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Irisin regulates thermogenesis and lipolysis in 3T3-L1 adipocytes

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ABSTRACT

Background: Adipose tissue plays a pivotal role in the development and progression of the metabolic syndrome which along with its complications is an epidemic of the 21st century. Irisin is an adipo-myokine secreted mainly by skeletal muscle and targeting, among others, adipose tissue. In brown adipose tissue it upregulates uncoupling protein-1 (UCP1) which is responsible for mitochondrial non-shivering thermogenesis.
Methods: Here we analyzed the effects of irisin on the metabolic activity of 3T3-L1 derived adipocytes through a mitochondrial flux assay. We also assessed the effects of irisin on the intracellular signaling through Western Blot. Finally, the gene expression of *ucp1* and lipolytic genes was examined through RT-qPCR.
Results: Irisin affects mitochondrial respiration and lipolysis in a time-dependent manner through the regulation of PI3K-AKT pathway. Irisin also induces the expression of UCP1 and the regulation of NF-κB, and CREB and ERK pathways.
Conclusion: Our data supports the role of irisin in the induction of non-shivering thermogenesis, the regulation of energy expenditure and lipolysis in adipocytes.
General significance: Irisin may be an attractive therapeutic target in the treatment of obesity and related metabolic disorders.

1. Introduction

Metabolic syndrome and its complications have become an epidemic in the twenty-first century and a major public health issue. Adipose tissue (AT) plays a pivotal role in the development and progression of the metabolic syndrome [33,34]. AT is the most plastic organ in the human body and has the ability to constantly expand and regress [21]. It is the major organ that regulates energy homeostasis in living organisms and responds to environmental cues. Brown-like adipocytes reside within white adipose tissue (WAT) and can emerge through trans-differentiation after cold exposure, physical activity, and/or drug stimulation. This process is called browning of AT [17,47] and it is characterized by the increase of non-shivering thermogenesis, which depends on uncoupling protein 1 (UCP1) and increased lipolysis [8]. Thus, AT browning leads to in heat production and/or energy dissipation and has been considered as a possible strategy for the treatment of obesity [14].

Adipocytes play a central role in energy balance. Alteration of the mechanical, metabolic and secretory functions of adipocytes contribute to the pathogenesis of metabolic diseases, including obesity. For years, adipocytes were considered only as storage cells for triglycerides, with a passive role in the development of obesity. Today they are instead appreciated for their mechanical support, their thermal insulation function as well as the production of several hormone-like molecules, identified as adipokines [26,44]. Notably, the release of adipokines changes, depending on AT localization, on its cellular composition, and on the interaction with vascular structures as well as following the onset of obesity [13].

Irisin is an adipo-myokine secreted mainly by skeletal muscle and by AT, after the proteolytic cleavage of fibronectin type III domain-containing protein 5 (FNDC5) [6]. Physical activity induces the expression of irisin through the activation of the transcriptional factor peroxisome proliferator-activated receptor γ (PPARγ), the coactivator-1α (PGC-1α) and of FNDC5 [15,23]. These pathways are involved in the browning of WAT [50]. The concentration of irisin into the bloodstream is also affected by diet and hormones, while its uptake and clearance are not yet well characterized [25].

The biological activity of irisin is not limited to adipocytes, since it can modulate several cellular responses, including proliferation,

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differentiation, and cell growth in normal and cancerous cells [37]. Irisin binding to α-integrin receptor [24] activates the intracellular integrin-dependent signaling pathway, including the phosphorylation of focal adhesion kinase (FAK), extracellular signal-regulated kinases (ERK and MAPK), and the activator of transcription 3 (STAT3) in several cells including cardiomyocytes, hippocampal neuronal cells, and osteoblasts [16,30,36]. Moreover, irisin is involved in whole-body metabolism and thermoregulation by affecting thyroid hormone secretion [38,46]. In brown adipose tissue (BAT), it upregulates the expression of PGC-1a, leading to increased UCP1 expression and mitochondrial respiration [19].

UCP1 expression leads to non-shivering thermogenesis which takes place in the inner mitochondrial membrane of brown adipose tissue (BAT), WAT, and skeletal muscle [46] and can constitute up to 5% of the basal metabolic rate [49]. The mitochondrial expression of UCP1 separates the oxidative phosphorylation from ATP synthesis with energy being dissipated as heat, a process referred to as the mitochondrial proton leak [8,10].

Despite the accumulating information about irisin, our knowledge of its mechanistic effects on adipocytes remains poor and limits our understanding of how the thermogenic process occurs after irisin release in the circulation. Here, we examine the role of hr-irisin on non-shivering thermogenesis and energy expenditure using the well-characterized 3T3-L1 derived adipocytes model [42]. We also monitor the time-dependent effects of hr-irisin on mitochondrial respiration and characterize the metabolic signaling pathways and the lipolytic process modulated by irisin. Our findings set the basis for the potential use of irisin as a therapeutic target in combating obesity and related metabolic disorders.

