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Grape pomace extract supplementation activates FNDC5/irisin in muscle and promotes white adipose browning in rats fed a high-fat diet.

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Abbreviations: AMP-activated protein kinase (AMPK), epididymal white adipose tissue (eWAT), (-)-epicatechin (EC), fibronectin type 3 domain containing protein 5 (FNDC5), grape pomace extract (GPE), high fat diet (HFD), high fat-fed group (HF), peroxisome proliferator-activated receptor gamma co-activator-1α (PGC-1α), peroxisome proliferator-activated receptor gamma (PPARγ), PR domain containing 16 (PRDM16), uncoupling protein-1 (UCP-1) and white adipose tissue (WAT).

Keywords: adipose tissue browning, grape pomace extract, irisin, (–)-epicatechin, skeletal muscle.

Abstract

Irisin is a myokine regulated by peroxisome proliferator-activated receptor gamma co-activator-1α (PGC-1α) in the exercising skeletal muscle and released into the bloodstream after cleavage of FNDC5. Circulating irisin can up-regulate UCP-1 expression in white adipose tissue (WAT) promoting the formation of brown-like adipocytes. The aim of this study was to evaluate if supplementation with a grape pomace extract (GPE) could activate the FNDC5/irisin pathway via PGC-1α in rats fed a high fat diet (HFD). For this purpose we characterized the activation of: i. the FNDC5/irisin pathway and AMPK in skeletal muscle and ii. proteins involved in the formation of brown-like cells in epididymal WAT (eWAT). Consumption of GPE activated the FNDC5/irisin pathway, increased AMPK phosphorylation in skeletal muscle and enhanced irisin plasma levels. In eWAT GPE increased the level of proteins involved in WAT browning, i.e. PGC-1α, PPARγ, PRDM16 and UCP-1. GPE also prevented HFD-induced adipocyte hypertrophy and systemic insulin resistance. Consistently, in L6 myotubes, (–)-epicatechin (EC), a flavonoid abundant in GPE, prevented palmitate-mediated downregulation of FNDC5/irisin protein expression and secretion, in part via PGC-1α activation. Consumption of GPE, a winemaking residue rich in bioactive compounds, could be a beneficial strategy to counteract the adverse effects of Western style diets through the promotion of WAT browning.

Introduction

Obesity has become an alarming worldwide epidemic, predominantly caused by an excessive energy intake, especially of saturated fats and sugars [1] and reduced physical exercise. Excessive white adipose tissue (WAT) expansion, and more importantly adipocyte hypertrophy, lead to a state of chronic and low grade inflammation. This in turn contributes to the development of insulin resistance, type 2 diabetes and cardiovascular disease. [2]

Irisin is a recently discovered myokine, produced by cleavage of the membrane fibronectin type 3 domain containing protein 5 (FNDC5), and released into the bloodstream by the exercising skeletal muscle. [3] Circulating irisin can upregulate the expression of the uncoupling protein-1 (UCP-1) which promotes WAT browning. [4]. UCP-1, a highly specialized protein located in the inner mitochondrial membrane, has the capacity to dissipate energy in the form of heat. The main transcriptional regulators of browning are peroxisome proliferator-activated receptor gamma co-activator-1α (PGC-1α), peroxisome proliferator-activated receptor gamma (PPARγ) and PR domain containing 16 (PRDM16). [5] Furthermore, current evidence supports the concept that irisin can activate AMP protein kinase (AMPK). This enzyme is highly expressed in skeletal muscle and is involved in GLUT-4 membrane translocation and glucose uptake. Thus, AMPK has a key role improving glucose metabolism and insulin sensitivity. [6]

Recent evidence showed that select polyphenolic compounds can stimulate irisin secretion. [7] Grape pomace extract (GPE) is obtained from grape pomace, a residue of the winemaking process. GPE is mainly constituted of berry skins and seeds, which still contain high amounts of phenolic compounds. The most abundant polyphenols identified in Malbec grape pomace are flavanols (catechin, epicatechin, procyanidins), flavonols (quercetin), stilbenes (resveratrol) and

anthocyanins. [8–10] Among these compounds, the flavan-3-ol (-)-epicatechin (EC) is among the most abundant in GPE, and has proved beneficial effects against obesity and high fat/fructose-induced insulin resistance. [11–13]. EC activates AMPK in muscle improving glucose uptake [12] and in palmitate-treated 3T3-L1 adipocytes. [9] In addition, EC increased the expression of PGC-1α improving mitochondrial biogenesis in the WAT of mice fed a high-fat diet (HFD) and in human adipocytes isolated from subcutaneous WAT of obese subjects. [14,15] We previously observed that GPE and EC were able to activate PGC-1α in epididymal WAT (eWAT) from spontaneously hypertensive rats and in 3T3-L1 adipocytes, respectively. [9] Taking into account that FNDC5 expression is activated by PGC-1α in exercising skeletal muscle [3], we hypothesize that GPE could activate the FNDC5/irisin pathway via PGC-1α. Thus, this work investigated in HFD-fed rats the effects of GPE dietary supplementation on: i. the FNDC5/irisin pathway and AMPK activation in skeletal muscle; and ii. proteins involved in eWAT browning. In addition, in L6 myotubes treated with palmitate, we investigated the mechanisms of FNDC5/irisin pathway activation by EC.

