Free Radical Biology and Medicine 227 (2025) 322–335

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Melatonin induces fiber switching by improvement of mitochondrial oxidative capacity and function via NRF2/RCAN/MEF2 in the vastus lateralis muscle from both sex Zücker diabetic fatty rats

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ARTICLE INFO

Keywords:
Skeletal muscle
Vastus lateralis
Fiber switching
Mitochondrial respirometry
Melatonin
MEF2
Obese-diabetic rats

ABSTRACT

The positive role of melatonin in obesity control and skeletal muscle (SKM) preservation is well known. We recently showed that melatonin improves vastus lateralis muscle (VL) fiber oxidative phenotype. However, fiber type characterization, mitochondrial function, and molecular mechanisms that underlie VL fiber switching by melatonin are still undefined. Our study aims to investigate whether melatonin induces fiber switching by NRF2/RCAN/MEF2 pathway activation and mitochondrial oxidative metabolism modulation in the VL of both sex Zücker diabetic fatty (ZDF) rats. 5-Weeks-old male and female ZDF rats (N = 16) and their age-matched lean littermates (ZL) were subdivided into two subgroups: control (C) and orally treated with melatonin (M) (10 mg/kg/day) for 12 weeks. Interestingly, melatonin increased oxidative fibers amounts (Types I and IIa) counteracting the decreased levels found in the VL of obese-diabetic rats, and upregulated NRF2, calcineurin and MEF2 expression. Melatonin also restored the mitochondrial oxidative capacity increasing the respiratory control ratio (RCR) in both sex and phenotype rats through the reduction of the proton leak component of respiration (state 4). Melatonin also improved the VL mitochondrial phosphorylation coefficient and modulated the total oxygen consumption by enhancing complex I, III and IV activity, and fatty acid oxidation (FAO) in both sex obese-diabetic rats, decreasing in male and increasing in female the complex II oxygen consumption. These findings suggest that melatonin treatment induces fiber switching in SKM improving mitochondrial functionality by NRF2/RCAN/MEF2 pathway activation.

1. Introduction

In recent decades, the prevalence of obesity and type 2 diabetes has been increasing worldwide. Skeletal muscle (SKM) dysfunction plays a crucial role in these metabolic disorders [1,2]. It has been shown that mitochondrial dysfunction and oxidative impairment of SKM led to insulin resistance and intramyocellular lipid accumulation [3], showing the key role of this organelle in obese-diabetic condition progression. Mammalian SKM contains different types of fibers: Type I fibers, also known as slow-twitch oxidative fibers, have a higher mitochondrial content and utilize oxidative metabolism for energy production. In contrast, type II fibers are less oxidative and rely on glycolysis for energy production [1]. These fibers are therefore responsible for energy metabolism and overall muscle function, which can be altered in muscle disease states. For example, results from large studies showed that the proportions of type I and type II are respectively reduced and increased in obese and type 2 diabetic subjects [4–6]. In addition, fiber I type proportion was correlated with insulin responsiveness [7]. Moreover, animal studies showed significant variations in insulin sensitivity and responsiveness among muscles with different fiber proportions [8]. While the majority of these studies have linked these metabolic characteristics to SKM fiber type, other studies have shown an independent effect [9]. Further research is required to understand how metabolic changes or altered muscle fiber type distribution may underlie obesity,

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https://doi.org/10.1016/j.freeradbiomed.2024.12.019
Received 9 July 2024; Received in revised form 19 November 2024; Accepted 4 December 2024
Available online 5 December 2024

and type 2 diabetes.

Indeed, the susceptibility of muscle fiber type transition is nowadays raising much interest as a therapeutic candidate. Numerous studies have investigated the factors and molecular mechanisms underlying muscle fiber type switching and its potential benefits for metabolic health [10]. For instance, a study by Wang and colleagues demonstrated that exercise-induced fiber type switching in mice with increased oxidative fibers was associated with glucose tolerance and resistance to high-fat-induced obesity [11]. In addition, it has been well documented the biological role of the activation of the nuclear regulator factor erythroid-derived 2 (NRF2) in energy metabolism, improving mitochondrial functionality and biogenesis in SKM and fiber type profile [12–15]. Based on its phosphorylation levels, other studies have investigated the role of the regulator of calcineurin (RCAN) in muscle fiber type switching and metabolic health [1]. Also, it was shown that activated calcineurin increases slow fibers and regulates the oxidative capacity of SKM [16], as well as increasing the enzymes responsible for lipid metabolism and oxidative phosphorylation (OXPHOS) [17,18]. The NRF2/RCAN pathway regulates the expression of key proteins involved in mitochondrial biogenesis, antioxidant defense, and energy metabolism [19]. The NRF2/RCAN pathway has been implicated in regulating SKM glycogen metabolism [19]. Moreover, a recent study in a "human knockout" model suggested that the RCAN1 gene may be involved in regulating exercise-induced mitochondrial function and energy metabolism in SKM [20,21]. Further to this pathway, the role of the myocyte enhancer-binding factor 2 (MEF2) as a new gene regulatory pathway is being highlighted in SKM growth and energy metabolism [22,23]. Overall, understanding the mechanisms behind the NRF2/RCAN-mediated fiber-type switching could have important implications for development of therapeutic strategies to improve muscle function and combat metabolic diseases. In synthesis, muscle fiber type switching from type II to type I has emerged as a potential therapeutic target for improving metabolic health in obesity. Molecular pathways such as NRF2, RCAN, calcineurin, and MEF2 signaling may offer promising avenues for future research in this field.

Several studies have shown that melatonin improves mitochondrial respiration in a variety of pathological conditions affecting a vast range of tissues [24,25] including the brain [26], liver [27–29], heart [30], adipose [31,32], and SKM [33]. This effect is accompanied by an increase of activities of main complexes I (C_I) and IV (C_{IV}) of the mitochondrial respiratory chain [34]. However, there is no data on the potential role of melatonin in VL mitochondrial respiratory function in obesity or diabetes. To approach this question, we studied the effect of chronic oral administration of melatonin on mitochondrial function in the VL from Zücker diabetic fatty (ZDF) rat, a model of obesity-related type 2 diabetes mellitus (diabesity).

Melatonin is a nighttime hormone produced by the pineal gland and by many other tissues as well [35–37]. We previously showed that oral administration of melatonin reduces body weight and improves metabolic profiles in male ZDF rats [38,39]. The experimental evidence has shown that melatonin curbs obesity in ZDF rats by several mechanisms such as activation of non-shivering thermogenesis (NST) of brown and beige adipose tissue [39–42] and of SKM [43]. This was associated to enhanced uncoupling protein 1 (UCP1) expression in adipose tissue and sarco-endoplasmic reticulum Ca^{+2}-ATPase (SERCA)-sarcolipin (SLN) uncoupling in SKM, where it also increased SKM mitochondrial dynamics, autophagy and ATP production, increasing the SKM oxidative enzymatic activity and oxidative MYH isoforms expression [44]. However, immunofluorescence characterization of fiber switching, whether melatonin is an inducer of oxidative fibers number and a VL mitochondrial respiratory function enhancer, and the molecular mechanism involved in these processes are still undefined. In the present work, we investigated the potential role of orally administered melatonin on VL switching in ZDF rats of both sexes.

2. Materials and methods

2.1. Reagents

All reagents used were of the highest purity available. Melatonin (cat#PHR1767) was obtained from Sigma Chemicals (Madrid, Spain).

2.2. Animals and experimental protocols

This study followed the European Union guidelines for animal care and protection and approved by the Ethical Committee of the University of Granada (Granada, Spain) under permit project number June 23, 2021/096-CEEA. Male (n = 16) and female (n = 16) ZDF rats and male (n = 16) and female (n = 16) lean littermates (ZL) were obtained at the age of 5 wks. Male animals were maintained on Purina 5008 rat chow (proteins 23 %, fats 14 %, carbohydrates (by difference) 51 %, fibers 4 %, and ash 8 %; Charles River) and female ones on Research Diet #D12468 chow (proteins 12 %, fats 25.5 %, carbohydrates (by difference) 51 %, fibers 6 % and ash 5.5 %), tap water ad libitum and housed 2 per clear plastic cage in a climate-controlled room at 28–30 °C and 30–40 % relative humidity, with a 12-h dark/light cycle (lights on at 07:00 h). The animals were acclimated to room conditions in the first week after arrival, and water intake was recorded. Then, both ZL and ZDF rats were subdivided into two groups, each composed of 8 rats: animals treated for 12 wk with melatonin in drinking water (melatonin-treated, M-ZDF, and M-ZL) and vehicle-treated controls (C-ZDF and C-ZL). Melatonin (10 mg/kg body weight/day) was dissolved in a minimum volume of absolute ethanol and diluted in the drinking water, with a final concentration of 0.066 % (w/v). Fresh melatonin and vehicle solutions were prepared three times a week, and the melatonin dose was adjusted to the body weight and water intake throughout the study period. Water bottles were covered with aluminum foil to be protected from light, and the drinking fluid was changed every two days to maintain melatonin stability and ensure the correct dosage. At the end of the treatment period, the animals were anesthetized with sodium thiobarbital (thiopental) and sacrificed.

