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(-)-epicatechin treatment did not modify the thermogenic pathway in the gastrocnemius muscle of male rat offspring obeses by programming

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Original Article

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Abstract

The aim of this study was to analyse the expression of genes related to the regulation of energy metabolism in skeletal muscle tissue by comparing male offspring in two age groups [at 110 and 245 postnatal days (pnd)] from a mother with obesity induced by a high-fat diet and (-)-epicatechin (Epi) administration. Four groups of six male offspring from different litters were randomly selected for the control groups [C and offspring of mothers with maternal obesity (MO)] or Epi intervention groups. We evaluated the effect of Epi on gastrocnemius tissue by analysing the mRNA and protein expression levels of Fndc5/irisin, Pgc-1α, Ucp3, and Sln. Epi significantly increased the Pgc-1α protein in the MO group of offspring at 110 pnd ($p < 0.036$, MO *vs.* MO+Epi), while at 245 pnd, Epi increased *Fndc5/irisin* mRNA expression in the MO+Epi group *versus* the MO group ($p = 0.006$).

No differences were detected in Fndc5/irisin, Ucp3 or Sln mRNA or protein levels (including Pgc-1α mRNA) in the offspring at 110 pnd or in Pgc-1α, Ucp3, or Sln mRNA or protein levels (including Fndc5/irisin protein) at 245 pnd among the experimental groups. In conclusion, (-)-epicatechin treatment increased *Fndc5/irisin* mRNA expression and Pgc-α protein levels in the gastrocnemius muscle of offspring at postnatal days 110 and 245. Furthermore, it is suggested that the flavonoid effect in a model of obesity and its impact on thermogenesis in skeletal muscle are regulated by a different pathway than Fndc5/irisin.

Introduction

Pregnancy and lactation are crucial stages during which obesity gives rise to important health consequences in the short, medium, and long term for both mothers and their offspring.[1] In México, an alarming increase in the prevalence of obesity in adult women has been observed; i.e., in women aged > 20–29 years, the prevalence of obesity was 26%, which increased 46% in women of aged 30–59 years (https://www.inegi.org.mx/contenidos/saladeprensa/aproposito/2020/EAP_Obesidad20.pdf). Therefore, a significant percentage of women of reproductive age have obesity. MO damages skeletal muscle morphology and the muscle fibre composition of offspring during postnatal life;[2,3] this is important since skeletal muscle is crucial for thermogenesis and energy expenditure.[4]

FNDC5 encodes a membrane protein that is cleaved and secreted as irisin, which is a myokine that is synthesised mainly by skeletal muscle in response to exercise and is regulated in both humans and rodents by the peroxisome proliferator-activated receptor-gamma coactivator 1 α (PGC1 α).[5] It has been suggested that irisin in skeletal muscle is capable of increasing energy expenditure via an increase in the expression of genes related to metabolism, such as PGC-1 α, and uncoupling protein 3 (UCP3), among others.[5,6] In this way, it could lead to an improvement in metabolic dysfunction.[7] In addition, in a model of obesity induced by a high-fat diet (HFD), an increase in Fndc5 and Pgc1-α protein levels in the gastrocnemius muscle was reported, suggesting that this increase might have a compensatory role opposing excess body weight.[8]

It is well known that regular exercise and good nutrition are relevant to general health; in this context, identifying specific components in food, such as natural phytochemicals, might help prevent several chronic disorders, including obesity and its comorbidities.[9] The flavonoid

(-)-epicatechin has been shown to have a beneficial effect on the skeletal muscle of the offspring of obese mothers fed a HFD.[10] Furthermore, this flavonoid, considered as an exercise mimetic, induced mitochondrial biogenesis *in vitro*[11] and *in vivo*.[12]

We previously demonstrated that the offspring of obese mothers are also obese and have metabolic disorders and decreased lean mass;[10,13,14] however, to our knowledge, the implications of obesity programming on muscle energy regulation through the irisin pathway have not been reported. In this context, we aimed to analyse the expression of genes related to the regulation of energy metabolism in skeletal muscle tissue by comparing two age groups (at 110 and 245 pnd) of male offspring of a model of MO induced by a HFD and to determine whether these disorders are modified by the administration of the flavonoid (-)-epicatechin. We hypothesised that the presence of MO would decrease the expression of proteins involved in the thermogenic pathway in musculoskeletal tissue and that postnatal intervention with the flavonoid (-)-epicatechin would be able to restore the expression profile of these proteins. Moreover, we hypothesised that these changes would be more pronounced in mature adult rats (245 days of age).