Experimental

Materials

The standard chow diet was obtained from GepsaFeeds (Buenos Aires, Argentina). Bovine and porcine fat was from Recreo Refrigerating Industries SAIC (Santa Fe, Argentina). Total cholesterol, HDL, and triglyceride assay kits were purchased from GTLab (Bs. As., Argentina). The glucometer was obtained from Accu-Chek Performa, Roche (Bs. As., Argentina). The Ultra-Sensitive Insulin ELISA Kit was purchased from Crystal Chem (Downers Grove, IL, USA) and irisin competitive ELISA Kit was purchased from AdipoGen SA (Liestal,

Switzerland). The antibody for UCP-1 (U6382) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). The antibody (sc-293168) and siRNA (sc-72151) for PGC-1α and the antibody for PPARγ (sc-7273) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). AMP kinases (AMPK) (#5832) and phospho (Thr172) AMPK (#2535) primary antibodies were from Cell Signaling Technology (Danvers, MA, USA). PRDM16 (ab106410) and FNDC5 (ab174833) primary antibodies were from Abcam (Cambridge, MA, USA). Alexa Fluor 594-conjugated anti-rabbit IgG and FITC-conjugated anti-mouse IgG were from Jackson ImmunoResearch Co. Laboratories (West Grove, PA, USA). Nitrocellulose membranes, the western blotting system and protein standards dual color were obtained from BIO-RAD (Hercules, CA, USA). DMEM, TRIzol, M-MLV reverse transcriptase, random primers, Taq polymerase and dNTPs were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). Lipofectamine was from Termofisher (Waltham, MA, USA). The L6 rat cell line was from American Type Culture Collection, (Manassas, VA). Unless otherwise noted, reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Grape pomace extract sampling and chemical characterization

GPE was obtained from *Vitis vinifera* L. cv. Malbec as we previously described [9,10]. Grape pomace (GP) was provided by a local winery located in Gualtallary, Mendoza, Argentina. The vinification procedure was conducted with mechanical daily pumping. The skins and seeds were in contact with the juice for 11 d. After that, must was pressed. Fresh GP samples were collected and placed in ice cooled boxes for transportation to the laboratory, and then, stored at -20°C until processing. The GPE was obtained by solid-liquid extraction according to the

method of Antoniolli et al. [8] Five mg of lyophilized GPE were dissolved in 5 mL of ethanol 50% (v/v) aqueous solution for further analysis of the total phenolic content by the Folin–Ciocalteu assay according to Antoniolli et al. [8], and the chemical characterization of low-molecular-weight polyphenols was done according to Fontana et al. as previously described. [16] The chemical characterization of low-molecular-weight polyphenols present in the GPE used in this study is described in our recent publication. [9]

Animals

All procedures were approved by the Institutional Animal Care and Use Committee of the School of Medical Science, University of Cuyo (Protocol approval N° 36/2014). Male Sprague Dawley rats (n=21) at 8 week (w) of age were housed under controlled conditions of temperature (23±1 °C) and light (12 h light/12 h dark cycle). Rats were randomly divided into three experimental groups (7 rats/group) treated during 6 w as follows: i. the control group (Ctrl) receiving a standard chow diet containing 6% of fat, ii. the high fat diet group (HF) containing approximately 40% (w/w) bovine and porcine fat added to the standard chow, as described previously [9,17] and iii. the HF group supplemented with 300 mg GPE/kg of body weight (bw) (HFGPE). The composition of the standard chow diet is described in a supplementary file. The base of both control and HF diets was a commercial chow grinded to a flour texture, which was re-pelleted after addition of the corresponding amount of fat and/or GPE, as lyophilized ground powder. HF and GPE-containing diets were prepared weekly to account for changes in body weight and food intake, and to prevent fat oxidation and/or polyphenol degradation. Once prepared, HF and GPE-containing diets were stored at -20°C and food pellets administered daily.

The GPE was given at an amount of 300 mg GPE/kg bw that provides 23 mg per kg per d of total phenolic compounds, approximately. The dose of GPE was chosen based on our previous studies [9,10] and taking into account that it provides a physiological amount of phenolic compounds that can be achievable through food consumption, with a human equivalent dose of 305 mg per d of total phenolic compounds. The body weight and food consumption were recorded weekly.

After 6 w in the dietary treatments and after an overnight fast, rats were weighed and anesthetized with ketamine (50 mg/kg bw) and acepromazine (1 mg/kg bw). Blood from the abdominal aorta was collected into EDTA-containing tubes, and plasma isolated after centrifugation at 1000×g for 15 min at 4 °C. eWAT and skeletal muscle (soleus) were weighed and flash frozen in liquid nitrogen and then stored at -80 °C until assayed. A piece of eWAT was immediately fixed in 4% formaldehyde (w/v) solution for 24 h and processed for histological analysis.

Biochemical determinations

Glucose was measured in blood collected from the tail using a glucometer. Glucose tolerance tests (GTT) was done after 4 w in the respective diets as previously described. [11] Briefly, for GTT overnight fasted rats were injected with D-glucose (2g/kg bw), and blood glucose was measured before and at 30, 60, 90 and 120 min post injection. At the end of the study, plasma total cholesterol, triglycerides, HDL, and insulin concentrations were measured following manufacturer's guidelines. Insulin resistance was evaluated using the homeostasis model assessment parameter (HOMA-IR) using the following formula: HOMA-IR (mg/dL × μU/mL) = fasting glucose (mg/dL) × insulin in fasting (μU/mL)/405. Irisin

concentration in plasma and cell culture medium was measured using the irisin competitive ELISA Kit following manufacturer's guidelines.

Cell culture and incubations

L6 cells were grown in low glucose DMEM supplemented with 10% v/v FBS and penicillin/streptomycin at 5% v/v CO_2 and 37 °C until cells reached 80% of confluence. Differentiation of myoblasts into myotubes was performed by incubating the confluent myoblasts with DMEM containing 2% v/v FBS and penicillin/streptomycin for 5 d. The medium was changed every other d. L6 myotubes were incubated without or with EC (1μM) in the absence or presence of 0.5 mM palmitate for 24 h.

