Biochimica et Biophysica Acta xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Metformin and caloric restriction induce an AMPK-dependent restoration of mitochondrial dysfunction in fibroblasts from Fibromyalgia patients

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ARTICLE INFO

Article history:
Received 20 December 2014
Received in revised form 4 March 2015
Accepted 7 March 2015
Available online xxxx

Keywords:
Fibromyalgia
AMPK
Mitochondria
Oxidative stress
Metformin
Caloric restriction

ABSTRACT

Impaired AMPK is associated with a wide spectrum of clinical and pathological conditions, ranging from obesity, altered responses to exercise or metabolic syndrome, to inflammation, disturbed mitochondrial biogenesis and defective response to energy stress. Fibromyalgia (FM) is a world-wide diffused musculoskeletal chronic pain condition that affects up to 5% of the general population and comprises all the above mentioned pathophysiological states. Here, we tested the involvement of AMPK activation in fibroblasts derived from FM patients. AMPK was not phosphorylated in fibroblasts from FM patients and was associated with decreased mitochondrial biogenesis, reduced oxygen consumption, decreased antioxidant enzymes expression levels and mitochondrial dysfunction. However, mtDNA sequencing analysis did not show any important alterations which could justify the mitochondrial defects. AMPK activation in FM fibroblast was impaired in response to moderate oxidative stress. In contrast, AMPK activation by metformin or incubation with serum from caloric restricted mice improved the response to moderate oxidative stress and mitochondrial metabolism in FM fibroblasts. These results suggest that AMPK plays an essential role in FM pathophysiology and could represent the basis for a valuable new therapeutic target/strategy. Furthermore, both metformin and caloric restriction could be an interesting therapeutic approach in FM.

1. Introduction

Mitochondria are essential organelles present in virtually all eukaryotic cells. One of the primary functions of mitochondria is ATP production via the oxidative phosphorylation (OXPHOS) pathway. Moreover, they play crucial roles in many other metabolic, regulatory and developmental processes [1]. The involvement of mitochondria in a variety of pathological mechanisms has been partially ascribed to their central role in reactive oxygen species (ROS) production and to the damaging effect mediated by ROS themselves on the same organelles [2]. In eukaryotic cells, mitochondrial biogenesis is triggered through modulation of the ATP/ADP ratio, activation of adenosine monophosphate activated protein kinase (AMPK) and the subsequent expression of peroxisomal proliferator activator receptor γ co-activator 1α (PGC-1α) and nuclear respiratory factor-1 (NRF1) transcription factors. The AMPK cascade is one of the intracellular pathways that have evolved to ensure that energy homoeostasis is maintained even under pathological conditions or stress [3]. AMPK has also been involved in the cellular defense against oxidative stress damage induced by mitochondrial ROS through the increase of MnSOD and catalase expression levels [4].

Fibromyalgia (FM) is a common chronic pain syndrome accompanied by other symptoms such as fatigue, headache, sleep disturbances, and depression. Despite the fact that it affects up to 5% of the general

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http://dx.doi.org/10.1016/j.bbadis.2015.03.005
0925-4439/

population worldwide, its pathogenic mechanism remains elusive. Because not all FM patients have a mitochondrial dysfunction, it has recently been hypothesized that oxidative stress and mitochondrial dysfunction may be important events in pathogenesis of a subgroup of FM patients [5–9]. There is evidence supporting this hypothesis, and thus, reduced mitochondrial mass and impaired bioenergetics have been described in blood cells derived from FM patients [8–10]. Furthermore, different antioxidant enzymes have been observed to be drastically reduced in FM patients [5–7,9,11]. Recently, we have also found reduced AMPK gene expression levels in blood mononuclear cells (BMCs) from FM patients [9].

As AMPK has a central regulatory role in cell metabolism, mitochondrial biogenesis and oxidative stress response, we evaluate if AMPK down-regulation could be at least in part responsible for the impaired oxidative stress response and mitochondrial dysfunction observed in FM. Here, we assessed this hypothesis in cultured skin fibroblasts from patients enrolled in a trial concerning the study of inflammation and mitochondrial dysfunction in BMCs (all patients had mitochondrial dysfunction in BMCs) [8].