Materials and methods

Animal model

The animal research protocol used in the present study was approved by the Animal Experimentation Ethics Committee of the Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán (CINVA: UIO-1892-17/19-1) and by the Research and Ethics Commissions of the Facultad de Medicina, Universidad Nacional Autónoma de México. All procedures followed the Guidelines for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources (http://www.nal.usda.gov/awic/animal-welfare-act), and the Mexican Official Standard Norm NOM-062-ZOO-1999.

The chow diet and the HFD, as well as the standardisation of the phenotype of the Wistar female rats (F0) used to produce the F1 offspring, were described previously.[10,13] All female albino Wistar F0 rats were maintained on a diet (chow or HFD) during pregnancy and lactation (Fig. 1). The Chow diet contained 23.9% protein, 5.0% fat, 57.9% carbohydrates, 5.1% fibre, 7.0% minerals and 1.0% vitamins (w/w) (4.07 kcal/g of energy) (Purina 5001, Labdiet, St Louis, MO, USA). In contrast, the HFD contained 23.5% protein, 20.0% animal lard, 5.0% fat, 20.2% polysaccharide, 20.2% simple sugars, 5.0% fibre, 5.0% mineral mix and 1.0% vitamin mix (wt/wt) (4.9 kcal/g of energy). The mineral and vitamin contents of the two diets (chow and HFD) were selected following the American Institute of Nutrition recommendation to support growth, pregnancy, and lactation phases (AIN-93G).[15]

Since sexual dimorphism in skeletal muscle has been described, for this study, we analysed only male offspring.[16] To standardise the phenotype of the male offspring (F1), food intake, weight, adiposity index, and (-)-epicatechin administration were described by De los Santos *et al.*[10,13] In brief, male offspring were weaned at postnatal day 21, housed with two rats *per* cage, and fed a chow diet throughout the duration of the study. Because genotypic and phenotypic changes in various rat age-related muscles have been observed,[17] in the present study, we analysed rats at 110 (young adult) and 245 pnd (mature adult). Six males *per* group from different litters were randomly selected to form the control groups or the Epi intervention groups, totalling four groups of male rats for each postnatal age group: male rats descended from mothers fed a control diet (C); male rats treated with Epi descended from mothers fed a control diet (C + Epi); male rats descended from mothers fed a HFD (MO); and male rats treated with Epi descended from mothers fed a HFD (MO + Epi) (Fig. 1).

At 95 or 230 days of age, male offspring were administered a vehicle (water) or Epi (1 mg/kg body weight) (Sigma-Aldrich, St Louis, MO, USA) by oral gavage twice daily (morning and afternoon) for 15 days. As previously described,[10,13] the interventions were carried out at the same time and by the same individual. No adverse effects were recorded due to this dietary intervention, and no rats died before the end of the study. At postnatal day 110 or 245, after 6 hours of fasting, the rats were anaesthetized with isoflurane (based on the instructions for the use of the isoflurane inhaler of the brand: Stoelting Co., Cat. No. 50,207 regarding animal weight) and euthanized; we subsequently obtained the gastrocnemius muscle tissues and stored them at −80°C for subsequent analysis. The gastrocnemius muscle was chosen as a muscle model, since it has been reported that this muscle modifies its thermogenesis capacity under different conditions.[18]

Total RNA extraction and gene expression analysis

Total RNA was extracted from eighty milligrams of gastrocnemius tissue using TRIzol reagent (Invitrogen, California, U.S.A.) according to the manufacturer's protocol. A nanophotometer (Implen GmBH, M FC;nchen, Germany) was used to determine RNA purity and concentration. RNA quality was assessed by determining the integrity of the 28S and 18S ribosomal RNA bands following electrophoresis on a 1.5% agarose gel. After RNA isolation, all the samples were frozen and stored at −80°C until use.

RNA (100 ng) was used for DNA synthesis (cDNA) using the AgPath-ID™ One-Step RT-PCR Kit Reagents (Applied Biosystems™, Thermo Fisher Scientific) following the instructions provided by the manufacturer.

The gene expression of *fibronectin type III domain containing 5* (*Fndc5*) (Rn01519161_m1), the *transcription coactivator peroxisome proliferator-activated receptor gamma* (*Ppar-γ*) *type 1 alpha* (also known as *Pgc-1α*) (Rn00580241_m1), *Ucp3* (Rn00565874_m1), and *sarcolipin* (*Sln*) (Rn02769377_s1) was analysed in gastrocnemius muscle tissue by reverse transcription–quantitative polymerase chain reaction (RT–qPCR) using the TaqMan tool from the Demand Gene Expression Probes of Thermo Fisher Scientific. The values were normalised to the relative amounts of *β-actin* (assay ID: Rn00667869_m1) for each sample.