2.3. Mitochondria preparation

VL samples (~200 mg) were excised. Mitochondria were isolated from these tissues using a serial centrifugation protocol that has the advantage of preserving the integrity of the mitochondrial membrane, with minor adaptations [45,46]. Tissues were removed, excised, washed with cold saline, and homogenized in an isolation medium (10 mM Tris, 250 mM sucrose, 0.5 mM Na_2EDTA, and 1 g/L free fatty acid bovine serum albumin (BSA), pH 7.4, 4 °C) with a Teflon pestle. The homogenate was centrifuged at 1000 g for 10 min at 4 °C, and the supernatant was centrifuged again at 15,000 g for 20 min at 4 °C. The resultant pellet was resuspended in 1 mL of isolation medium without BSA, and an aliquot was frozen for protein measurement. The remaining mitochondrial suspension was centrifuged at 15,000 g for 20 min at 4 °C and resuspended in 50 μL of respiration buffer (20 mM HEPES, 0.5 mM EGTA, 3 mM $MgCl_2$, 20 mM taurine, 10 mM KH_2PO_4, 200 mM sucrose, and 1 g/L free fatty acid BSA). The mitochondrial suspension was kept on ice for 10–15 min before following experiments.

2.4. Mitochondrial respirometry measurement

Mitochondrial respiration was evaluated using the high-resolution oxygraph Oroboros-O2k equipment (Oroboros Instruments, Innsbruck, Austria). It consists of two chamber respirometers with a Peltier thermostat and electromagnetic stirrers [47]. The analyses were performed in 2 mL of respiration buffer. In each chamber, the medium was formerly equilibrated with air at 30 °C and stirred at 750 rpm until a stable signal at air saturation was obtained. A final concentration of 0.2–0.3 mg/mL fresh proteins from the mitochondrial suspension was used for the

experiments. The mitochondria were suspended in respiration buffer supplemented with pyruvate 5 mM, glutamate 5 mM, and malate 2.5 mM, succinate 5 mM or fatty acid Octanoyl-CoA 0.5 mM in the presence of rotenone as energizing substrates. Oxygen flux (JO_2) was recorded at 30 °C in a constantly stirred oxygraph vessel after consecutive additions of 1 mM ADP (state 3 or OXPHOS capacity), and, at the end of the measurement, 0.75 mM oligomycin as ATPase inhibitor (state 4 or leak respiration). For each respiratory state, the results were expressed as pmol of oxygen consumed/min/mg protein. Measurements were taken at 0.2-s intervals for 15–20 min and recorded using a computer-driven data acquisition system (DatLab, Innsbruck, Austria). The respiratory control ratio (RCR) (the ratio of state 3 to state 4) was used as a general measure of mitochondrial function [48]. Also, the phosphorylation coefficient (ADP/O ratio) was measured.

2.5. Respiratory chain complex activities measurement

To obtain submitochondrial components, the mitochondrial pellet (resulting from the isolation procedure) was frozen, dissolved twice, and resuspended by sonication, in 500 μL of the medium corresponding to the complex to be measured, as described below [34]. Specific activity was expressed as nmol/min/mg protein.

2.5.1. Complex I (C_I)

Submitochondrial components (0.3 mg/mL) were incubated for 5 min in a medium containing 250 mM sucrose, 50 mM KH_2PO_4, 1 Mm KCN, and 0.5 mM decylubiquinone, pH 7.4. The reaction was initiated by the addition of NADH. The specific activity was obtained by determining the decrease in absorbance at 340 nm as a consequence of NADH oxidation [49,50].

2.5.2. Complex II (C_{II})

Submitochondrial components (0.03 mg/mL) were added to the reaction buffer containing 1 M KH_2PO_4, 1 M succinate, 0.1 % 2,6-dichlorophenolindophenol (DCIP), 1 mM EDTA, and 1 % Triton X-100, with a pH of 7.4. Later, decylubiquinone 0.5 % (in ethanol) was added to initiate the reaction. Then, the specific activity represented by the reduction of DCIP was determined at 600 nm [50,51].

2.5.3. Complex III (C_{III})

Submitochondrial fractions (0.03 mg/mL) were mixed with the reaction medium containing 0.1 M KH_2PO_4, 15 mM decylubiquinone, 0.1 M NaN_3, and 10 % BSA pH 7.5. The reaction was initiated by adding 1 % oxidized cytochrome *c* [52,53]. The specific activity represented by cytochrome *c* reduction was then measured at 550 nm.

2.5.4. Complex IV (C_{IV})

The specific activity represented by cytochrome *c* oxidase (COX) was determined at 550 nm. Submitochondrial fractions (0.5 mg/mL) were mixed in a reaction buffer containing 75 mM KH_2PO_4, pH 7.4 at 25 °C [49]. This reaction was initiated by adding reduced cytochrome *c* with sodium borohydride.

2.6. Immunofluorescence

The optimal cutting temperature compound (OCT)-embedded tissue samples were frozen in 2-methylbutane cooled in liquid nitrogen. Cryosections were made using a LEICA CM1510S Cryrostat at −22 °C. After cutting the samples into 10 μm sections, they were mounted on slides and air dried 5 min. For immunofluorescence the sections were fixed for 5 min in 4 % paraformaldehyde (PFA), then washed three times for 5 min in Phosphate Buffer Saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.4). After washing, heat-induced antigen retrieval was performed with Tris-EDTA Buffer (10 mM Tris, 1 mM EDTA, and 0.5 % Tween-20, pH 9.0) at 95 °C for 10 min. When slices were cooled, samples were placed on PBS for 5 min at room temperature and then in permeabilization buffer consisting of PBS with 0.1 % Triton X and 0.1 M glycine for 10 min. Sections were then washed three times with PBS for 5 min and blocked in blocking solution (PBS containing 5 % normal donkey serum and 0.3 % Triton X-100) for 1 h. The slides were incubated with the first antibody raised in mouse against MyH2 (cat#SC-53095, Santa Cruz Biotechnology, USA), MyH1/2 (cat#SC-53088, Santa Cruz Biotechnology, USA), and MyH7(cat#SC-53090, Santa Cruz Biotechnology, USA) at a dilution of 1: 50 in blocking solution overnight at 4 °C. After three washings with PBS for 5 min, the slides were incubated with the anti-mouse secondary antibody conjugated to CF™ 594 (cat#SAB4600105, Sigma Chemicals, Spain) in a dilution of 1:1000 in PBS for 1 h in dark. The slides were washed again three times with PBS for 5 min and incubated with 1 μg/ml Hoechst (cat#H1399, Thermofisher Scientific, Spain) for 5 min. Negative controls included sections incubated with secondary antibodies only, omitting primary antibodies. After several washing steps with PBS, the slides were mounted with fluorescence Ibidi Mounting Medium (cat#50001, Ibidi, Germany). Fluorescence micrographs were taken with the high spectral confocal laser scanning model Nikon A1 coupled to an inverted microscope Nikon Eclipse Ti-E (Nikon Corporation, Japan) from the Center for Scientific Instrumentation (CIC) at the University of Granada; and analyzed using Fiji (ImageJ) and Nikon Software. Images of tissue cross-sections were captured at 20 × objective. The number of positive fibers for each MyH isoform were counted and expressed as a relative amount (percentage) of the total number of fibers in the field. Type IIx fibers were obtained from the subtraction of MyH2 positive cells to the total MyH1/2 positive cells. Type IIb fibers were obtained as the negative staining cells in primary antibody cocktail MYH1/2/7-stained sections. An average of ten transverse sections per animal, and three different animals of each experimental group were used for the measurements. Multiple fields of view (20 × magnification) within each section were selected for the average fiber counting. Each field corresponds to approximately 180–200 fibers.

2.7. Real-time polymerase chain reaction (q-PCR)

For the gene expression analysis by Reverse Transcriptase-Quantitative-Polymerase Chain Reaction (RT-q-PCR), a total cellular RNA was extracted from 25 mg VL tissue using the RNeasy Mini Kit (cat#74104, QIAGEN, Germany). To assess the quantity and the integrity of the isolated RNA, we utilized a Nanodrop one/one (cat#ND-ONE-W, Thermofisher Scientific, Spain) employing spectrophotometric absorption at 230, 260, and 280 nm. Then, 1.0 μg of RNA was used to synthesize the first-strand complementary DNA (cDNA) by GoScript Reverse Transcriptase Kit (cat#A5001, Promega, USA). The reverse transcription was performed in a final volume of 20 μL. GoTaq qPCR Master Mix (cat#A6002, Promega, USA) was used for q-PCR, following the manufacturer's protocol and the amplification measurement's by QuantStudio 3 Real-Time PCR System thermocycler (cat#A28567, Thermofisher Scientific, Spain). For the amplification of the target genes (NRF2, MEF2, RCAN1.1, RCAN1.4, and ACTB), the primers listed in Table 1 were used and were generated using Primer-Blast platform from the National Center for Biotechnology Information (NCBI).

We used β-actin (ACTB) cDNA as an internal control. We also used appropriate controls and standard curves by amplifying first-strand cDNA for 16 to 31 cycles to ensure PCR linearity and to validate cDNA

Table 1
List of primers pair used in RT-q-PCR

Gene	Forward sequence (5' → 3')	Reverse sequence (5' → 3')
RCAN1.1	CCGGGCCAAATTTGAATCCCT	GACAGGGGGTTGCTGAAGTT
RCAN1.4	CCAGGGCCAAATTTGAATCCC	GACAGGGGGTTGCTGAAGTT
NRF2	ATCCAGGGCAAGCGACTCAT	GGTTGCCCACATTCCCAAAC
MEF2	GACAGGTGACTTTTACAAAG	GTACTCGGTGTACTTGAG
ACTB	ACCCGCCACCAGTTCGCCAT	CGGCCCACGATGGAGGGGAA

quantification. The amplicons were separated on 1 % agarose gel and stained with Sybr Safe (cat#S33102, Thermofisher Scientific, Spain) to verify the reverse transcription q-PCR quality.