2. Materials and methods

2.1. Reagents

All reagents were of analytical grade. DMEM media and fetal calf serum were purchased from GIBCO Life Technologies (Grand Island, NY). Insulin, dexamethasone, 3-isobutyl-methyl-xanthine, Oil Red O, EtOH Triton-X100, BriJ, and protease inhibitor were purchased from Sigma-Aldrich (St. Louis, MO). Recombinant human irisin was purchased from (Cayman Chemical, Michigan, USA). Anti-phospho-NF-κB [sc101748; working dilution (wd) 1:1000], anti-NF-κB (sc109, wd 1:1000), anti-AKT (sc 1619, wd 1:1000), anti UCP- (sc 6529, wd 1:1000), anti-CREB (sc 186, wd 1:1000) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-ERK1,2 (#4695, wd 1:2000), anti-phospho-AKT (#4060, wd 1:2000) and anti-phospho-ERK1/2 (#4370, wd 1:2000) antibodies were purchased from Cell Signaling Technology (Beverly, MA). Donkey HRP-labeled secondary antibodies (anti-mouse Cat #SA1–100, wd 1:5000; anti rabbit Cat #31458, wd 1:5000; anti goat Cat #A16005, wd 1:5000) were purchased from Thermo Fisher Scientific.

2.2. Cell lines

Murine 3T3-L1 fibroblasts (ATCC, Manassas, VA) were maintained at no higher than 90% confluence in DMEM with 10% Bovine Serum (BS) and antibiotics (preadipocyte medium). To induce adipocyte differentiation, cell lines reaching the confluence received a differentiating medium, consisting of DMEM supplemented with 10% fetal bovine serum (FBS), 10 μg/ml insulin, 1 μM dexamethasone and 0.5 mM 3-isobutyl-methyl-xanthine (IBMX), for two days. The medium was then changed to 10% FBS-DMEM containing 10 μg/ml insulin. After the course of three to five days, differentiated adipocytes could be observed under the light microscope (accumulated lipid droplets in the cytoplasm) until day 12 to 15 of differentiation.

2.3. Oil red O staining

Staining with Oil red O was performed to confirm the differentiation of 3T3-L1 cells. On day 12 of differentiation the cell monolayer was fixed for 24-hours (h) with phosphate buffered formalin (10%). After rinsing with water and EtOH 70%, the monolayer was stained with the Oil Red O solution (water-based solution of 60% saturated Oil red O in isopropanol) for 15-min. Excess stain was removed with EtOH 70% followed by a last wash with water before observation under an inverted photomicroscope (Zeiss, Axiovert 200 M).

2.4. Extracellular mitochondrial flux assay

3T3-L1 preadipocytes were seeded on Seahorse XFe24 culture plates (Agilent, Santa Clara, CA, USA) in a density of 30,000 cells/well and differentiated into mature adipocytes as described above. Then, cells were treated with 20 nM hr-irisin [6] (Cayman Chemical, Michigan, USA) for 2-h and 4-h. Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) measurements were performed at 9-min intervals (3-min mixing, 3-min waiting and 3-min measuring) using a Seahorse XFe24 Extracellular Flux Analyzer (XFe Wave software) [22]. Seahorse XF Mito-Stress Test (Agilent, # 103015–100) was used to measure key parameters of mitochondrial function. Sequential treatments with oligomycin (1 μM), FCCP (0.5 μM) and rotenone/antimycin A (0.5 μM) were performed to enable quantification of basal OCR, ATP-coupled OCR, proton leak, and maximal respiration. Finally, cells were lysed and total proteins quantified by Bradford assay. Results are expressed as pmol of OCR / min for μg of proteins.

2.5. ATP measurement

ATP quantification was performed on 1×10^3 cells in growth medium with ATP Determination Kit (Molecular Probes, Thermo Fisher Scientific, #A22066) following manufacturer's instructions. The bioluminescent signal was measured with EnSight Multimode Plate Reader (PerkinElmer, Waltham, Massachusetts, United States).