Reverse transcription-quantitative PCR was performed on a LightCycler® 480 instrument (Roche Diagnostics Ltd., Switzerland), and relative mRNA concentrations were calculated with LightCycler Relative Quantification analysis Software. All the samples were analysed in duplicate.

Western blotting

Between 120 and 150 mg of gastrocnemius muscle tissue was homogenised in RIPA lysis buffer (Santa Cruz Biotechnology, Inc., Dallas, U.S.A.) (sc-2494A) supplemented with protease and phosphatase inhibitors. Fifty milligrams of protein was separated via SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Cat. #1620115, Germany); after being blocked with 5% fat-free milk, the membranes were incubated with different primary antibodies. We used anti-Fndc5/irisin (ab174833 Abcam, dilution 1:1000), anti-Pgc1 α (ab3242 EMD Millipore,

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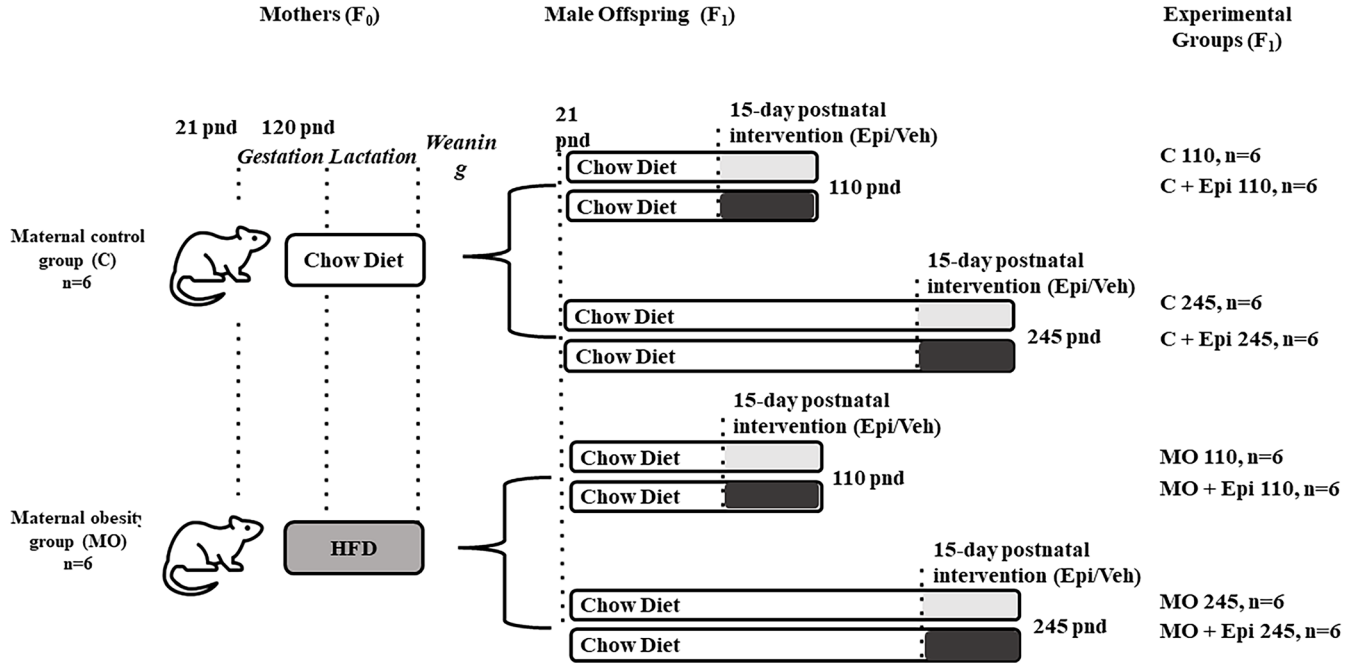

Figure 1. Timeline for the study of male offspring of obese mothers (F1) of 110 or 245 postnatal days. For the obesity model, six female rats (F0) *per group* were fed a control or high-fat diet at weaning and during pregnancy and lactation. At postnatal day 21, twelve males *per* group aged 110 or 245 (C and MO) from different litters were randomly allocated to be administered either water or (-)-epicatechin twice *per day* for 2 weeks (C, C + Epi, MO, MO + Epi, n = 6 rats *per* group). Pnds= postnatal days, Epi= (-)-epicatechin; Veh= Vehicle, *C* = male rat offspring of control mothers at 110 or 245 pnds; C + Epi= male rat offspring of control mothers treated with Epi at 110 or 245 pnds; MO = male rat offspring of obese mothers at 110 or 245 pnds; MO + Epi= male rat offspring of obese mothers treated with Epi at 110 or 245 pnds.

dilution 1:750), anti-Ucp3 (ab3046 EMD Millipore, dilution 1:1000), and anti-Sln (18,395-1-AP Proteintech, dilution 1:500) antibodies. For the loading control, anti-Gadph (PA1-988; dilution 1:14000) (Thermo Fisher Scientific, Rockford, U.S.A.) was used.