2.8. Protein isolation and calcineurin quantification by western-blot

The total VL proteins were extracted from approximately 0.2 g of samples using RIPA Lysis Buffer (150 mM NaCl, 50 mM Tris-HCl, 1 %

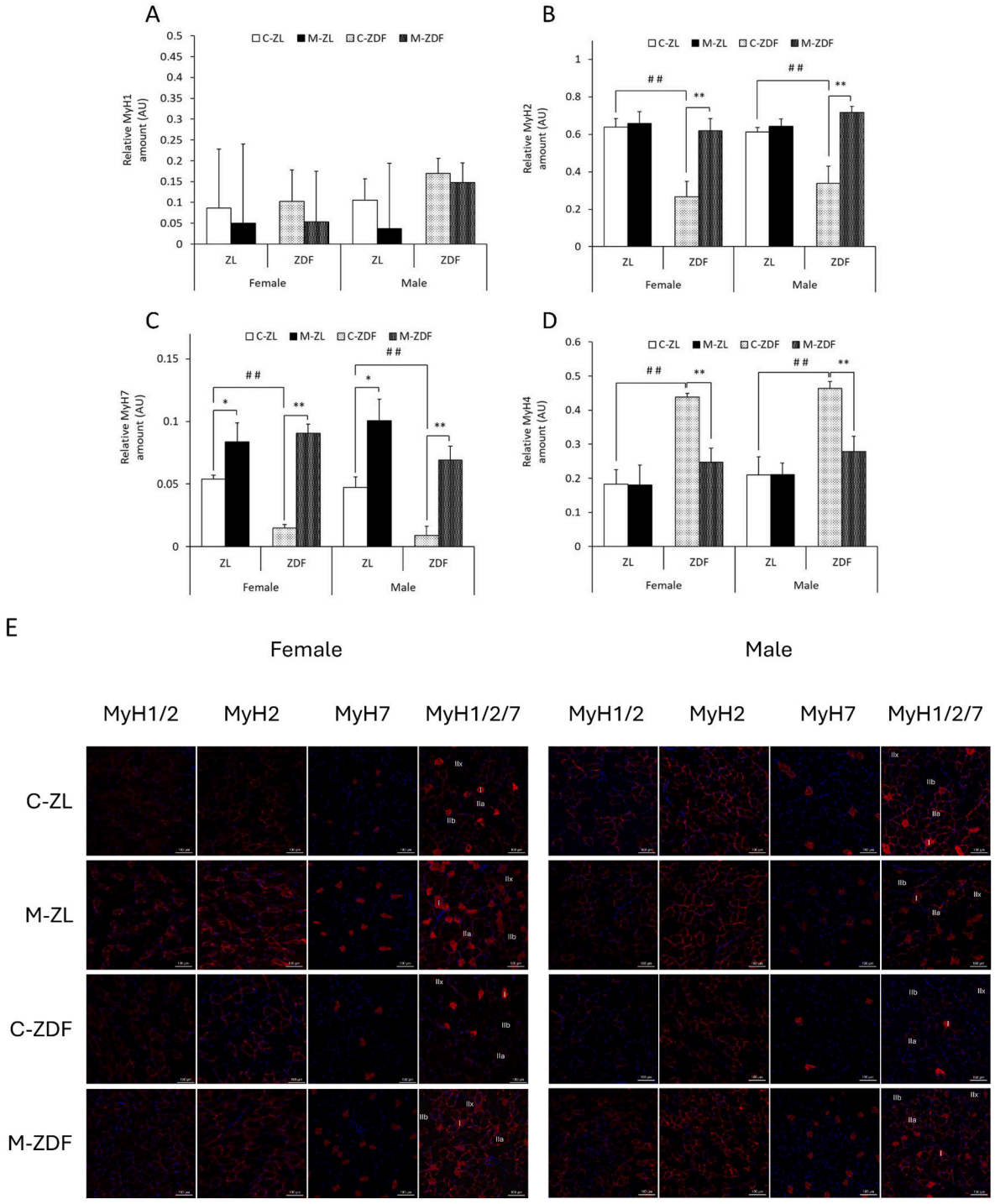

Fig. 1. Melatonin effects on VL fiber type composition in female and male Zücker lean (ZL) and Zücker diabetic fatty (ZDF) rats. **A** Relative MyH1 positive cells number expressed as a percentage of the total number of fibers in the field, obtained from the subtraction of MyH2 positive cells to the total MyH1/2 positive cells. **B** Relative MyH2 positive cells number expressed as a percentage of the total number of fibers in the field. **C** Relative MyH7 positive cells number expressed as a percentage of the total number of fibers in the field. **D** Relative MyH4 positive cells number expressed as a percentage of the total number of fibers in the field, obtained from the subtraction of MyH1/2/7 cocktail positive cells to the total number of fibers in the field. **E** Representative microscopy image showing in blue labeled the nucleus (Hoechst) and in red labeled the MYH isoforms (CF594). Results are expressed as the means ± S.E.M. of ten transverse sections per animal in triplicate. One-way ANOVA followed by Tukey's post-test was performed for static analysis (*$P < 0.05$ and **$P < 0.01$ melatonin vs control rats; ##$P < 0.01$ C-ZDF vs C-ZL rats). Original magnification at × 200. Scale bar: 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Nonidet P-40, 0.5 % sodium deoxycholate, and 0.1 % sodium dodecyl sulfate (SDS)) supplemented with 1 % Protease Inhibitor Cocktail (cat#P8340-1 ML, Sigma-Aldrich, Spain) and 1 % Phosphatase Inhibitor Cocktail (cat#P5726-1 ML, Sigma-Aldrich, Spain) in a Teflon pestle homogenizer (6 cycles of 4 s each, 4 °C). Subsequently, centrifugation was performed at 13,000 g for 30 min at 4 °C, followed by protein quantification of the supernatant using the Protein Assay Kit II (BioRad, Spain). A total of 100 μg of total protein from each sample was subjected to analysis via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). For immunoblot analyses, the gels were transferred onto a nitrocellulose membrane (BioRad Trans-Blot SD; BioRad Laboratories, Hercules, CA, USA). The membranes were subsequently blocked in Blocking Buffer (PBS, supplemented with 1 % Tween-20 (PBS-T) and 5 % BSA) and then incubated overnight at 4 °C with primary antibody anti-Calcineurin A (cat#H00005530-M03, Abnova, USA) produced in mouse at a dilution of 1:2000 in Blocking Buffer. The anti-α-Tubulin antibody produced in mouse (cat#SC-5286, Santa Cruz Biotechnology, USA) was used as control. Following overnight incubation, the membranes were washed 3 times in PBS-T and a 1 h incubation with horseradish peroxidase-conjugated anti-mouse secondary antibody (cat#MBS674947, Donkey Anti-Mouse IgG, MyBiosource, USA) was carried out at room temperature. Immune reactive signals were detected via enhanced chemiluminescence using a Clarity Western ECL Substrate kit (BioRad, Spain). The blots were digitally recorded using a Kodak Image Station 4000 PRO and analyzed for molecular weight determinations through image analysis software, ImageJ. Fold changes in relative expression were calculated based on the signal intensities obtained from ImageJ analysis.

2.9. Statistical analysis

All analyses were performed in three independent experiments and repeated at least in duplicate in each rat to ensure reliability. All results were expressed as means ± standard deviation (SD) values. Comparisons between experimental groups were analyzed using one-way ANOVA followed by the post-hoc Tukey's Test. Differences between groups were considered statistically significant if the P-value <0.05. SPSS version 22 for Windows (SPSS, Michigan, IL, USA) was used for statistical data analyses.

3. Results

3.1. Effects of melatonin on VL fiber type composition and fiber switching

To evaluate the activation of the fiber switching to a more oxidative phenotype (Type IIb fibers to Type IIa and/or Type I fibers), we performed immunofluorescence to characterize the VL myofiber types and measure the changes in MyH isoforms expression (Type IIx fibers, MyH1; Type IIa fibers, MyH2; Type I fibers, MyH7; and Type IIb fibers, MyH4). No significant changes in MyH1 expression patterns were observed in any of the experimental groups as observed in Fig. 1A–E. Obese-diabetic rats from both sexes presented lower number of oxidative Type IIa (MyH2) and Type I (MyH7) fibers and increased number of glycolytic fast-twitch Type IIb (MyH4) fibers as shown in Figure E (female, MyH2, 0.27 ± 0.08, MyH7, 0.015 ± 0.003, and MyH4, 0.43 ± 0.01; male, MyH2, 0.34 ± 0.09, MyH7, 0.009 ± 0.008, and MyH4, 0.46 ± 0.02) compared to their respective lean control rats (female, MyH2, 0.64 ± 0.05, MyH7, 0.054 ± 0.003, and MyH4, 0.18 ± 0.04; male, MyH2, 0.62 ± 0.02, MyH7, 0.047 ± 0.009, and MyH4, 0.21 ± 0.05; $P <$ 0.01, Fig. 1B, C, and D respectively). Melatonin treatment completely reversed the effects of obesity in VL fiber type composition in both sex ZDF rats, increasing the number of oxidative Type IIa and I fibers while decreasing the number of glycolytic Type IIb fibers as indicated in Fig. 1E (female, MyH2, 0.62 ± 0.06, MyH7, 0.091 ± 0.009, and MyH4, 0.25 ± 0.04; male, MyH2, 0.72 ± 0.03, MyH7, 0.070 ± 0.011, and MyH4, 0.28 ± 0.04; $P < 0.01$, Fig. 1B, C, and D respectively). Moreover, melatonin treatment also increased Type I fibers in M-ZL rats from both sexes (female, MyH7, 0.083 ± 0.015; male, MyH7, 0.101 ± 0.017; $P <$ 0.05, Fig. 1C–E).