The concentration used of EC (1 μM) was chosen based on our previous *in vitro* studies in 3T3-L1 adipocytes [9,18,19] and due to this concentration is compatible with those found in human tissues (high nanomolar-low micromolar range) after dietary consumption. [20]

Cell transfection

L6 myotubes seeded in 6-well plates were transfected with 100 nM siRNA for PGC-1α for 6 h according to the manufacturer's protocol. Then, the myotubes medium was replaced by fresh medium and incubated for additional 24 h. Then, L6 myotubes were incubated without or with EC (1μM) in the absence or presence of 0.5 mM palmitate for 24 h.

Histological analysis

The degree of adipocyte hypertrophy was evaluated in eWAT sections, respectively, from paraffin-embedded samples, and stained with hematoxylin

and eosin. Images were analyzed using a CCD camera (Nikon, Japan) 20x magnification and adipocyte area was measured using the Image J software.

Western blot analysis

Skeletal muscle (soleus), L6 cell homogenates and eWAT were prepared as previously described [11,18] in order to evaluate FNDC5/irisin pathway (PGC-1α and FNDC5), total and phosphorylated AMPK and proteins involved in WAT browning (PGC-1α, PPARγ, UCP-1 and PRDM16), respectively. Aliquots of total homogenates containing 25–40 μg protein were denatured with Laemmli buffer, separated by reducing 8–12.5% polyacrylamide gel electrophoresis, and electroblotted onto nitrocellulose membranes. Membranes were blocked for 2 h in 5% (w/v) nonfat milk and subsequently incubated in the presence of the corresponding primary antibodies (1:1000 dilution) overnight at 4 °C. After incubation for 90 min at room temperature in the presence of secondary antibodies (either HRP or biotinylated antibodies, followed by 1 h incubation with streptavidin), the conjugates were visualized and quantified by chemiluminescence detection in a Luminescent Analyzer Image Reader (LAS-4000) (Fujifilm, Japan). The densitometric analysis was performed using the Image J Program.

Immunocytochemistry

L6 myotubes were seeded on coverslips in 12-well plates treated in the absence or the presence of 0.5 mM palmitate and with or without EC (1uM) for 24 h as described above. Cells were fixed in 4% (w/v) solution of paraformaldehyde in PBS for 1 h, rinsed with PBS, permeabilized with 0.1% (v/v) Triton X-100 in 0.1 M PBS for 5 min, blocked for 2 h in 5% (v/v) BSA PBS,

and incubated overnight at 4 °C with primary antibodies for PGC-1α (1:500) and FNDC5 (1:500). Sections were washed in PBS and incubated for 2 h at room temperature with Alexa Fluor 594-conjugated anti-rabbit IgG and FITC-conjugated anti-mouse IgG. After immunostaining, cell nuclei were stained with Hoechst 33342 and sections were imaged using an Olympus FluoView FV1000 confocal microscope (Japan) equipped with multiple filter sets, 30 cells for each condition were analyzed with ImageJ and Adobe illustrator. Images are representative of three independent experiments. Scale bars represent 10 μm.

Quantitative RT-PCR

Total RNA from L6 cells was isolated using TRIzol isolation reagent following the manufacturer's instructions. The expression of genes was detected using a 2X reverse transcription master mix (Applied biosystem) with gene specific primers pairs. The results were quantified after normalization with actin. Primers for rat genes were designed as follow: *Pgc1a* (forward) 5' TGTGGAACTCTCYGGAACTGC 3', (reverse) 5' GCCTTGAAAGGGTTATCTTGG 3'; *Fndc5* (forward) 5' GCCTGTGCTCTTCAAGACCC 3', (reverse) 5' CATGAACAGGACCACGAC 3'; *Actin* (forward) 5' AAGGCCAACCGTGAAAAGAT 3', (reverse) 5' AACAGAGGCATACAGGGACA 3'.

Statistical analysis

Data are shown as mean ± S.E.M. Statistical significances were assessed by one-way ANOVA followed by Bonferroni's Multiple Comparison Test. GraphPad Prism version 5.00 for Windows (Graph-Pad Software, San Diego, CA, USA) was used for all statistical analysis. A P value < 0.05 was considered statistically significant.

Results

GPE supplementation improves metabolic parameters

Daily food intake in the groups fed a HFD was significantly lower than in the Ctrl group **(Table 1)**. The energy intake was higher in the HFGPE group compared with Ctrl and HF groups. After 6 w in the respective diets, the body weight was similar among groups. No differences were observed in plasma triglycerides, total and HDL cholesterol, and glucose levels among groups. Chronic consumption of HFD significantly increased fasted plasma insulin concentrations and the index of insulin resistance (HOMA:IR) in the HF compared to the Ctrl group. Co-administration of GPE attenuated and prevented these alterations, respectively. GTT tests showed that HF rats exhibit impaired glucose tolerance compared with Ctrl rats **(Fig. 1 A)**. The area under the curve for the GTT was significantly higher in the HF group compared to Ctrl and HFGPE groups **(Fig. 1 B).** Overall, GPE prevented HFD-induced altered glucose homeostasis.

GPE supplementation activated the FNDC5/Irisin pathway and AMPK in skeletal muscle from rats fed a HFD

We next investigated if GPE could activate the FNDC5/irisin pathway in the soleus muscle of rats fed a HFD. GPE supplementation significantly increased the protein content of PGC-1α and FNDC5 compared to Ctrl (50% and 115%, respectively) and HF (67% and 169%, respectively) groups **(Fig. 2 A-B)**. Irisin plasma concentration was 130% and 140% higher in the HFGPE group compared to Ctrl and HF groups, respectively **(Fig. 2 C)**.