Afterwards, we incubated the sections with HRP-conjugated goat anti-rabbit IgG (111-095-003) and anti-mouse IgG (715-035-150) (Jackson ImmunoResearch Laboratories, I.N.C.) as secondary antibodies. The proteins were detected with a SuperSignal^{tm} West Femto Maximum Sensitivity Substrate Kit (Thermo Fisher Scientific, Rockford, U.S.A.). The band intensities were digitally quantified using LI-COR Image Studio software (http://www.licor.com/bio/products/software/image_studio_lite/) and ImageJ software (U.S. National Institutes of Health, Bethesda, Maryland, U.S.A.).

Statistical analysis

The results are expressed as the median ± range of 6 individual experimental observations and were analysed using GraphPad Prism 9.3.1 software (GraphPad Software, San Diego, CA). The sample size and power were calculated according to Dell *et al.*[19] We tested the normality of the distribution of the data using the Kolmogorov-Smirnov test, and a Kruskal-Wallis nonparametric test was performed with Dunn's *post hoc* test. Differences were considered significant at $p < 0.05$.

Results

Effect of (-)-epicatechin treatment on the mRNA expression of genes related to the thermogenic pathway in the gastrocnemius muscle tissue of male offspring of obese Wistar rats of 110 and 245 postnatal days

To evaluate the expression of thermogenic genes, the total RNA in the gastrocnemius muscle of male offspring of obese Wistar rats of 110 and 245 pnd was obtained and analysed via qRT-PCR. We did not observe that Epi treatment modified the expression of *Fndc5*, *Pgc-1α*, *Ucp3*, or *Sln* of the mRNA level in the male offspring at 110 postnatal days in the experimental groups (C, MO, C + Epi, and MO + Epi) (Fig. 2, Panels A–D).

Regarding the male offspring of 245 pnd, Epi treatment in the MO group (MO + Epi) induced an increase in the expression of *Fndc5* mRNA in comparison to that in the MO group ($p < 0.006$; Fig. 3, Panel A). We did not observe differences in the expression of *Pgc-1α*, *Ucp3,* or *Sln* in the other groups of male offspring of 245 pnd (Fig. 3, Panels B–D).

(-)-Epicatechin treatment affects proteins related to the thermogenic pathway in the gastrocnemius muscle tissue of male offspring of obese Wistar rats of 110 and 245 postnatal days

Epi administration to male offspring of the MO model (MO + Epi) of 110 pnd resulted in an increase in the expression of Pgc-1 α protein in comparison to that in the MO group ($p < 0.036$; Fig. 4, Panels A and C). Regarding Fndc5, Ucp3, and Sln protein levels, we did not find differences in the experimental groups of male offspring of 110 pnd (Fig. 3, Panels A–E).

Moreover, we did not observe that MO or Epi treatment modified the protein levels of Fndc5, Pgc-1 α, Ucp3, or Sln in the male offspring of the four experimental groups (C, MO, C + Epi, and MO + Epi) of 245 pnd (Fig. 5, Panels A–E).

Discussion

Obesity has a detrimental effect on skeletal muscle, as it can lead to muscle atrophy, inflammation, and changes in skeletal muscle fibre composition, among others.[20] Importantly, studies in animal

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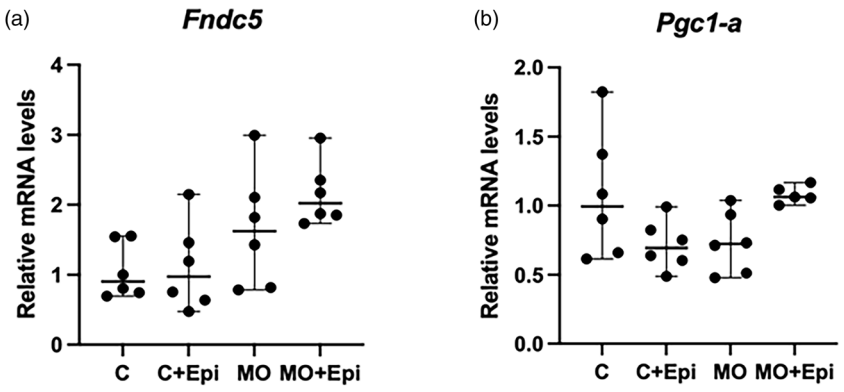

Figure 2. Effect of Epi on the relative mRNA expression of genes related to thermogenesis in the gastrocnemius muscle of male offspring of obese mothers of 110 postnatal days. (***a***) *Fndc5/irisin*, (***b***) *Pgc-1α*, (***c***) *Ucp3*, and (***d***) *Sln*. We did not observe differences in the experimental groups (C, C + Epi, MO, MO + Epi). The data are expressed as medians and ranges and were analysed by the Kruskal-Wallis test followed by the *post hoc* Dunn test for pairwise comparisons ($n = 6$ rats *per* group). Epi= (-)-epicatechin; C = male rat offspring of control mothers; C + Epi= male rat offspring of control mothers treated with Epi for 2 weeks; MO = male rat offspring of obese mothers; MO + Epi= male rat offspring of obese mothers treated with Epi for 2 weeks.