3.2. Effects of melatonin on VL activation of fiber switching pathway

To evaluate the activation of the fiber-switching pathway to a more oxidative phenotype, we performed q-PCR and western-blot techniques for measuring the fiber-switching pathway genes and protein regulation by its changes in expression. Obese-diabetic rats from both sexes presented higher gene expression levels of RCAN1.1 and 1.4 (female, RCAN1.1, 1.66 ± 0.23 and RCAN1.4, 3.26 ± 0.64; male, RCAN1.1, 1.85 ± 0.05 and RCAN1.4, 2.43 ± 0.38) compared to their respective lean controls (female, RCAN1.1, 0.87 ± 0.05 and RCAN1.4, 0.99 ± 0.26; male, RCAN1.1, 1.08 ± 0.03 and RCAN1.4, 1.32 ± 0.05; $P < 0.01$, Fig. 2A, B respectively). Therefore, calcineurin protein expression was decreased in both sex ZDF rats (female, 0.45 ± 0.08; male, 0.42 ± 0.02) compared to ZL control ones (female, 0.80 ± 0.05; male, 0.71 ± 0.04; $P <$ 0.05) as showed in Fig. 2C and D. Furthermore, NRF2 and MEF2 gene expression was also lower in obese diabetic female (NRF2, 0.55 ± 0.03 and MEF2, 0.96 ± 0.05) and male rats (NRF2, 0.48 ± 0.02 and MEF2, 0.89 ± 0.04) than in lean control lean ones (female, NRF2, 0.85 ± 0.05 and MEF2, 1.43 ± 0.03; male, NRF2, 0.85 ± 0.04 and MEF2, 1.24 ± 0.04; $P < 0.05$, Fig. 2E, F respectively). RCAN gene expression was downregulated by melatonin in both sex ZDF rats (female, RCAN1.1, 0.41 ± 0.03 and RCAN1.4, 1.54 ± 0.12; male, RCAN1.1, 1.03 ± 0.03 and RCAN1.4, 1.12 ± 0.19; $P < 0.01$), and also as well melatonin decreased RCAN1.1 messenger RNA (mRNA) levels in both sex ZL ones (female, 0.68 ± 0.03, $P < 0.05$; male, 0.38 ± 0.03; $P < 0.01$) as showed in Fig. 2A, B. Melatonin also increased calcineurin expression in both sex obese (female, 1.03 ± 0.06, $P < 0.01$; male, 0.66 ± 0.04; $P < 0.05$) and lean rats (female, 1.07 ± 0.09; male, 0.95 ± 0.03; $P < 0.05$, Fig. 2C and D). Furthermore, as showed in Fig. 2E, F, after melatonin treatment NRF2 and MEF2 gene expression was higher in ZDF female (NRF2, 2.19 ± 0.23; MEF2, 1.58 ± 0.03; $P < 0.01$) and male rats (NRF2, 1.69 ± 0.06, $P < 0.01$; MEF2, 1.27 ± 0.03; $P < 0.05$), and also MEF2 gene expression was upregulated in both sex ZL rats (female, 1.72 ± 0.06; male, 1.48 ± 0.03; $P < 0.05$).

3.3. Effects of melatonin on VL mitochondrial respiratory function

SKM is a mitochondria-rich organ and optimal mitochondrial function is key for SKM energy metabolism and plasticity [54], so we investigated whether melatonin treatment influences mitochondrial respiration in isolated mitochondria from VL samples in both sex and phenotype animals. O_2 consumption rate, proportional to oxygen flux (expressed as pmol/min/mg protein) increased sharply after the addition of ADP, followed by a rapid reduction due to the depletion of ADP, which has been phosphorylated to ATP in response to respiratory substrates (state 3, coupled OXPHOS capacity). In obese ZDF rats, while female animals presented decreased state 3 compared to their lean controls (C-ZDF, 22.87 ± 1.29; C-ZL, 26.39 ± 0.83; $P < 0.05$), male animals showed increased state 3 component (C-ZDF, 29.77 ± 2.39; C-ZL, 23.19 ± 1.06; $P < 0.05$), and thus, this is also higher in male C-ZDF rats compared to female C-ZDF ones ($P < 0.05$; Supplementary Fig. 1). Melatonin completely reversed the obesity effects on state 3 in both sex ZDF rats (female, 27.87 ± 1.21; male, 20.19 ± 0.83; $P < 0.05$, Fig. 3A). Furthermore, state 4 or leak respiration (O_2 consumption rate in the absence of ATP formation) was found to be increased in both sex ZDF rats (female, 10.66 ± 0.80; male, 10.77 ± 1.02) compared to their lean littermates (female, 8.28 ± 0.13; male, 8.04 ± 0.67; $P < 0.05$, Supplementary Fig. 1), and after melatonin treatment, state 4 was lowered in both sex and phenotype animals (female M-ZDF, 8.32 ± 0.62; and M-ZL, 6.24 ± 0.19; male M-ZDF, 6.01 ± 1.61; and M-ZL, 5.01 ± 1.04; $P <$ 0.05) as showed in Fig. 3B.

The RCR, which corresponds to the state 3/state 4 ratio, is considered

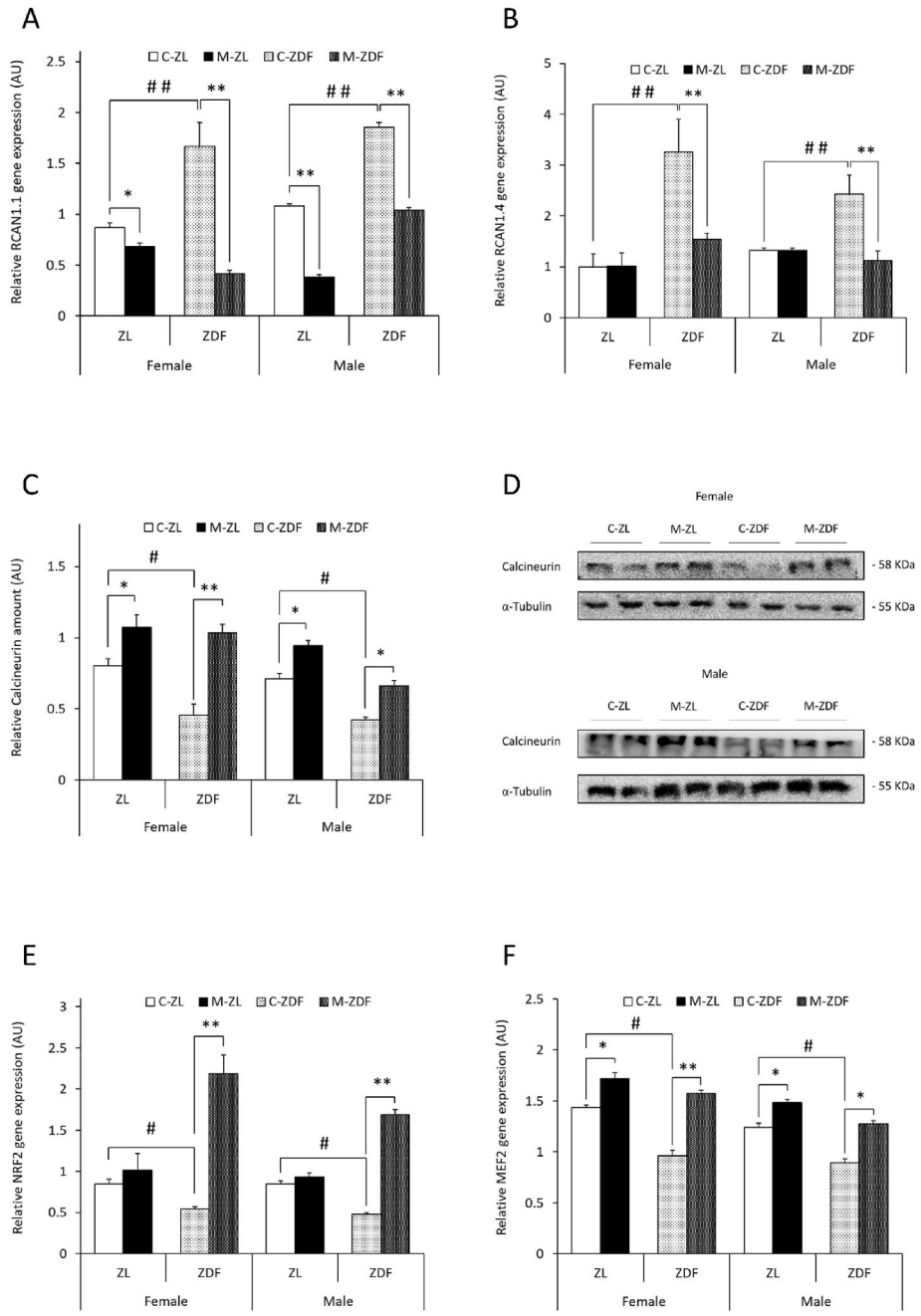

Fig. 2. Melatonin effects on the activation of the fiber switching pathway in the VL from female and male Zücker lean (ZL) and Zücker diabetic fatty (ZDF) rats. **A** Relative RCAN1.1 gene expression (mRNA levels quantification). **B** Relative RCAN1.4 gene expression (mRNA levels quantification). **C** Densitometry quantification of Calcineurin expression. **D** Representative blots of Calcineurin protein expression. **E** Relative NRF2 gene expression (mRNA levels quantification). **F** Relative MEF2 gene expression (mRNA levels quantification). Results are expressed as the means ± S.E.M. of three independent experiments in duplicate. One-way ANOVA followed by Tukey's post-test was performed for static analysis (*$P < 0.05$ and **$P < 0.01$ melatonin vs control rats; #$P < 0.05$ and ##$P < 0.01$ C-ZDF vs C-ZL rats).