2.6. RT-qPCR

Total RNA was extracted using TRIzol Reagent (Invitrogen, Thermo Fisher Scientific) according to the manufacturer's instructions. Two μg of total RNA were retro-transcribed with MMLV reverse transcriptase (Invitrogen, Thermo Fisher Scientific) using random hexaprimers. Then, cDNA was analyzed by quantitative PCR using the following primers:

Plin1_For_TTACCTAGCTGCTTTCTCGGTG, Plin1_Rev_CACAGGC AGCTGCAGAACTC; Lipe_For_GCTGGGCTGTCAAGCACTGT, Lipe_Rev_GTAACTGGGTAGGCTGCCAT; ATGL_For_ACAGGGCTACAGAGATG GACT, ATGL_Rev_AGGCTGCAATTGATCCTCCTC; mGAPDH_For_-CATGGCCTTCCGTGTTCCTAC, mGAPDH_Rev_TTGCTGTTGAAGTCG-CAGGAG. mUCP1_For_AGGCTTCCAGTACCATTAGGT, mUCP1_Rev_ CTGAGTGAGGCAAAGCTGATTT.

Quantitative RT-PCR was performed using the iTaqTM Universal SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA), by ViiA7 Real-Time PCR System (Life Technologies), and data were analyzed with Viia7 Real-Time Software (Life Technologies). 2-ΔΔCt was calculated using murine_GAPDH as housekeeping. Data are expressed as relative expression ratios. All samples were analyzed in triplicates.

2.7. Western blot

Whole-cell lysates were prepared in lysis buffer containing 1% Triton-X100, 0.1 BriJ, 1 mM sodium orthovanadate, and protease inhibitor cocktail. 50 μg of total proteins were separated by SDS-PAGE and probed with specific antibodies and donkey HRP-labeled secondary antibodies. Chemiluminescent signal was acquired by ChemiDoc Imaging System (BioRad). All experiments were been performed in

duplicates.

2.8. Statistical analysis

Statistical analysis was performed using SPSS 22.0 (IBM, Armonk, NY, USA). Paired samples *t*-tests were used to assess the differences in proton leak, basal OCR and mean ATP production. Repeated measures ANOVA was performed to assess differences in protein expression, gene expression, and ATP production. Post-hoc tests incorporating Bonferroni adjustment were performed for multiple comparisons. All values are reported as mean ± SD. The level of significance was set at $p < 0.05$.

3. Results

3.1. 3T3-L1 differentiated cells show higher cellular respiration

In a first set of experiments, we differentiated murine 3T3-L1 cells into adipose cells and analyzed their metabolic potential. To this, confluent cells were treated with differentiating medium for 12 days. As show in Fig. 1A-C, the morphological changes of differentiated 3T3-L1 cells are clearly visible. These cells are filled with lipid droplets (Oil red O stained) visible under the microscope (Fig. 1C).

Seahorse Mito-Stress Test was employed to measure key parameters of mitochondrial function, including basal respiration, ATP-linked respiration, spare capacity, proton leak, and maximal respiration by directly measuring the OCR of undifferentiated and 3T3-L1 derived adipocytes (Fig. 1D-J). As expected, differentiation induced an increase in the metabolic capacity of 3T3-L1 cells as demonstrated by the significantly higher basal OCR (Fig. 1E) and a simultaneous increase of the ECAR. Differentiation also significantly increased the maximal mitochondrial potential as demonstrated by the higher OCR measured upon treatment with the uncoupling agent FCCP (Fig. 1H) and the proton leak (Fig. 1G). These results confirm that our protocol was suitable to drive 3T3-L1 cell differentiation into mature adipocytes.