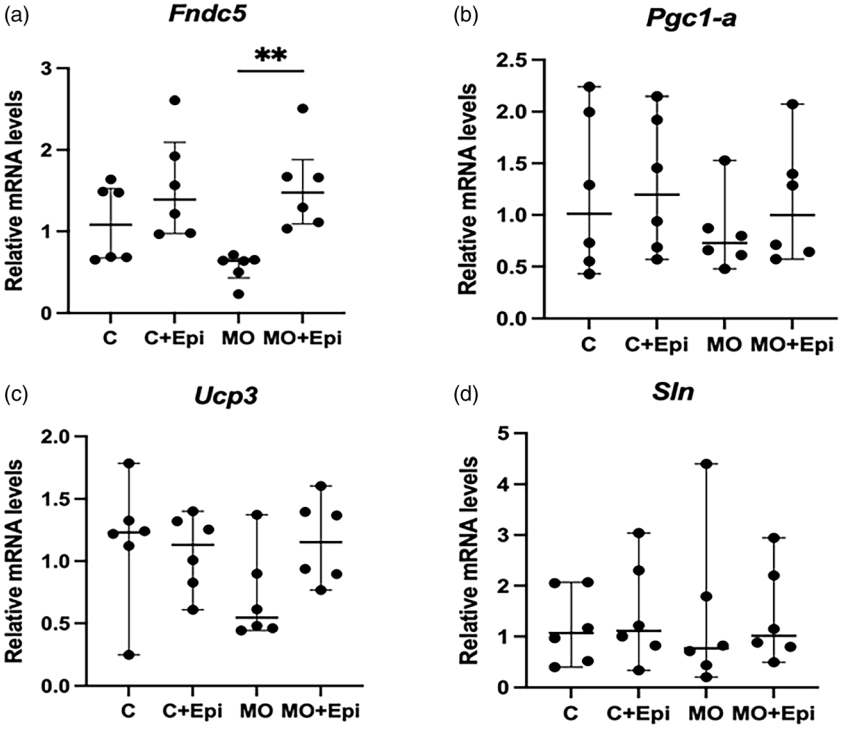

Figure 3. Effect of Epi on the relative mRNA expression of genes related to thermogenesis in the gastrocnemius muscle of male offspring of obese mothers at 245 postnatal days. (***a***) *Fndc5/irisin*, (***b***) *Pgc-1α*, (***c***) *Ucp3*, and (***d***) *Sln*. (***a***) Epi significantly increased the expression of *Fndc5/irisin* in the MO group. The data are expressed as medians and ranges and were analysed by the Kruskal-Wallis test followed by the *post hoc* Dunn test for pairwise comparisons ($n = 6$ rats *per* group). $^{**}p < 0.006$, MO *vs*. MO + Epi. Epi= (-)-epicatechin; C = male rat offspring of control mothers; C + Epi= male rat offspring of control mothers treated with Epi for 2 weeks; MO = male rat offspring of obese mothers; MO + Epi= male rat offspring of obese mothers treated with Epi for 2 weeks.

models have shown that obesity in mothers has an impact on the skeletal muscle development and metabolism of their offspring[10,21,22] as well as muscle strength in adulthood, despite following a control diet from weaning.[23] In addition, the impact of obesity on morphological changes observed in all types of muscles is more accelerated in aged mice than in young mice, with type II glycolytic muscle fibres being the most affected.[24] This is important since, due to the large volume of skeletal muscle tissue, this is the most critical determinant of thermogenic capacity and total body energy expenditure.[25] Furthermore, it is well known that the basal metabolic rate decreases with age due to skeletal muscle loss, which could lead to obesity.[26,27]