the best mitochondrial function indicator [48]. In all experimental conditions tested, RCR values were greater than 2.2, which indicated an optimal mitochondrial isolation and sample preparation and well-coupled isolated mitochondria [31]. However, as Fig. 3C indicates, both sex ZDF rats presented lower RCR (female, 2.24 ± 0.25; male, 2.23 ± 0.14) than their respective lean controls (female, 3.19 ± 0.35; male, 2.92 ± 0.15; $P < 0.05$), and melatonin was shown to improve RCR in both sex ZDF (female, 3.35 ± 0.56; male, 3.36 ± 0.10; $P < 0.05$) and ZL rats (female, 4.24 ± 0.41; male, 4.54 ± 0.34; $P < 0.05$). Moreover, the phosphorylation coefficient (ADP/O ratio) was decreased in both sex obese rats compared to ZL control ones (female C-ZDF, 1.36 ± 0.29; and C-ZL, 2.53 ± 0.22; male C-ZDF, 1.29 ± 0.41; and C-ZL, 2.17 ± 0.03; $P <$ 0.05), and after melatonin treatment, the ADP/O ratio was increased in both sex and phenotype rats (female M-ZDF, 2.14 ± 0.22; and M-ZL, 3.12 ± 0.24; male M-ZDF, 2.42 ± 0.29; and M-ZL, 2.61 ± 0.18; $P <$ 0.05) as illustrated in Fig. 3D.

The overall bioenergetics of OXPHOS respiration depends on the efficient regulation of the electron transport chain (ETC) composed of different respiratory enzymes, and the main electron entry points in the respiratory chain are C_I and C_{II} that use different substrates as electron sources such as NADH from pyruvate, malate, and glutamate (C_I); or $FADH_2$ from succinate (C_{II}) [55,56]. Because of this, we aim to study the coupled OXPHOS capacity (state 3) in isolated VL mitochondria from ZDF rats using different substrates to separately identify the

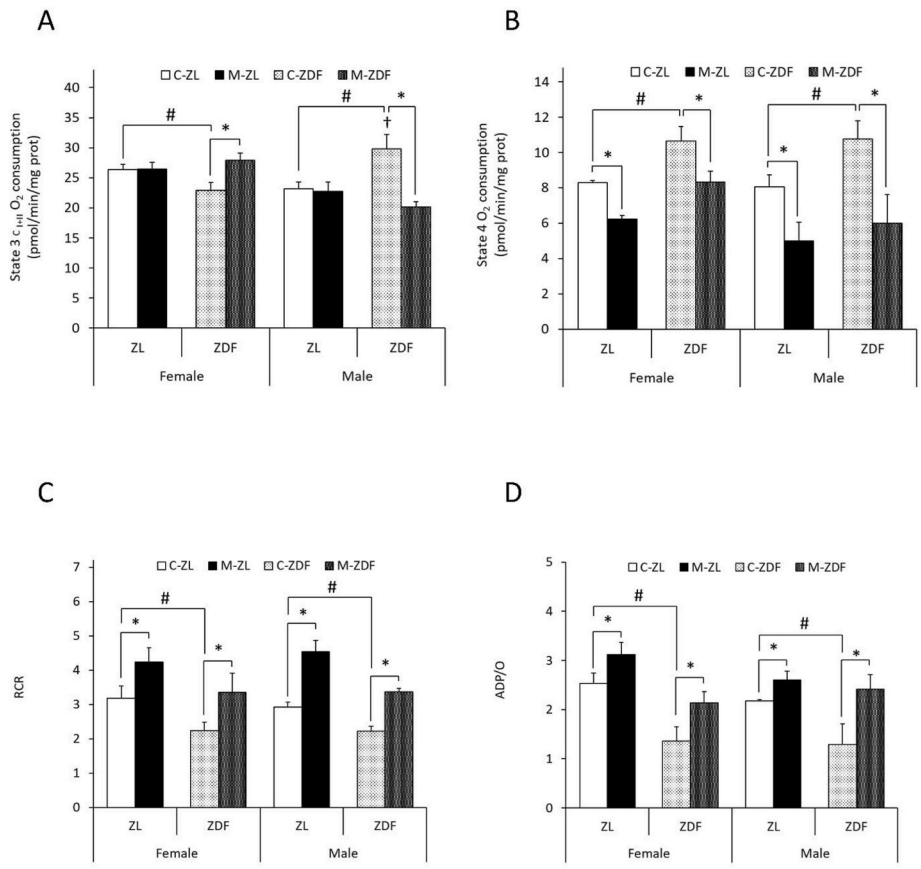

Fig. 3. Melatonin effects on respiratory parameters in the VL isolated mitochondria from female and male Zücker lean (ZL) and Zücker diabetic fatty (ZDF) rats. **A** State 3 oxygen consumption (oxygen flux (JO_2) while producing ATP in response to ADP in the presence of respiratory substrates for complex I and II (pyruvate, malate, glutamate, and succinate)), coupled OXPHOS capacity. **B** State 4 oxygen consumption (oxygen flux (JO_2) without ATP synthesis in the presence of oligomycin), leak respiration. **C** Respiratory control ratio (RCR) defined as the ratio state 3/state 4. **D** Respiratory phosphorylation coefficient (ADP/O ratio). Results are expressed as the means ± S.E.M. of three independent experiments in duplicate. One-way ANOVA followed by Tukey's post-test was performed for static analysis (*$P < 0.05$ melatonin vs control rats; #$P < 0.05$ C-ZDF vs C-ZL rats; †$P < 0.05$ female C-ZDF vs male C-ZDF).

contribution of each complex and even the contribution of the fatty acid β-oxidation (FAO), adding octanoyl-L-carnitine, to the total OXPHOS respiration. In the VL isolated mitochondria from obese-diabetic rats, state 3 C_I O_2 consumption was decreased compared to their control female (C-ZDF, 5.40 ± 1.31; C-ZL, 13.31 ± 1.65; $P < 0.01$) and male rats (C-ZDF, 6.44 ± 1.40; C-ZL, 15.57 ± 0.73; $P < 0.01$). After melatonin treatment, the effect of obesity on state 3 C_I O_2 consumption was reversed in both sex ZDF rats (female, 19.90 ± 2.22; male, 17.67 ± 1.06; $P < 0.01$), and also increased in both sex ZL rats (female, 17.78 ± 1.46; male, 20.06 ± 0.60; $P < 0.05$, Fig. 4A). Moreover, state 3 C_{II} O_2 consumption was found to be decreased in females (C-ZDF, 9.44 ± 1.64) and increased in male ZDF rats (C-ZDF, 20.84 ± 0.42) compared to their respective ZL lean controls (female, 22.12 ± 1.08; male, 7.09 ± 0.84; $P < 0.01$), and therefore, male C-ZDF rats presented higher state 3 C_{II} component than female C-ZDF ones ($P < 0.01$). Melatonin modulated state 3 C_{II} in ZDF rats by improving in females (M-ZDF, 19.97 ± 1.19; $P < 0.01$) and reducing in males (M-ZDF, 8.53 ± 1.21; $P < 0.01$) the mitochondrial O_2 consumption as showed in Fig. 4B. Concerning β-oxidation of fatty acid, state 3 FAO O_2 consumption was highly decreased in both sex ZDF rats (female, 2.41 ± 3.61; male, 5.61 ± 1.40) compared to both sex ZL control ones (female, 19.26 ± 3.14; male, 19.78 ± 1.26; $P < 0.01$, Supplementary Fig. 1). Melatonin treatment ameliorated the state 3 FAO component by raising the mitochondrial O_2 consumption in both phenotype females (M-ZDF, 25.77 ± 3.63; and M-ZL, 28.93 ± 2.56; $P < 0.01$ and $P < 0.05$, respectively) and male rats (M-ZDF, 22.21 ± 2.53; and M-ZL, 38.39 ± 3.24; $P < 0.01$, Fig. 4C).