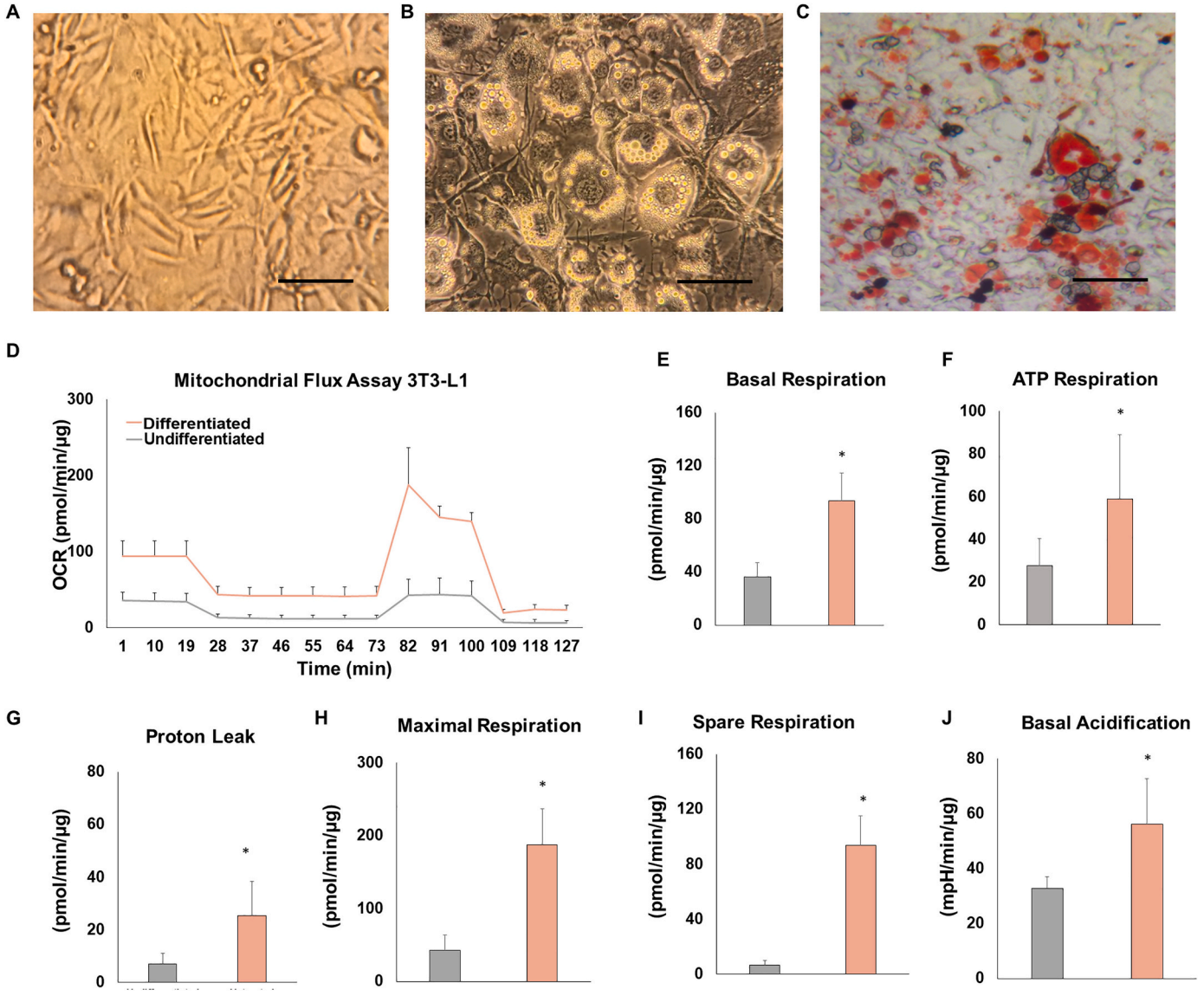

Fig. 1. The differentiation of 3T3-L1 cells into adipose cells increases their mitochondrial metabolism. A-B) Representative images of undifferentiated (A) and differentiated 3T3-L1 cells (B) stuffed with lipid droplets (scale bar 20 µm). C) Lipid droplets stained with Oil red O (scale bar 20 µm). D) Seahorse Mito Stress Test performed on undifferentiated and differentiated 3T3-L1 cells. Sequential treatments with Oligomycin, FCCP and Rotenone/Antimycin A were performed to enable quantification of E) basal respiration, F) ATP-linked respiration, G) proton leak, H) maximal respiration, I) spare respiration and J) basal ECAR. Data were analyzed according to the Agilent Seahorse XF Cell Mito Stress Test Report Generator. * indicates statistically significant differences ($p < 0.05$). Experiments were performed in triplicates. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Irisin regulates browning of 3T3-L1 derived adipocytes through PI3K-Akt pathway

To understand the effects of irisin on 3T3-L1 derived adipocytes, cells were treated for different times with 20 nM of hr-irisin. Then, 50 μg of the whole lysate were assessed for the activated intracellular signaling (Fig. 2). Irisin rapidly increased the phosphorylation level of the transcriptional factor cAMP response element-binding protein (CREB) (Fig. 2B), while nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Fig. 2C) was downregulated. Also, the activation of their upstream modulators including protein kinase B (AKT) and extracellular regulated kinase (ERK), were reduced after the treatment with irisin (Fig. 2D-E). Irisin treatment induced a fast and transitory increase of the expression of the brown adipogenic marker UCP1 both at mRNA and protein levels (Fig. 2F-G and S1), supporting the role of irisin in thermogenesis. Furthermore, the prolongation of irisin stimulation restored the UCP1 expression in 3T3-L1-derived adipocytes (Fig. S1).

3.3. Irisin modulates cellular thermogenesis by reducing cellular respiration

The effects of irisin on UCP1 expression prompted us to delve into the molecular mechanisms involved in the modulation of AT metabolism. To this, we analyzed the OCR, an index of OXPHOS, in 20 nM irisin-treated adipocytes by Seahorse Mito-Stress Test (Fig. 3A). Results showed that irisin rapidly affected the adipocyte metabolism. Indeed, the basal OCR was significantly reduced in the first 2-h of treatment [53 ± 19.3 *vs.* 110 ± 51.2 pmol/min/μg of untreated cells ($p < 0.05$)] and returned to basal levels upon prolonged stimulation 74 ± 7.6 pmol/min/μg (Fig. 3A-D). Importantly, to this point the amount of irisin in cell supernatant did not change over the stimulation (Fig. S2).