AMPK has been recently involved in the irisin-mediated improvement of glucose tolerance. Animals supplemented with GPE showed a 3-fold increase in skeletal muscle AMPK phosphorylation at Thr172 compared to the HF group **(Fig. 2 D-E)**. These results show that GPE promoted muscle irisin production and secretion, and AMPK activation.

GPE supplementation enhances proteins involved in eWAT browning and protected against diet-induced adipose tissue hypertrophy.

GPE-mediated increase in irisin circulating levels could promote WAT browning. Thus, we investigated the effects of GPE on PGC-1α, PPARγ, UCP-1 and PRDM16 protein levels in the eWAT by Western blot **(Fig. 3 A)**. PGC-1α protein levels were 4.6-fold higher in the HFGPE compared to the HF group and 2.3-fold higher than the Ctrl group. PPARγ and PRDM16 protein levels were 5.1- and 3.3-fold higher in the HFGPE group compared to the HF and Ctrl groups, respectively. UCP-1 protein levels were 5.2- and 3-fold higher in HFGPE than in HF and Ctrl groups, respectively **(Fig. 3 B)**.

We next investigated if GPE could modulate adiposity in rat eWAT. Consumption of a HFD significantly increased adipocyte size **(Fig. 3 C-D)** and eWAT weight **(Fig. 3 E)** compared to the Ctrl group. The addition of GPE to the HFD did not modify eWAT weight, but significantly reduced (32%) adipocyte size. Together, the above data indicate that GPE supplementation increased the expression of proteins involved in eWAT browning, which is associated with the presence of smaller adipocytes.

EC activates the FNDC5/Irisin pathway in L6 myotubes via PGC-1α

To elucidate if PGC-1α is involved in GPE-induced activation/secretion of irisin, we next evaluated the effects of EC, one of the main flavonoids present in GPE, in L6 myotubes challenged with palmitate, to mimic a condition of fatty acids oversupply. PGC-1α and FNDC5 were assessed by Western blot and immunofluorescence. Protein levels of PGC-1α and FNDC5 were 3- and 2.6-fold higher, respectively, in the EC and palmitate-treated cells, compared with palmitate-treated cells **(Fig. 4 A-B)**. The same tendency was observed by immunocytochemical analysis of PGC-1α, while no differences were observed in FNDC5 fluorescence intensity among treatments **(Fig. 5 A-B)**. In addition, EC

caused a 1-fold increase in the medium irisin concentration compared with cells treated with palmitate **(Fig. 4 C)**. In order to assess if PGC-1α is involved in the EC-mediated upregulation of FNDC5, a PGC-1α siRNA transfection experiment was conducted. As expected, Pgc1a siRNA transfection abrogated the effects of EC on Fndc5 protein expression **(Fig. 5 C)**. Together, this data suggests that EC-dependent FNDC5/irisin pathway activation is in part mediated by PGC-1α.

Discussion

This study present evidence that GPE, rich in phenolic compounds, activates the FNDC5/irisin pathway in muscle and increases irisin plasma levels in rats fed a HFD. These changes were accompanied by increased levels of proteins involved in eWAT browning, which may in part explain GPE capacity to prevent HFD-induced adipocyte hypertrophy and glucose intolerance. In L6 myotubes, the flavan-3-ol EC, one of the main flavonoids found in GPE, increased the expression and secretion of irisin via PGC-1α.

Western lifestyle, characterized by a sedentary life and high consumption of saturated fats and simple sugars, is a main contributor to the alarming increased rates of overweight, obesity and their associated pathologies. Thus, the identification of food bioactives that can attenuate the adverse consequences of obesogenic diets would be of major relevance.

PGC-1α is a transcriptional coactivator that mediates several biological effects related to energy metabolism. In skeletal muscle, PGC-1α is induced by exercise which in turn stimulates irisin secretion to the circulation, affecting the function of other tissues; e.g. WAT and muscle. [3] Irisin can promote AMPK activation in muscle and the ‘browning’ of WAT. [3,7] Interestingly, recent studies showed that certain bioactive compounds, including phenolic compounds, stimulate irisin

expression in muscle [21,22], L6 myotubes and inguinal WAT. [7,23] Accordingly, GPE supplementation activated the FNDC5/irisin pathway in skeletal muscle increasing circulating irisin levels in HFD-fed rats. As far as we know, this is the first study showing that a GPE could mimic the effects of exercise activating the FNDC5/irisin pathway.

AMPK is a key protein present in skeletal muscle and WAT. It is involved in glucose uptake and insulin sensitivity. [24] Exercise and muscle contraction are important physiological activators of AMPK in humans and rodents, being this energy sensor activated by irisin in skeletal muscle. [7] GPE increased AMPK phosphorylation in the muscle. Enhanced AMPK phosphorylation could contribute to the attenuation of HFD-induced glucose intolerance by GPE. Accordingly, we previously observed that GPE also improve insulin signaling and glucose tolerance in high-fat-fructose fed Wistar rats. [10]

In accordance with *in vivo* results, EC increased the mRNA and protein levels of PGC-1α and FNDC5 in L6 myotubes treated with palmitate. In addition, EC significantly stimulated irisin secretion in both, control and palmitate-treated cells. As we mentioned before, PGC-1α is the main transcriptional factor involved in the regulation of FNDC5 expression. Accordingly, Pgc1a siRNA transfection in L6 myotubes showed that PGC-1α is required for EC-mediated increase in Fndc5 expression, supporting the involvement of PGC-1α in EC-mediated irisin increased production/release. These effects of EC, at concentrations found in plasma after oral ingestion, can in part explain similar *in vivo* effects of GPE.