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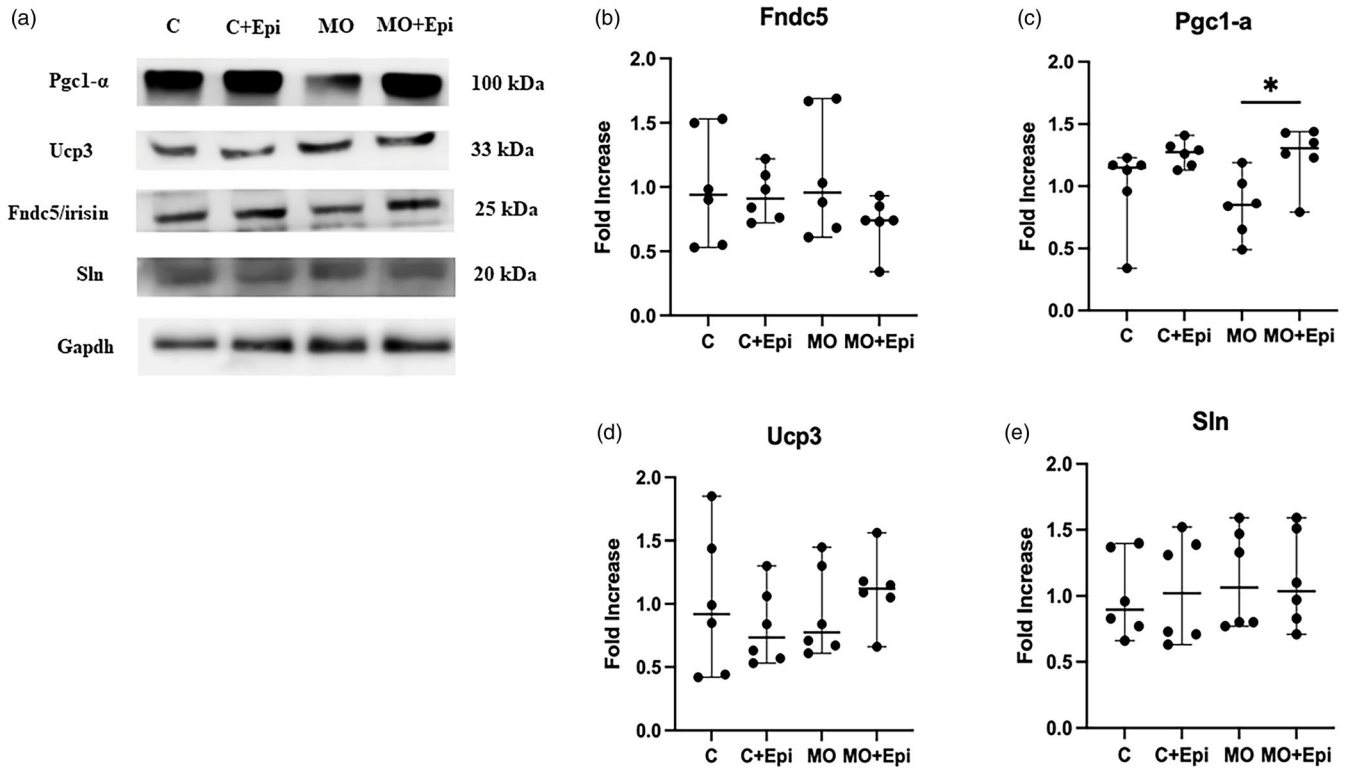

Figure 4. Effect of Epi on the expression of proteins related to thermogenesis in the gastrocnemius muscle of male offspring of obese mothers at 110 postnatal days. (***a***) Representative immunoblotting of Fndc5/irisin, Pgc-1 α, Ucp3, and Sln proteins; GAPDH was used as the loading control. (***b***–***e***) Densitometry analysis of Fndc5/irisin, Pgc-1 α, Ucp3, and Sln. (C) Epi significantly increased the protein expression of Pgc-1 α in the MO group. The data are expressed as medians and ranges and were analysed by the Kruskal-Wallis test followed by the *post hoc* Dunn test for pairwise comparisons ($n = 6$ rats *per* group). *$p < 0.036$, MO *vs*. MO + Epi. Epi= (-)-epicatechin; C = male rat offspring of control mothers; C + Epi= male rat offspring of control mothers treated with Epi for 2 weeks; MO = male rat offspring of obese mothers; MO + Epi= male rat offspring of obese mothers treated with Epi for 2 weeks.

In addition, it has been described that the fetal skeletal muscle of a non human primate's descendant of a model of MO presented a reduced mitochondrial content, oxidative capacity, and oxidative phosphorylation efficiency.[28] Besides, Dias-Rocha et al.[29] demonstrated that offspring adults of a model of MO by HFD exhibited increased energy metabolism markers and induced mitochondrial alterations in skeletal muscle.