The energy map combining OCR with ECAR, a readout of cell glycolytic activity (Fig. 3B) indicated that 2-h of treatment with irisin results in a more quiescent metabolic profile, suggesting that irisin suppresses ATP production. Again, the energy phenotype was partially rescued at 4-h of treatment (Fig. 3A). Also, proton leak and ATP-linked respiration show the same rapid and transitory increase (Fig. 3C-E). Accordingly, irisin stimulation induced the expression of lipogenic genes including adipose triglyceride lipase (ATGL) and of the hormone-sensitive lipase (HSL). Perilipin (Plin1) continued to increase until 24-h after irisin treatment (Fig. 4A). The production of ATP increased during irisin stimulation (Fig. 4B). Of note, 20 nM of irisin ensured a stimulation with a large excess of soluble stimulus. Indeed, the amount of irisin in cell supernatant was almost constant during the experimental timeframe (Fig. S2).

4. Discussion

Upon its discovery, irisin has been a subject of intense investigation [15]. Since irisin is an exercise-induced hormone [6] and passes a signal to AT, it is important to identify the molecular mechanisms that mediate its effects. Irisin holds an essential role in the upregulation of UCP1 [5,11,50], which is abundant in the inner membrane of the BAT mitochondria. However, the mechanism through which irisin causes the upregulation of UCP1 remains unclear. Furthermore, the increase of irisin after physical exercise is another factor that needs to be clarified, since our current knowledge is based on measurements that took place across different time points, utilizing various exercise protocols [7]. As a result, controversial data about the clearance of irisin from the circulation exist in the literature. The present study addresses these gaps by defining a timeframe for the effect of irisin on adipocytes.

We found that irisin regulates *UCP1* gene in adipose derived 3T3-L1 cells inducing a fast and transitory increase in its expression, while it induces a slow and long-term upregulation of lipolytic genes. These data suggest that prolonged treatment with irisin could induce AT browning. Classically, *UCP1* gene expression is under the control of free fatty acids