Irisin can upregulate UCP-1 expression in white adipose cells both *in vitro* and *in vivo* leading to the development of brown-like adipocytes. [3] In contrast to white adipocytes, brown-like adipocytes have the capacity to burn fatty acids and dissipate the energy in the form of heat. Mice with transgenically increased PGC-

1α expression in muscle show an increased expression of brown fat selective genes in inguinal subcutaneous WAT, which is particularly prone to browning, but not in eWAT or interscapular brown adipose tissue. [3] Interestingly, in accordance to other studies showing the presence of brown-like cells in eWAT from mice or rats under different experimental conditions [25,26], we observed that GPE supplementation in HFD-fed animals increased browning proteins in eWAT compared to the HF group. These results have a particular relevance taking into account the adverse effects of increased visceral adipose tissue depots on obesity-associated pathologies. Current findings are in accordance to our previous study in which GPE and EC were able to stimulate the main transcriptional regulators of brown-like cell development, i.e. PGC-1α, PPARγ, PRMD16 and UCP-1 in the eWAT of spontaneously hypertensive rats fed a HFD and in 3T3-L1 adipocytes, in part through the activation of the downstream β-adrenergic signaling cascade. [9] Several stimuli can induce WAT browning such as cold, exercise or pharmacological treatments, and PPARγ agonists. [27] All these stimuli share the capacity to activate the β-adrenergic receptors. Thus, components of GPE could induce eWAT browning through different mechanisms, including β-adrenergic activation and/or irisin-mediated effects. More studies are necessary to better understand the underling mechanisms related to GPE-mediated WAT browning. Similar to our findings, supplementation with raspberry extracts and other phenolic compounds such as resveratrol and quercetin have been shown to induced browning of WAT in obese mice with the appearance of characteristic smaller adipocytes. [7,23,28] This is relevant taking into account that smaller adipocytes are related to lower levels of inflammation and improved insulin sensitivity. Chronic consumption of a HFD led to the development of adiposity and systemic insulin resistance. While GPE did not affect eWAT weight, it significantly reduced the area

of adipocytes and prevented glucose intolerance. We have previously reported that GPE or flavonoids such as catechin and quercetin can reduced adiposity. [10,11,18] These protective effects could be attributed to the capacity of GPE to enhance proteins that participate in adipogenesis, angiogenesis and/or adipose tissue browning. [9]

Conclusions

In summary, GPE supplementation activated the FNDC5/irisin pathway in skeletal muscle and increased irisin secretion in rats, at least in part via PGC-1α upregulation. In addition, GPE enhanced proteins involved in WAT browning, reduced HFD-induced adipocyte hypertrophy and improved glucose tolerance. Thus, utilization of winemaking residues such as GPE, rich in bioactive compounds, constitute an interesting and sustainable strategy to counteract the adverse effect associated with the Western lifestyle. GPE could be used as a functional ingredient that mimic/support the beneficial effects of exercise. Future human studies are necessary in order to evaluate the effects of grape pomace on irisin production and glucose homeostasis.

Conflict of interest

The authors have declared no conflict of interest.

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Legends

Figure 1. GPE supplementation protected against HFD-induced glucose intolerance. Rats were fed a control diet (Ctrl), or a HFD without (HF) or with supplementation with GPE (HFGPE) for 6 w. (A) glucose tolerance test (GTT), blood glucose levels were measured at time 0, 30, 60, 90 and 120 min, and (B) area under the curve GTT. Results are shown as means ± SEM of seven animals/ treatment. Values having different superscripts are significantly different ($p < 0.05$, one-way ANOVA).

Figure 2. GPE supplementation activated the FNDC5/irisin pathway and AMPK in muscle from rats fed a HFD. Rats were fed a control diet (Ctrl), or a HFD without (HF) or with supplementation with GPE (HFGPE) for 6 w. (A) representative Western blot bands for PGC-1α and FNDC5 in skeletal muscle, (B) after quantifying the bands of PGC-1α and FNDC5 proteins levels were normalized to β-actin content (loading control), (C) irisin plasma levels (μg/ml) were determined by ELISA, (D) representative Western blot bands for phosphorylated an total AMPK and (E) after quantifying the bands the p-AMPK/AMPK ratio was calculated. Results are shown as means ± SEM of seven animals/ treatment. Values having different superscripts are significantly different ($p < 0.05$, one-way ANOVA).

Figure 3. GPE supplementation enhances proteins involved in eWAT browning and protected against HFD-induced adipose tissue hypertrophy. Rats were fed a control diet (Ctrl), or a HFD without (HF) or with supplementation with GPE (HFGPE) for 6 w. (A) representative Western blot bands for PGC-1α, PPARγ**,** PRDM16 and UCP-1 in eWAT, (B) after quantifying bands of PGC-1α, PPARγ**,** PRDM16 and UCP-1 proteins levels were normalized to β-actin content (loading control), (C) representative histological images of eWAT stained with hematoxylin/eosin and eosin, (D) mean adipocyte diameter (μm) and