Interestingly, *in vitro* and *in vivo* studies have demonstrated that Epi stimulates mitochondrial biogenesis, which is essential for energetically supporting the growth, differentiation, and function of skeletal muscle;[11,30] moreover, this flavonoid can reduce cellular oxidative stress in skeletal muscle.[30,31] Furthermore, Epi increased the expression of growth-regulating proteins and muscle differentiation in obese mice induced by a HFD.[32]

To our knowledge, there are no reports in the literature related to the expression of irisin or genes related to thermogenesis in skeletal muscle in the context of induced obesity.

Previously, we demonstrated that these offspring were obese and had metabolic disorders.[10,13] In addition, the Fndc5/irisin protein can induce mitochondrial biogenesis in skeletal muscle.[5,33] Likewise, it has been described that Fndc5/irisin expression only at the mRNA level[34] or in both mRNA and protein expression[35,36] are decreased in skeletal muscle of a model of obese animal induced by HFD.

Although we did not find a significant difference in *Fndc5/irisin* mRNA expression in pups of 245 days of the MO group, we observed that the descendants of this group presented lower expression of this myokine than did those in the control group (Fig. 3, Panel A). Notably, irisin expression is regulated by exercise,[35] and Epi is considered a mimetic of exercise.[12] In this context, we found that Epi treatment in the MO pups of 245 pnd significantly increased *Fndc5/irisin* mRNA expression. This increase was similar to the expression of this gene in the offspring of the control group treated with this flavonoid. Interestingly, differences in the expression of Fndc5/irisin were not maintained at the protein level in any of the groups analysed.

Moreover, we did not detect differences in mRNA or protein levels of Fndc5/irisin in the offspring of any of the four experimental groups of 110 pnd.

On the other hand, the increase in thermogenesis in skeletal muscle caused by exercise was related to the expression of PGC-1 α, which increases irisin secretion and, by this pathway, could stimulate mitochondrial oxidative metabolism.[37] Similarly, as described for irisin expression in an obesity model, PGC-1 α mRNA/protein levels are decreased in the skeletal muscle of animals with obesity induced by a HFD.[35,36]

Concerning our model of obesity induced by programming, compared with those in the MO group, the offspring of 110 pnd showed a significant increase in Pgc-1 α in the MO + Epi group but only at the protein level. It has been demonstrated that Epi can mitigate oxidative stress by acting on cell signalling of those genes that regulate mitochondrial function in skeletal muscle, including Pgc-1 α, among other proteins.[30]

The discrepancies observed in the correlation between *Fndc5/irisin* mRNA expression and protein levels or between the Pgc-1 α protein and mRNA could be due to transcriptional,

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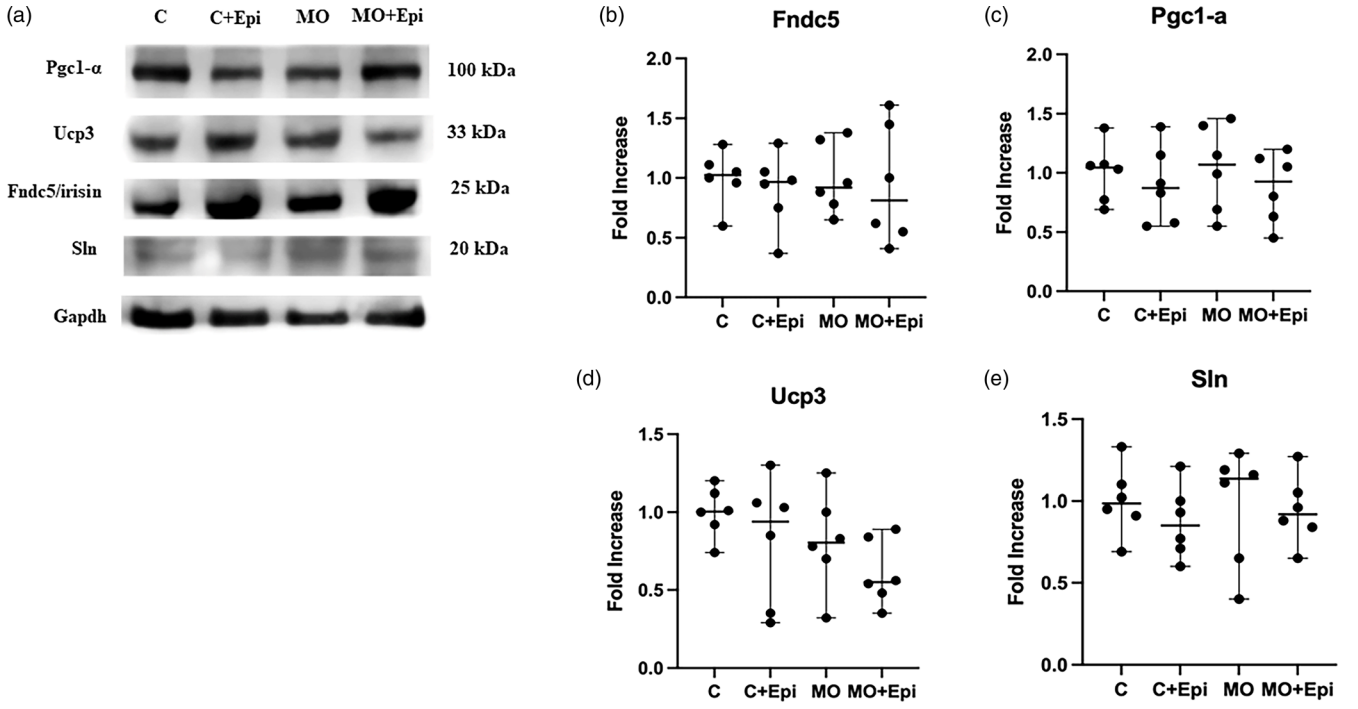