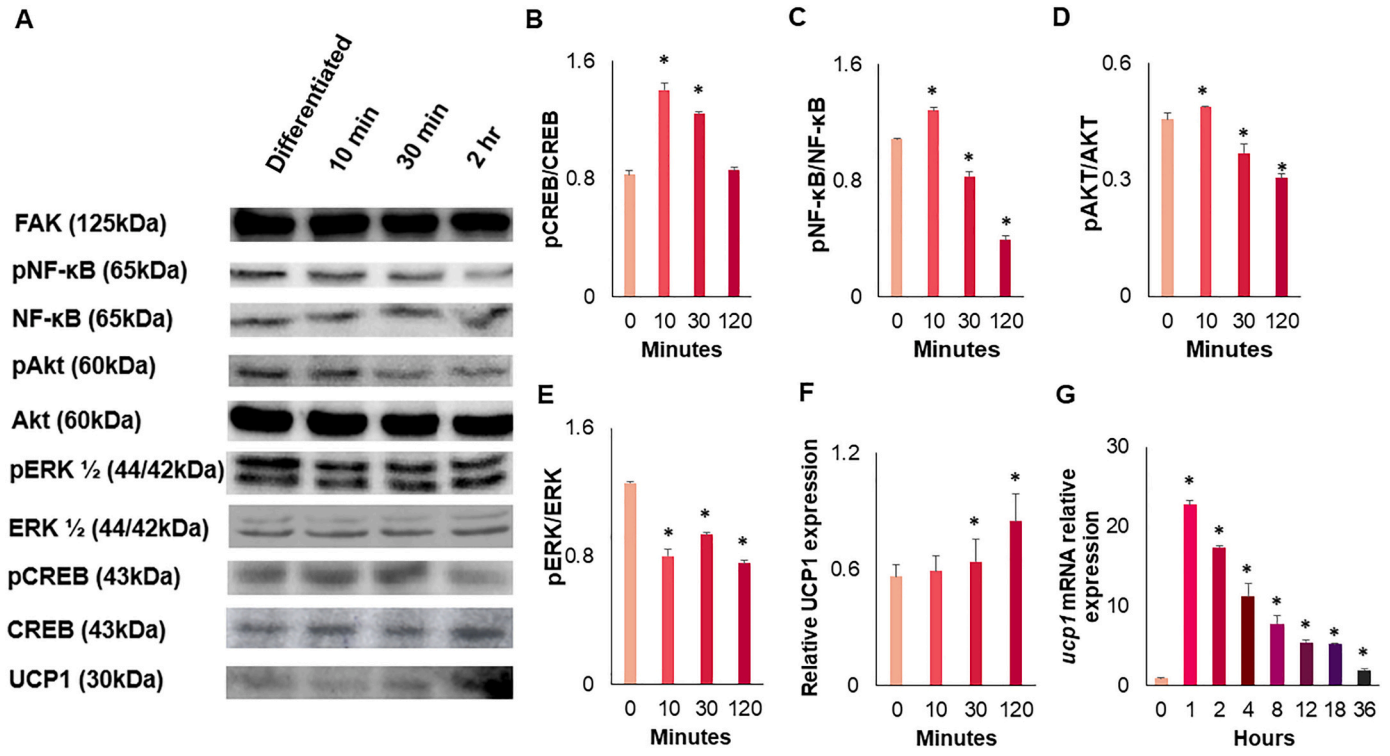

Fig. 2. Irisin downstream effect on protein expression. A) Western blot analysis of 50 μg of total protein lysates (all experiments were performed in duplicates). B-E) Phosphorylated *vs* unphosphorylated relative ratios for pCREB/CREB, pNF-κB/NF-κB, pAKT/AKT, and pERK/ERK were calculated by WB densitometry. F) Relative densitometric analysis of UCP1 protein normalized to FAK. G) mRNA expression of *ucp1* calculated by RT-qPCR. * indicates statistically significant differences ($p < 0.05$) from untreated cells.