To test the effect of melatonin treatment on the mitochondrial respiratory enzymes involved in the ETC, the activities (nmol/min/mg protein) of C_I, C_{II}, C_{III}, and C_{IV} were examined in isolated VL mitochondria from both sex and phenotype rats. Both sex ZDF rats presented lower C_I activity than their respective controls (female C-ZDF, 45.71 ± 16.08; and C-ZL, 108.82 ± 6.98; male C-ZDF, 35.01 ± 3.48; and C-ZL, 91.35 ± 4.36; $P < 0.01$), and melatonin treatment restored obesity effects in both sex ZDF rats (female, 156.32 ± 17.69; male, 67.60 ± 3.83; $P < 0.01$ and $P < 0.05$, respectively) and also increased C_I activity in both sex ZL rats (female, 141.37 ± 5.57; male, 144.63 ± 4.71; $P < 0.05$ and $P < 0.01$, respectively) as indicated in Fig. 5A. As for C_{II}, obesity reduced the complex activity in female ZDF rats (C-ZDF, 47.84 ± 6.66) and augmented in male ones (C-ZDF, 146.57 ± 7.66) compared to their respective lean control animals (female, 105.61 ± 16.72; male, 116.27 ± 2.02; $P < 0.01$ and $P < 0.05$, respectively), thus being higher the C_{II} activity in male C-ZDF than female C-ZDF rats ($P < 0.01$, Fig. 5B). Melatonin regulated C_{II} in ZDF rats by enhancing in females (M-ZDF, 114.26 ± 14.55; $P < 0.01$) and diminishing in males (M-ZDF, 99.46 ± 10.69; $P < 0.05$) the activity of the complex as showed in Fig. 5B.

C_{III} and C_{IV} activities were also reduced in both sex ZDF rats (female C_{III}, 74.54 ± 12.93; and C_{IV}, 71.24 ± 2.84; male C_{III}, 72.26 ± 4.26; and C_{IV}, 74.21 ± 4.98) compared to their respective both sex ZL ones (female C_{III}, 114.50 ± 8.42; and C_{IV}, 119.03 ± 12.20; male C_{III}, 109.20 ± 8.18; and C_{IV}, 133.59 ± 10.11; $P < 0.05$, Fig. 5C, D respectively). After melatonin treatment, C_{III} activity was improved in both phenotype female (M-ZDF, 150.42 ± 17.06; and M-ZL, 212.20 ± 16.76; $P < 0.01$) and male rats (M-ZDF, 103.06 ± 5.56; and M-ZL, 194.29 ± 8.60; $P < 0.05$ and $P < 0.01$, respectively) as illustrated in Fig. 5C. Also, melatonin

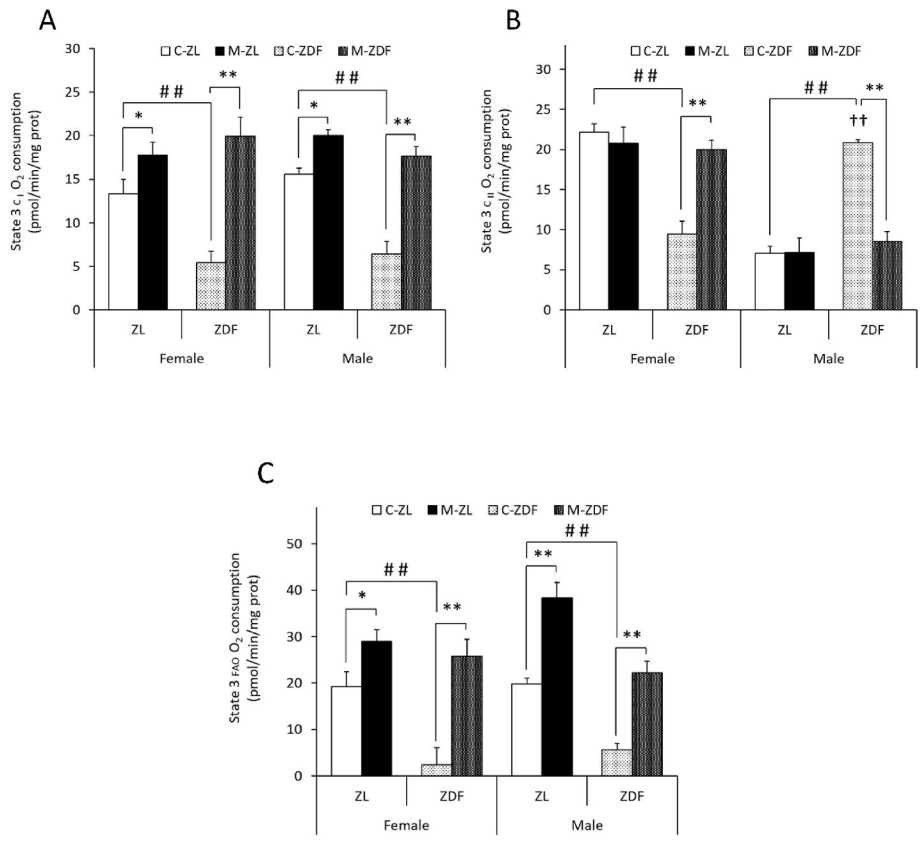

Fig. 4. Melatonin effects on respiratory State 3 with different substrates in the VL isolated mitochondria from female and male Zücker lean (ZL) and Zücker diabetic fatty (ZDF) rats. **A** State 3 Complex I (C_I) oxygen consumption (oxygen flux (JO_2) while producing ATP in response to ADP in the presence of pyruvate, malate and glutamate). **B** State 3 Complex II (C_{II}) oxygen consumption (oxygen flux (JO_2) while producing ATP in response to ADP in the presence of succinate). **C** State 3 fatty acid oxidation (FAO) oxygen consumption (oxygen flux (JO_2) while producing ATP in response to ADP in the presence of octanoyl-L-carnitine). Results are expressed as the means ± S.E.M. of three independent experiments in duplicate. One-way ANOVA followed by Tukey's post-test was performed for static analysis (*$P < 0.05$ and **$P < 0.01$ melatonin vs control rats; ##$P < 0.01$ C-ZDF vs C-ZL rats; ††$P < 0.01$ female C-ZDF vs male C-ZDF).

increased C_{IV} activity in both sex and phenotype rats (female M-ZDF, 111.63 ± 18.13; and M-ZL, 205.22 ± 29.87; male M-ZDF, 117.24 ± 5.59; and M-ZL, 181.59 ± 4.56; $P < 0.05$, Fig. 5D).

4. Discussion

We recently reported that melatonin enhanced the fiber oxidative phenotype in the deep VL of the same rat strain by increasing the mitochondrial oxidative enzymatic activity of NADH reductase and also the expression of MyH7 and MyH2 decreasing MyH4 in male ZDF rats [44]. In the present study, we reported that melatonin further modulates the VL fiber type composition remodeling the VL fibers from a glycolytic to an oxidative type in both sex obese-diabetic rats increasing oxidative Type I and IIa amount. Those results proved that chronic melatonin treatment drives VL to acquire an oxidative phenotype and melatonin acts as a switcher of fiber types, leading to an improved mitochondrial function of VL in both male and female ZDF rats via the NRF2/RCAN/MEF2 signaling pathway.

It is well known that performance and metabolism of SKM rely on its plasticity, i.e., on its fiber type proportions [57]. Also, abnormal metabolic profile of SKM in obesity is shown to be correlated with an alteration of oxidative muscle fiber composition [54,57]. As we mentioned above, SKM fibers are heterogeneously distributed and classified as oxidative and glycolytic types, being the first type known to be fatigue-resistant, while the second type is vulnerable [1].

Our results from IHF analyses indicate that obese-diabetic animals exhibited a lower proportion of type I and IIa fiber markers that rely on oxidative metabolism, and a higher proportion of type IIb, which are known to use glycolytic metabolism. These results are compatible with our previous work in males [44] and with other studies in human subjects, that reported fewer type I muscle fibers and extra type II in obese and type 2 diabetics in comparison with leans [5,58]. Interestingly, the analyses revealed that melatonin increased oxidative fibers amount (type I and type IIa), marked by MyH7 and MyH2 respectively, and reduced glycolytic ones (type IIb), marked by MyH4; thereby reversing the effects of obesity and leading to higher fiber oxidative capacity. Surprisingly, this change in fiber proportion between type I and type II was simultaneous, which is explained by the switching from glycolytic to oxidative phenotype within the VL with no significant variations in type IIx fibers. In line with these findings, a recent study showed that small hibernating mammals during interbout period, in which endogenous melatonin levels are augmented [59], exhibited enhanced cold adaptation by switching SKM fibers, increasing the number of oxidative type IIa fibers and MyH2 expression [60]. This brings to light the close relationship between SKM NST, SKM fiber switching, SKM metabolic plasticity, and melatonin, being in coherence with our previous results showing the effects of melatonin on increasing SKM NST in the same rat strain of both sexes [43]. Likewise, several studies in rodents and exercise or exercise-mimicking signals showed similar results to those obtained in the present study in terms of fiber switching showing an increase in MyH2/7-positive fibers (fast oxidative type IIa and slow oxidative type I fibers) and decreasing MyH4-positive fibers (fast glycolytic type IIb fibers) [61,62]. However, the absence of exercise presented a different fiber characterization pattern with an increase in the number of IIb fibers and a decrease in oxidative I and IIa fibers [63] as ZDF rats of both sexes of the current study presented.