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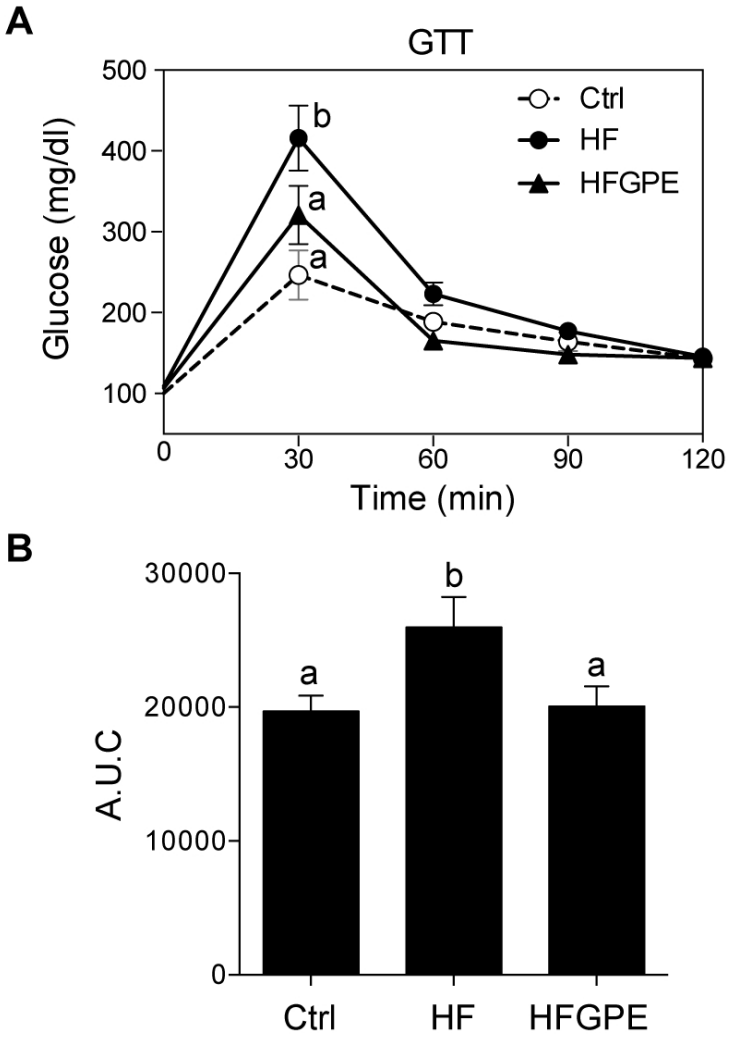
Figure 4. EC activates the FNDC5/Irisin pathway in L6 myotubes. L6 myotubes were incubated without or with EC (1μM) in the absence or presence of 0.5 mM palmitate for 24 h. (A) representative Western blot bands for PGC-1α and FNDC5, (B) after quantifying bands PGC-1α and FNDC5 proteins levels were normalized to β-actin content (loading control), (C) irisin concentration in the cell culture media. Data represent means ± SEM of three independent experiments. Values having different superscripts are significantly different ($p < 0.05$, one-way ANOVA).

Fig 5. EC activates the FNDC5/Irisin pathway in L6 myotubes via PGC-1α. L6 myotubes were incubated without or with EC (1μM) in the absence or presence of 0.5 mM palmitate for 24 h. (A) representative immunofluorescence images for PGC-1α (green fluorescence) and FNDC5 (red fluorescence). Nuclei were visualized with Hoechst staining (blue fluorescence), (B) quantification of fluorescence intensity was done as described in experimental section and (C) mRNA levels of Pgc1a and Fndc5 after PGC-1α siRNA transfection (see experimental section). Pgc1a and Fndc5 mRNA levels were normalized to those of GAPDH. Data represent means ± SEM of three independent experiments. Values having different superscripts are significantly different ($p < 0.05$, one-way ANOVA).

Table 1. Metabolic parameters

Parameter	Ctrl	HF	HFGPE
Food intake (g/d)	28.5 ± 1.4[a]	18.7 ± 0.9[b]	22.3 ± 0.9[b]
Energy intake (Kcal/d)	90.7 ± 4.3[a]	96.9 ± 5.1[a]	115 ± 4.8[b]
Body weight (g)	450 ± 12.9	478.6 ± 10.3	492 ± 11.6
Glucose levels (mg/dl)	100 ± 1.9	109 ± 2.3	106.5 ± 4.0
Insulin levels (ng/ml)	0.3 ± 0.0[a]	1.3 ± 0.5[b]	0.6 ± 0.4[c]
HOMA:IR	1.1 ± 0.1[a]	5.6 ± 0.6[b]	1.7 ± 0.4[a]
Triglycerides (mg/dl)	49.6 ± 6.8	67.2 ± 4.3	51.1 ± 3.8
Total cholesterol (mg/dl)	65.5 ± 1.6	63.8 ± 3.3	63.1 ± 1.9
HDL cholesterol (mg/dl)	33.6 ± 0.8	30.2 ± 1.3	35.4 ± 4.1

Food intake and metabolic parameters from rats fed a control diet (Ctrl), or a HFD without (HF) or with GPE (HFGPE) for 6 w. The biochemical determinations were measured in plasma after an overnight fast. HOMA:IR: homeostatic model assessment of insulin resistance (mg/dL × μU/mL). Results are shown as the mean ± SEM of seven animals/treatment. Values having different superscripts are significantly different ($p < 0.05$, one-way ANOVA).



