patients. *Diabetes* **2008**, *57*, 2943–2949.

(30) Lenaers, E.; De Feyter, H. M.; Hoeks, J.; Schrauwen, P.; Schaart, G.; Nabben, M.; Nicolay, K.; Prompers, J. J.; Hesselink, M. K. Adaptations in mitochondrial function parallel, but fail to rescue, the transition to severe hyperglycemia and hyperinsulinemia: a study in Zucker diabetic fatty rats. *Obesity (Silver Spring)* **2010**, *18*, 1100–1107.

(31) van Dijk, C.; Driessen, A. J.; Recourt, K. The uncoupling efficiency and affinity of flavonoids for vesicles. *Biochem. Pharmacol.* **2000**, *60*, 1593–1600.

(32) Biasutto, L.; Sassi, N.; Mattarei, A.; Marotta, E.; Cattelan, P.; Toninello, A.; Garbisa, S.; Zoratti, M.; Paradisi, C. Impact of mitochondriotropic quercetin derivatives on mitochondria. *Biochim. Biophys. Acta* **2010**, *1797*, 189–196.

(33) Echtay, K. S.; Pakay, J. L.; Esteves, T. C.; Brand, M. D. Hydroxynonenal and uncoupling proteins: a model for protection against oxidative damage. *Biofactors* **2005**, *24*, 119–130.

(34) Aguirre, E.; Cadenas, S. GDP and carboxyatractylate inhibit 4-hydroxynonenal-activated proton conductance to differing degrees in mitochondria from skeletal muscle and heart. *Biochim. Biophys. Acta* **2010**, *1797*, 1716–1726.

(35) Liu, Q.; Gauthier, M. S.; Sun, L.; Ruderman, N.; Lodish, H. Activation of AMP-activated protein kinase signaling pathway by adiponectin and insulin in mouse adipocytes: requirement of acyl-CoA synthetases FATP1 and Acsl1 and association with an elevation in AMP/ATP ratio. *FASEB J.* **2010**, *24* (11), 4229–4239.

(36) Schrauwen, P.; Schrauwen-Hinderling, V.; Hoeks, J.; Hesselink, M. K. Mitochondrial dysfunction and lipotoxicity. *Biochim. Biophys. Acta* **2010**, *1801*, 266–271.

(37) Kim, J. K.; Gimeno, R. E.; Higashimori, T.; Kim, H. J.; Choi, H.; Punreddy, S.; Mozell, R. L.; Tan, G.; Stricker-Krongrad, A.; Hirsch, D. J.; Fillmore, J. J.; Liu, Z. X.; Dong, J.; Cline, G.; Stahl, A.; Lodish, H. F.; Shulman, G. I. Inactivation of fatty acid transport protein 1 prevents fat-induced insulin resistance in skeletal muscle. *J. Clin. Invest.* **2004**, *113*, 756–763.

(38) Lin, J.; Della-Fera, M. A.; Baile, C. A. Green tea polyphenol epigallocatechin gallate inhibits adipogenesis and induces apoptosis in 3T3-L1 adipocytes. *Obes. Res.* **2005**, *13*, 982–990.

(39) Hock, M. B.; Kralli, A. Transcriptional control of mitochondrial biogenesis and function. *Annu. Rev. Physiol.* **2009**, *71*, 177–203.

(40) Wu, Z.; Puigserver, P.; Andersson, U.; Zhang, C.; Adelmant, G.; Mootha, V.; Troy, A.; Cinti, S.; Lowell, B.; Scarpulla, R. C.; Spiegelman, B. M. Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. *Cell* **1999**, *98*, 115–124.

(41) Rasbach, K. A.; Schnellmann, R. G. Isoflavones promote mitochondrial biogenesis. *J. Pharmacol. Exp. Ther.* **2008**, *325*, 536–543.

(42) Rodgers, J. T.; Lerin, C.; Gerhart-Hines, Z.; Puigserver, P. Metabolic adaptations through the PGC-1 alpha and SIRT1 pathways. *FEBS Lett.* **2008**, *582*, 46–53.

(43) Garcia-Ramirez, M.; Francisco, G.; Garcia-Arumi, E.; Hernandez, C.; Martinez, R.; Andreu, A. L.; Simo, R. Mitochondrial DNA oxidation and manganese superoxide dismutase activity in peripheral blood mononuclear cells from type 2 diabetic patients. *Diabetes Metab.* **2008**, *34*, 117–124.