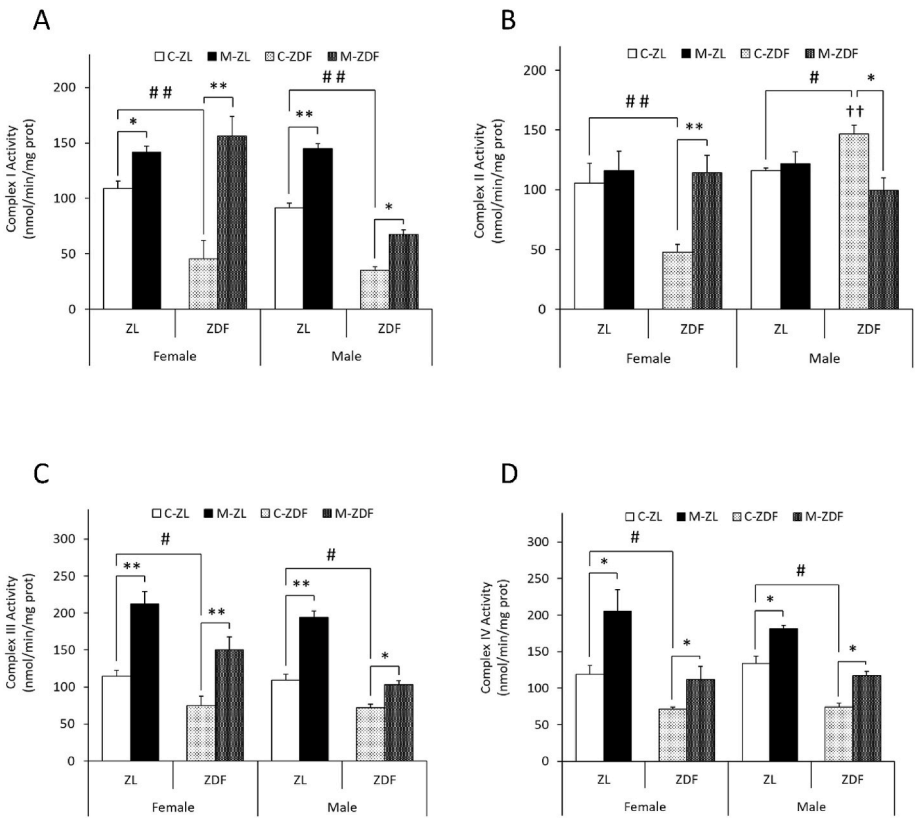

Fig. 5. Melatonin effects on the electron transport chain (ETC) complexes in the VL isolated mitochondria from female and male Zücker lean (ZL) and Zücker diabetic fatty (ZDF) rats. **A** Complex I activity. **B** Complex II activity. **C** Complex III activity. **D** Complex IV activity. Results are expressed as the means ± S.E.M. of three independent experiments in duplicate. One-way ANOVA followed by Tukey's post-test was performed for static analysis (*$P < 0.05$ and **$P < 0.01$ melatonin vs control rats; #$P < 0.05$ and ##$P < 0.01$ C-ZDF vs C-ZL rats; ††$P < 0.01$ female C-ZDF vs male C-ZDF).

Consistent with the results found in the present study, SKM is a very moldable organ in which different signaling pathways are involved in muscle plasticity [64]. For example, a study showed that an increasing number of slow fibers in SKM by overexpression of peroxisome proliferator-activated receptor gamma (PPARγ) coactivator 1-alpha (PGC-1α) was dependent on the transcription factor MEF2 [65,66]. Moreover, several studies in the SKM of transgenic mice have shown the potential involvement of MEF2 activation in switching fast/glycolytic fibers to slow/oxidative ones through a calcium-dependent pathway [67,68]. In addition, recent findings have proved the implication of calcineurin pathway genes as RCAN in skeletal fiber type switching [69], and mitochondrial biogenesis by NRF2 activation [70]. Therefore, finding new drugs to target these pathways would have a big insight into SKM and its related diseases.

NRF2, RCAN, calcineurin, and MEF2 signaling pathways have gained much attention as a key signaling target in mediating changes in mitochondrial content and metabolism in some cell types, as well as in SKM [19,71]. Therefore, our study aims to examine if melatonin may have a regulating effect on the gene expression of these proteins and its relationship to fiber switching within SKM. Emerging evidence has revealed the contribution of NRF2 in the regulation of mitochondrial function and biogenesis in different tissue types [72–75]. We pursued to investigate the role of melatonin on NRF2 expression in SKM in ZDF and ZL animals. In this study, we showed that obese and diabetic ZDF rats display altered oxidative metabolism in their VL tissue, as evidenced by lower expression of NRF2 in both males and females when compared to their lean littermates. These results are in accordance with those obtained in a model of high-fat-diet mice, where type 2 diabetes decreases NRF2 mRNA expression and its target genes [76,77]. Also, other studies have shown down-regulation of NRF2 in obesity and type 2 diabetes in the cardiac muscle of ZDF rats [78,79]. In addition, it has been mentioned that NRF2 deficiency is related to aged-SKM mitochondria [80], which was shown to be as well associated with obesity [81] and other metabolic disorders. Our work is the first to display that melatonin has a significant effect on enhancing the NRF2 gene expression in SKM from ZDF rats. This finding is of great importance, given the fundamental role of the presence and the activation of NRF2 on mitochondrial function, fiber type profile, and muscle performance in the SKM of mice [12,13,71]. More recently, a study showed the role of another treatment with *Gynostemma pentaphyllum* (GP), on stimulating the expression of the NRF2 gene and promoting mitochondrial metabolism and myotube differentiation in SKM [82]. Further, exercise played a significant role in increasing NRF2 mRNA levels and SKM metabolism and mitochondrial functionality in wild-type mice [83].

From our study, obese and diabetic ZDF rats display impaired metabolic regulation in their VL tissue as evidenced by higher expression of RCAN1.1 and RCAN1.4 and consequently, decreased expression of calcineurin in both males and females when compared to their lean littermates. These results are compatible with other findings obtained in mice models deficient for RCAN1, these mice were resilient to high-fat diet induced obesity and to insulin resistance, and mitochondrial dysfunction in type 2 diabetes [84,85]. These studies suggest the contributor role of RCAN1 to the development of obesity by acting as a mediated suppressor of energy expenditure and thermogenic processes [84]. Importantly, our data suggest that melatonin treatment modulated the RCAN gene expression by downregulating the mRNA levels RCAN1.4 from VL in males and females from obese rats and decreasing RCAN1.1 expression and increasing calcineurin protein levels in both sex and phenotype rats. This fact shows the tight relationship between RCAN1.1 and calcineurin in obesity and melatonin treatment. In addition, our finding is in line with other studies on the modulating effect of exhausting exercise on the expression of RCAN1.4 in extensor digitorum

longus (EDL) rat muscle [86]. However, no effect was observed on RCAN1.1 suggesting an independent expression from one gene isoform to another.

On the other hand, we found that obese ZDF rats exhibit lower MEF2 gene expression in both males and females compared to their lean littermates. Observations obtained in this work are supported by previous results showing that the SKM of a model of obesity-prone (OP) rats exhibits a lower expression of MEF2 when compared to obesity-resistant (OR) animals [87]. Furthermore, in a study on the effect of TNF-like weak inducer of apoptosis cytokine (TWEAK), a regulator of SKM mass and function on gene expression, it was observed significant repression of MEF2 mRNA levels in the SKM of mice that was related to metabolic abnormalities and obesity [88]. Our study reveals that MEF2 gene expression exhibited a significant increase in response to melatonin treatment in both ZL and ZDF rats from both sexes. In addition, MEF2 was also increased, following exercise training of human SKM [89]. Also, from a large number of studies on muscle-specific transgenic mice, it was mentioned the implication of the transcription factor MEF2 with exercise training in regulating a larger subgroup of metabolic gene targets that increase SKM glucose metabolism, mitochondrial biogenesis, and dynamics [90,91]. These signaling pathways involved in SKM plasticity are closely related to mitochondrial biogenesis [70]. Several publications have shown the important role of NRF2 signaling in the process of mitochondrial biogenesis, either directly or by regulating the expression of other antioxidant genes [92]. In this sense, NRF2 activation by calcium-dependent pathways such as calcineurin/RCAN [70], and consequently MEF2 [93], could be key activators of muscle metabolism switching via mitochondrial biogenesis regulation. In the present study, we only studied the activation of the NRF2/RCAN/MEF2 pathway by increased mRNA amounts, however, post-transcriptional regulation in VL of this pathway and protein expression is key to further understanding its role in fiber switching.

It is well known that SKM is a mitochondria-abundant tissue [94]. As expected, we showed that obese and diabetic ZDF rats display mitochondrial respiration alterations in their VL as demonstrated by higher oxygen consumption in state 4 and lower in state 3 when compared to their lean littermates, indicating a disturbance between the different states of mitochondrial respiration. Also, a reduction in the efficiency of the ADP/O ratio was noticed. These findings are compatible with results from other studies, that revealed an altered mitochondrial function and metabolism under diabetes and obesity in SKM of rodents [44,95], and in other thermogenic tissues such as brown and beige adipose tissue [31,32], or insulin-sensitive tissues such as the liver [34] in the same ZDF rat strain, and in humans in other different tissues [96,97]. In addition, favorable mitochondrial function is essential for energy metabolism [98], in our study we investigated the effect of melatonin treatment on mitochondrial respiration in isolated mitochondria from VL, in both ZL and ZDF rats. Here, we report that the RCR, which is the best indicator of mitochondrial function, was increased by melatonin [48]. Our data show that melatonin treatment raised the RCR of mitochondria from VL in both lean and obese animals from the two sexes by reducing the proton leak component of respiration (state 4), as marked by the downregulation of total oxygen consumption. Teodoro et *al.* described the important role of melatonin in mitochondrial dysfunction and insulin resistance in SKM of rats [99]. These results are supported by what we found in our previous work in adipose tissue [31,32], and liver from obese and diabetic fatty rats [34] and in streptozotocin-diabetic rats from another published study [27], where melatonin enhanced the RCR of mitochondria and improved its function. These findings prove that VL mitochondria have a higher oxidative capacity, and that melatonin may contribute to its functionality and dynamics.