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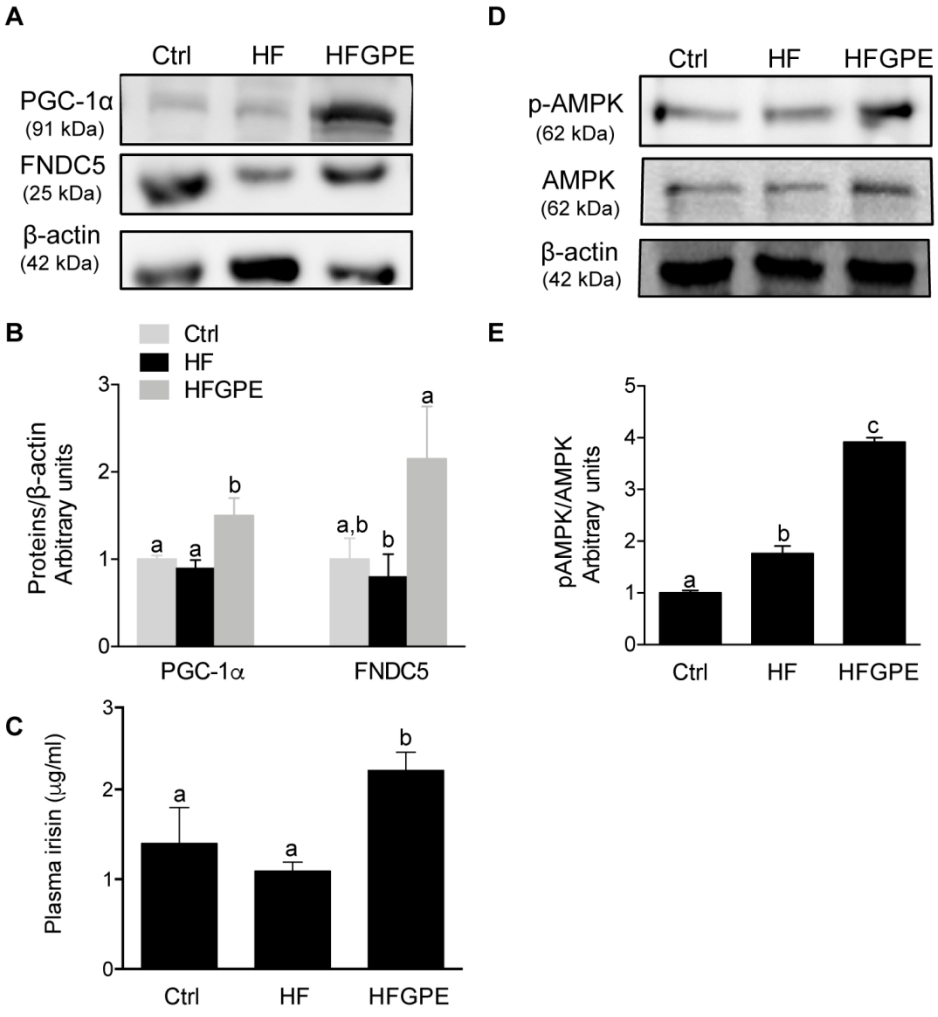

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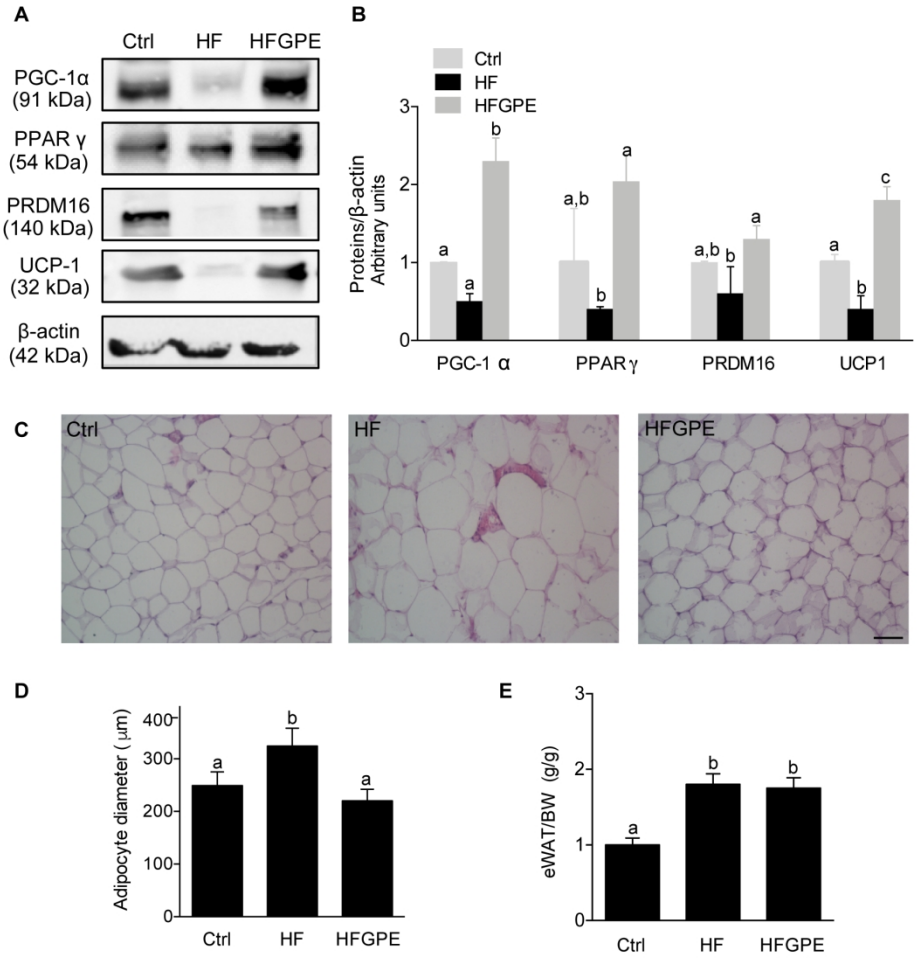