Figure 5. Effect of Epi on protein expression related to thermogenesis in the gastrocnemius muscle of male offspring of obese mothers at 245 postnatal days. (***a***) Representative immunoblotting of Fndc5/irisin, Pgc-1 α, Ucp3, and Sln proteins; GAPDH was used as the loading control. (***b***–***e***). Densitometric analyses of Fndc5/irisin, Pgc-1 α, Ucp3, and Sln. We did not observe differences in the experimental groups (C, C + Epi, MO, MO + Epi). The data are expressed as medians and ranges and were analysed by the Kruskal-Wallis test followed by the *post hoc* Dunn test for pairwise comparisons (n = 6 rats *per* group). Epi= (-)-epicatechin; C = male rat offspring of control mothers; C + Epi= male rat offspring of control mothers treated with Epi 2 weeks; MO = male rat offspring of obese mothers; MO + Epi= male rat offspring of obese mothers treated with Epi 2 weeks.

post-transcriptional, translational, or posttranslational regulation[38] as well as protein stability.[39] At postnatal day 110, there was an increase in the Pgc-1 α protein but not in the irisin protein. In the case of the offspring at postnatal day 245, an increase in irisin but not in Pgc-1 α was observed, which could indicate that the regulation of thermogenesis in skeletal muscle is mediated by another pathway.[40]

Sarcolipin plays a role in metabolism and muscle thermogenesis through Ca^{2+}-ATPase of the endoplasmic reticulum (SERCA),[41] and in an animal model of obesity induced by a HFD, sarcolipin/SERCA expression is decreased. However, irisin treatment increased the expression of both genes. Hence, it has been proposed that irisin modulates Sln, which can improve the function and thermogenesis of skeletal muscle.[42] In addition, in 2022, Son et al. demonstrated in a mouse model of MO that the expression of sarcolipin and Ucp3 in skeletal muscle is decreased in offspring with diet-induced obesity and that this change is due to pathway regulation other than Find5/irisin. According to our results, we did not find that obesity by programming or Epi treatment modified the expression of Sln or Ucp3 in the offspring of 110 or 245 pnd; as mentioned for Fndc5/irisin or Pgc-1 α, the regulation of thermogenesis in skeletal muscle could occur through pathways other than Fndc5/irisin.[40,43]

Notably, we performed the analysis only in the gastrocnemius muscle since, in obese mice by HDF, a reduced area of muscle myofibers and satellite cells, with a decrease of the expression and activity of mitochondrial enzyme genes in this muscle was observed.[24]

In conclusion, (-)-epicatechin treatment increased Fndc5/irisin mRNA expression and Pgc-α protein levels in the gastrocnemius muscle of offspring of obese mother of 245 and 110 postnatal days, respectively. Furthermore, we believe that the effect of this flavonoid in a programming model of obesity and its impact on thermogenesis in skeletal muscle are regulated by a different pathway than that of Fndc5/irisin.

Data availability statement. The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Author contribution. Tejeda M. E.: Conceptualisation; Data curation; Formal analysis; Investigation; Visualisation; Manuscript elaboration.

De los Santos S: Conceptualisation; Data curation; Formal analysis; Investigation; Visualisation.

Coral-Vazquez RM: Conceptualisation; Formal analysis; Investigation; Visualisation. Manuscript elaboration.

Méndez JP: Conceptualisation; funding acquisition; Manuscript elaboration.

Álvarez-Chávez A: Data curation; Formal analysis; Investigation; Visualisation.

Palma-Flores C: Data curation; Formal analysis.

Zambrano E: Conceptualisation; funding acquisition; Manuscript elaboration.

Canto P: Conceptualisation; Formal analysis; Funding acquisition; Investigation; Project administration; Supervision. Manuscript elaboration.

All authors read and approved the final manuscript.

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Competing interests. None.

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