In the present work, mitochondrial respiration study was executed in complexes separately focusing exactly on the function of each mitochondrial complex by respirometry, measuring state 3 (coupled O_2 consumption state of the SKM mitochondria) corresponding to mainly C_I and/or C_{II}, depending the substrates used (NADH from pyruvate, malate and glutamate; or $FADH_2$ from succinate); and even the β-oxidation of fatty acid, by adding acyl-L-carnitine (octanoyl-L-carnitine) as a respiration substrate [55,56]. On the one hand, C_I is suggested to be the main respiration complex in mitochondrial ATP production by OXPHOS at physiological conditions in SKM [100–102], being the major source of energy as we found in lean male and female ZDF rats.

On the other hand, C_{II} activity is recently gaining importance because of its implications in cell reactive oxygen species (ROS) production regulation in health and disease [103], being a cell death regulator and an apoptosis sensor [104]. Recent studies in SKM showed that under ROS conditions, such as obesity and diabetes, C_I activity was reduced, as our results in both sex ZDF rats showed, and consequently, as a compensatory mechanism to sustain energy demands, C_{II} activity was found to be increased [105–107]. All these data are consistent with results obtained in male ZDF compared to lean control rats. Differences in C_I and C_{II} activities in female compared to male rats were shown, where male rats were found to present higher O_2 consumption rates in C_{II} compared to female ones maybe due to sex-based pathophysiological differences in obesity [108]. Also, in our previous studies, we found that female ZDF rats showed higher obesity risk and complications [43], supporting the idea that women have more obesity complications even though women are more resistant to weight gain than men [109,110], and being coherent with our data where female ZDF C_{II} activity and oxygen consumption were detected to be decreased, while male ones had developed a compensatory mechanism by increasing C_{II} functionality to supply energy deficiency caused by the decreased C_I activity observed in both sex ZDF rats. In another study, after SKM injury, a decreased C_{II} activity and function was observed as an acute response, and an increased C_{II} activity was observed after 2 days of recovery showing the important role of C_{II} activity in ROS regulation [111]. Also, previous studies showed that melatonin in rodents' myotubes [112] and other insulin-sensitive tissues [31,32,34] is crucial for modulating the C_I and C_{II}, separately and/or together, respiration capacity counteracting cellular induced-oxidative stress by uric acid and obesity, respectively. All these findings are coherent with data obtained in the present study, where melatonin was found to modulate both C_I and C_{II} O_2 consumption in both sex ZDF rats recovering physiological levels. Also, in sedentary humans' SKM, exercise training increased C_I coupling ameliorating also NS-state global OXPHOS capacity [55]. C_{II} coupling to C_I and C_{III} is crucial for ROS production control [113,114] and this mechanism could be the key component for understanding C_{II} implications in ROS production in obesity. Complexes coupling has not been taken into account in the present study, so further studies in C_I and C_{II} coupling to C_{III}, and melatonin effects on *in vivo* complexes functionality are needed to better understand this process.

Obesity is characterized by the accumulation of ectopic lipids and decreased metabolic plasticity of SKM [54,57,115] which makes drugs increasing FAO a good therapeutic target. SKM lipid accumulation may be caused by the reduced fatty acid β-oxidation found in this organ, accompanied by a decreased OXPHOS capacity derived from mitochondrial dysfunction and a reduced number of mitochondria, which leads to the metabolic inflexibility observed in SKM from obese rodents [116]. In humans, type 2 diabetes and obesity were shown to decrease FAO in SKM-isolated mitochondria, and in whole SKM tissue, respectively [117,118]. These data are consistent with those of the present study, which demonstrate that FAO was reduced in both sex ZDF rats. However, in humans, no differences in O_2 consumption after FAO were observed in isolated mitochondria from obese subjects compared to lean ones [118], bringing to light the three main hypotheses that may explain this incoherence: 1) the mitochondrial isolation method altered the mitochondrial survival, as authors mentioned; 2) obesity decrease mitochondria number which lowers the total O_2 consumption measured without affecting mitochondrial FAO; and 3) fatty acid transport into the mitochondria is reduced in obesity, lowering FAO without affecting mitochondrial respiratory capacities [119]). It is also possible that the fatty acid β-oxidation is not coupled, which could also explain the

conflicting data obtained in different studies [120]. Further studies are needed in this field to better understand and clarify the effects of obesity and melatonin on mitochondrial FAO coupling in the SKM. Chronic melatonin treatment, as showed in the present work, restores the FAO in both sex ZDF rats and also increases the O_2 consumption derived from β-oxidation in both sex lean ones. In coherence with this, a study in weaned piglets showed that melatonin regulates SKM lipid metabolism by activating the gene expression of proteins related to FAO and transport into the mitochondria [121]. Also, in humans, other thermogenic drugs, and spices were found to increase SKM lipid metabolism and FAO [122]. Moreover, physiological stimuli such as long-term exercise training and more than 6 h of fasting also increase SKM FAO by increasing the FAO and lipid transport into the mitochondria [55,123,124]. In rats, this increased lipid metabolism and mitochondrial FAO were related to enhanced 5′AMP-activated protein kinase (AMPK) and PPARγ activation [125]. Our previous work demonstrated that melatonin also activates AMPK and PPARγ [43] being coherent with results obtained in the present work. However, further studies on melatonin effects and biochemical pathway activation in FAO, lipid metabolism, and mobilization are required as they have not been studied in the present study and should be considered to clarify these processes.

Restoring normal mitochondrial function requires preserved mitochondrial respiratory chain complex activities [126]. However, a growing body of evidence has demonstrated a causal link between type 2 diabetes state, ROS generation via the mitochondrial respiratory chain, and mitochondrial dysfunction [127,128]. In our study, we also demonstrated a reduction in the mitochondrial complex activities in diabetic fatty rats in comparison with leans. Therefore, stimulation of the respiratory chain complexes by melatonin treatment could be a way to decrease the production of ROS and bind the molecular damage in VL mitochondria from both sex ZDF rats. Although the high metabolic rate and attainability of SKM [129], few studies have been conducted on the effect of melatonin on mitochondrial dysfunction in this tissue. However, previous studies showed improved activities of both C_I and C_{IV} after melatonin treatment in other tissues such as liver and brain mitochondria from rodents [130,131], and from the same rat strain in hepatic C_{IV} [34].

The protective effect of long-term melatonin treatment on the mitochondrial respiratory chain activity may derive from its ability to maintain the intracellular redox balance [26], by exhilarating the production of antioxidant enzymes [132] or through restraining overstimulation of cellular respiration [133], which improves the antioxidant potential in the mitochondrial compartment and protects from oxidative stress. Indeed, several evidences have widely reported the protective effect of melatonin in mitochondria from oxidative damage by decreasing oxygen consumption and improving the respiratory chain, as well as ATP production [50]. However, SKM is a very complex tissue with different fiber type profiles and plasticity. More studies are needed in different SKM types to better understand the implication of fiber type profile and chronic melatonin treatment in SKM remodeling and function in diabesity.

5. Conclusion

Current findings demonstrated that oral chronic melatonin treatment induces VL fiber switching to an oxidative slow phenotype in both sex ZDF rats by activating the RCAN/NRF2/MEF2 pathway and improving mitochondrial oxidative metabolism and function. A previous study from our group showed the beneficial melatonin effects on VL, preserving SKM structure, reducing lipid accumulation, and improving mitochondrial dynamics, autophagy and ATP production, even reducing mitochondrial nitro-oxidative stress in the same rat strain [44]. Taken together present and previous results, melatonin may represent a new therapeutic strategy against diabesity by ameliorating SKM mitochondrial health, dynamic and function, increasing lipid oxidative metabolism and preserving SKM structure and function. Further studies on melatonin's effects on SKM are needed to better understand these processes and support the application of melatonin in clinical treatments against human diabesity.

CRediT authorship contribution statement

Diego Salagre: Writing – original draft, Visualization, Methodology, Investigation. **Habiba Bajit:** Writing – original draft, Investigation, Data curation. **Gumersindo Fernández-Vázquez:** Writing – review & editing, Validation, Formal analysis. **Mutaz Dwairy:** Software, Resources, Data curation. **Ingrid Garzón:** Visualization, Data curation. **Rocío Haro-López:** Methodology, Investigation. **Ahmad Agil:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Conceptualization.

Ethical statement

The study was performed according to the guidelines of the Declaration of Helsinki and approved by the Ethical Committee of the University of Granada (Granada, Spain) according to the European Union guidelines. The protocol reference number is June 23, 2021/096-CEEA.

Funding

This study was supported by grant PID2021-125900OB-I00 funded by MCIN/AEI/10.13039/501100011033 and, by ERDF "A way of making Europe".

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors thank Vanessa Blanca for her technical assistance, Habiba Bajit, and Mutaz Dwairy visiting professor as postdoctoral fellow. Authors are also grateful to the Unit of Excellence for the Study of Aging Disorders (UNETE), University of Granada, and Ana Santos from the Center for Scientific Instrumentation (CIC) of the University of Granada for their technical support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.freeradbiomed.2024.12.019.

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