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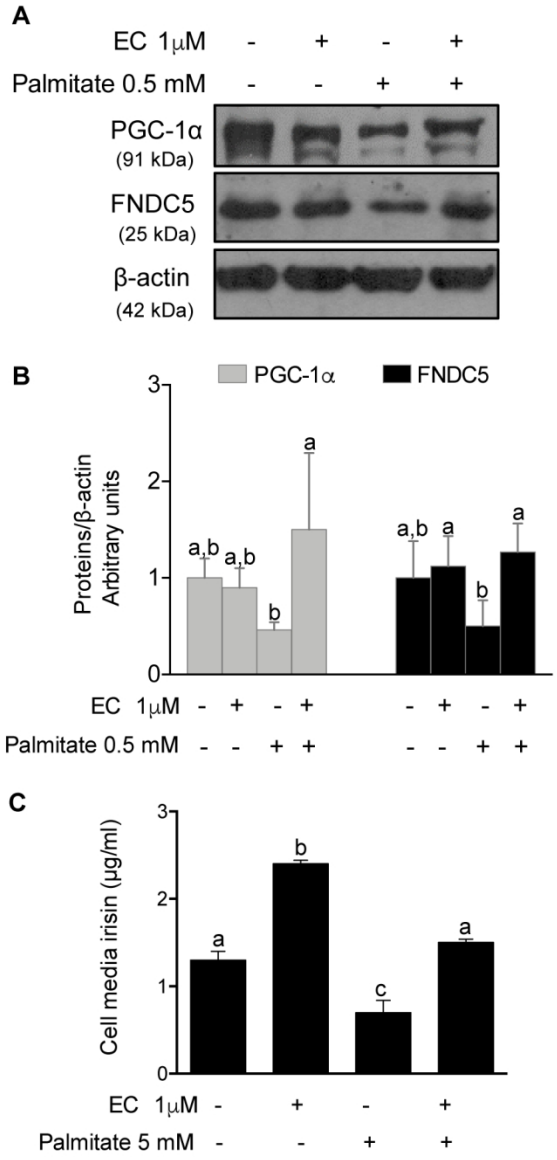

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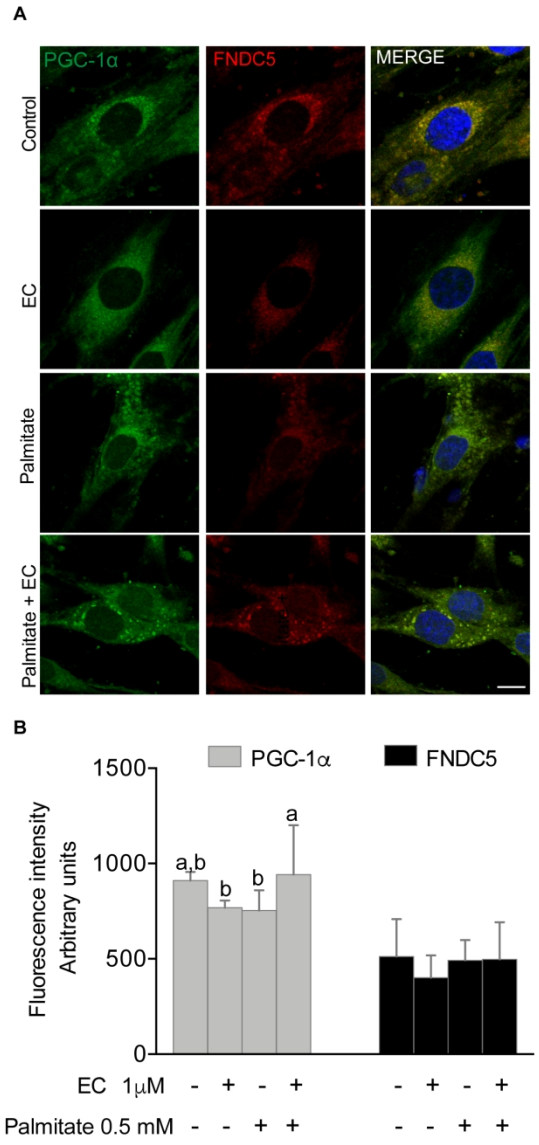

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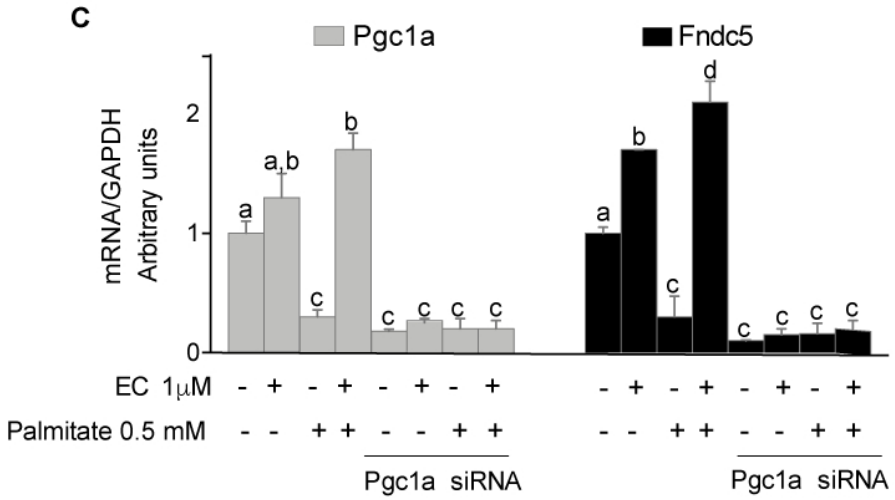

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