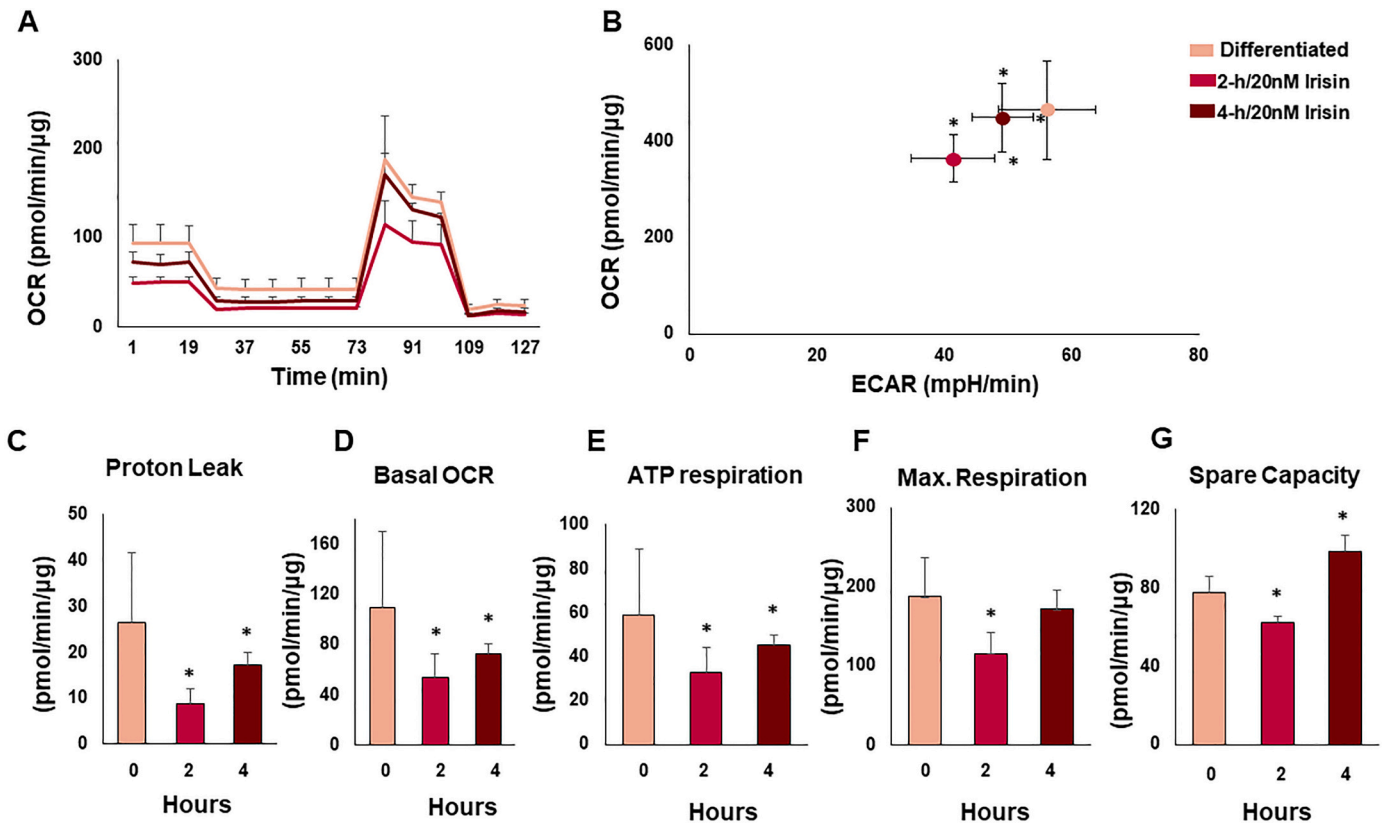

Fig. 3. Irisin reduces mitochondrial activity in adipose-derived 3T3 cells A) Seahorse Cell Mito Stress Test performed on adipose-derived 3T3 cells in the absence or in the presence of 20 nM of irisin. Oxygen consumption rate (OCR) was recorded over time before and after sequential addition of 1 μM oligomycin (Oligom), 0.5 μM FCCP and 1 μM Rotenone/Antimycin-A (Rot/Anti-A). B) Stressed energy phenotype of 3T3-L1 differentiated adipocytes with different periods of treatment with hr-irisin. Normalized OCR and ECAR were plotted to reveal overall basal metabolic profiles for differently treated cells. (C, D, E) Proton leak, basal OCR and mean ATP-linked OCR were calculated. Basal OCR was calculated by subtracting non-mitochondrial respiration from baseline OCR. Note: * indicates statistical significance ($p < 0.05$) from untreated cells. Experiments were performed in triplicates.

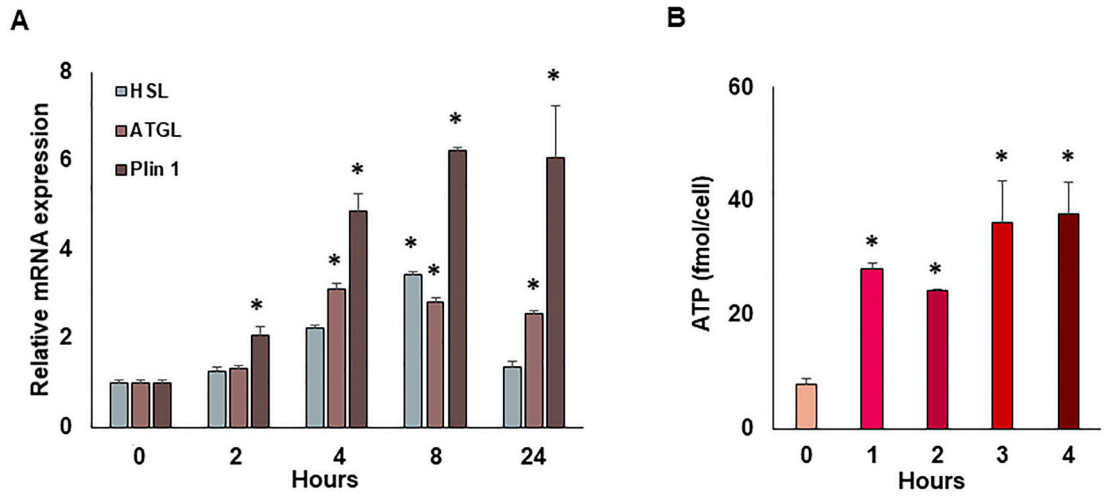

Fig. 4. Irisin affects the expression of lipogenic genes A) Relative expression of HSL, ATGL and Plin1 during treatment with irisin (0-h to 24-h). B) ATP concentration at different time points during 4-h. * indicates statistically significant difference ($p < 0.05$) from untreated cells. Experiments were performed in triplicates.

released upon extensive lipolysis [18]. However, more recent studies demonstrated that thermogenesis and *UCP1* expression is independent from lipolysis, suggesting that the two processes may be involved in AT browning through distinct mechanisms [43]. Our results support this notion, showing that irisin induces a fast upregulation of *ucp1* gene that precedes, and is likely independent from, the upregulation of lipolytic genes. Moreover, we elucidated the intracellular signaling activated by irisin, which involves NF-κB (p65), AKT, pCREB, and pERK.

Adipocyte differentiation is under the control of PPARγ, which is responsible for regulating the expression of genes involved in adipogenesis and lipid storage [6,41]. PPARγ, which has an important role in adipocyte differentiation [2], interacts with the p65 subunit of NF-κB by inhibiting NF-κB transcriptional activity [12]. NF-κB is a physiological regulator of mitochondrial respiration [29]. We observed that

phosphorylation of the p65 subunit of NF-κB, which enhances the selective NF-κB-mediated gene expression [40], was attenuated at 30-min and 2-h of hr-irisin treatment (Fig. 3B). The decreased phosphorylation of p65 after irisin administration in the medium indicates that irisin induces adipocyte differentiation through the regulation NF-κB signaling pathway. The concomitant increase of UCP1 expression observed in this study further demonstrates the possible role of irisin in the induction of the browning process.

Irisin is involved in the molecular pathway that drives the expression of UCP1 which decreases the proton gradient developed in oxidative phosphorylation and generates heat [39]. As revealed in our results, 2-h of irisin treatment on 3T3-L1 adipocytes moved the energy phenotype of the cells to a more quiescent state, indicating that the metabolic processes that produce energy in the form of ATP (oxidative phosphorylation and glycolysis) are initially restricted upon the presence of irisin. Of note, the ATP levels that we measured represent accumulated (produced and non-consumed) ATP in the cell medium. Accordingly, we found that ATP accumulates in the medium during irisin stimulation Taken together, our findings suggest that during exercise, and consequent release of irisin, 3T3-L1 cells release ATP in the extracellular environment. This extracellular ATP could have an autocrine effect (*e.g.*, on insulin stimulated glucose uptake) [1] or be taken up by the muscle tissue as an extra fuel source [20].

The quiescent state that the cells are transitioning to after irisin administration could also indicate a role of irisin on fatigue development. Here we examined the effects of irisin on adipocytes, but if the present results are also confirmed in muscle cells, this would suggest that irisin acts as an autocrine agent to induce fatigue, potentially as a protective mechanism. Also, it would be promising to use a systemic or an *in vitro* co-culture approach to understand the cross-talk between the muscle and adipose tissues and its potential role on fatigue development [31].

AKT regulates glycolysis and oxidative phosphorylation in AT through the glucose transporter [4], which mediates β3-adrenergic signaling followed by increased lipolysis and glucose uptake [32]. Our results are in line with previous findings demonstrating that reduced AKT levels in AT can induce browning of WAT [45]. Increased UCP1 expression accompanied by reduced activation of AKT upon irisin stimulation indicates that irisin targets the PI3K-AKT pathway in AT. The CREB is a downstream target of PI3K-AKT pathway [35] and a transcriptional regulator of UCP1 [9]. CREB was activated rapidly after irisin treatment, and its activation was present even after 2-h of treatment. This confirms the involvement of irisin in UCP1 upregulation and its role in thermogenesis. Accordingly, irisin induces the expression of lipolytic genes, including ATGL, HSL and Plin1, which are necessary for thermogenesis.

Extracellular signal kinases (ERK ½) mediate an inhibitory effect of the expression of UCP1 as a response to their phosphorylation of tumour necrosis factor-alpha (TNF-α) [48]. During 2-h stimulation with irisin, we observed that ERK ½ phosphorylation was reduced which indicates that irisin may modulate TNF-α activity. As we did not directly measure the expression of TNF-α, potential confirmation by future studies will demonstrate the role of irisin in the browning of white adipocytes through ERK signaling. Finally, we also measured the expression of FAK which participates in adipocyte differentiation, and its disruption impairs adipocyte survival *in vitro* in 3T3-L1 adipocytes [27,28]. We did not observe significant changes in the expression of FAK following irisin stimulation, indicating that it is not a molecular target of irisin in adipose tissue.

Following previous methodology [6], we treated our cells with 20 nM hr-irisin. This may be considered relatively high compared to the levels of irisin in human blood plasma reported in some studies. The literature data on circulating irisin levels are neither concordant nor conclusive, as the results depend on the affinity of the antibody used [3]. Further analyses are necessary to establish the normative range of irisin in human blood plasma induced by physical activity [3]. Therefore, we based our experimental design on the available literature of *in vitro* experiments [6], considering that *in vitro* experiments require higher concentrations of stimuli as there is no constant source of the cytokine as in the *in vivo* environment.

5. Conclusion

In summary, in this study we demonstrate that irisin regulates the mitochondrial respiration in 3T3-L1 derived adipocytes and exerts its effects 2-h after induction in the cellular environment. The effects of irisin occur through the modulation of intracellular signaling pathways, the expression of lipolytic genes, as well as the upregulation of UCP1. Taken together, our findings demonstrate the potential role of irisin in the induction of non-shivering thermogenesis and the regulation of energy expenditure and lipolysis in WAT adipocytes. Since AT metabolism holds a crucial role in combating obesity and related metabolic disorders, irisin can be an attractive therapeutic target that needs to be investigated in future studies.

Data availability statement

The datasets generated during and/or analyzed during the current study are available by the corresponding author on a reasonable request.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbagen.2022.130085.

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