2. Material and methods

2.1. Ethical statements

The approval of the ethical committee of the University of Seville was obtained, according to the principles of the Declaration of Helsinki and all the International Conferences on Harmonization and Good Clinical Practice Guidelines. All participants in the study gave their written informed consent before initiating it.

2.2. Patients

The inclusion criterion was Fibromyalgia, based on current ACR diagnostic criteria [9], and diagnosed 2 to 3 years previously. The clinical characteristics of each group are shown in Supplementary Table 1. Exclusion criteria were: acute infectious disease within the previous 3 weeks; past or present neurological, psychiatric, metabolic, autoimmune, allergy-related, dermal or chronic inflammatory disease; undesired habits (*e.g.,* smoking and alcohol); medical conditions that required glucocorticoid treatment, analgesics or antidepressant drugs; past or current substance abuse or dependence; pregnancy or current breastfeeding. Three FM female patients and two healthy female volunteers matched for age range, gender, ethnicity and demographic features (completion of at least 9 years of education and member of the middle socioeconomic class), were included in the study. Healthy controls had no signs or symptoms of FM and had not taken any medication for at least 3 weeks prior to commencing the study. None of the patients or controls had taken any drug or vitamin/nutritional supplements during the 3 weeks prior to blood sample collection. All patients and controls followed a standard balanced diet (carbohydrate 50–60%, protein 10–20% and fat 20–30%) for 3 weeks prior to blood collection, as established by a diet program. Clinical data were obtained from a physical examination and subjects were evaluated using the Fibromyalgia Impact Questionnaire (FIQ), the visual analogues scale (VAS) and depression with the Beck Depression Inventory (BDI). Tender points were identified by digital pressure at the 18 locations recommended by ACR which included a minimum of 11 out of 18. Coagulated blood samples were collected from patients and controls after 12 h fasting, centrifuged at 3800 ×*g* for 5 min, and the serum was stored at −80 °C until testing. Serum biochemical parameters were assayed by routine analytical methods. Routine laboratory test yielded normal results for glucose, uric acid, creatine kinase, aspartate aminotransferase, alanine aminotransferase, cholesterol, and triglycerides (Supplementary Table 2).

2.3. Reagents

Trypsin and metformin were purchased from Sigma Chemical Co., (St. Louis, Missouri). Monoclonal Antibodies specific for mitochondrial respiratory chain complex subunits [Anti-human Complex I (39 kDa subunit), Complex II (30 kDa subunit I), Complex III (Core 1 subunit) and Complex IV (COX II)], Mitosox™, PicoGreen, and Hoechst 3342, were purchased from Invitrogen/Molecular Probes (Eugene, Oregon). Anti-cytochrome c antibodies were purchased from PharMingen (BD Bioscience, San Jose, California). Anti-GAPDH monoclonal antibody, clone 6C5, was purchased from Research Diagnostic, Inc., (Flanders, New Jersey). Complex I 8 kDa subunit and Complex II 70 kDa subunit, anti-PGC1-alpha and OGG-1 antibodies were from Abcam (Cambridge, UK); anti-AMPK-p antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); and MnSOD antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A cocktail of protease inhibitors (complete cocktail) was purchased from Boehringer Mannheim (Indianapolis, IN). The ImmunStar HRP substrate kit was from Bio-Rad Laboratories Inc. (Hercules, CA).

2.4. Caloric restriction assay

For all experiments, only male mice were used. Eight-week-old male C57/BL6/J mice weighing 25–30 g were maintained on a 12 h light/dark cycle. All studies were performed in accordance with the European Union guidelines (86/609/EU) and Spanish regulations for the use of laboratory animals in chronic experiments (BOE 67/8509-12, 1988). All experiments were approved by the local institutional animal care committee. Calorie restriction (CR) regimen was progressively implemented: it was initiated with 10% restriction diet during the first week, followed by 20 and 30% during the second and third weeks, respectively, and maintained at 30% until the end of treatment. After testing, mice were sacrificed by decapitation. Blood samples were collected frozen at −80 °C. In several experiments, fibroblasts were cultured using 10% mice serum fed *ad libitum* (AL) or CR. Cells were incubated at 37 °C in a 5% CO_2 atmosphere. Serum was heat activated for 30 min at 55 °C.

2.5. Behavioral assays

Behavioral analyses were performed in a testing room with homogeneous noise and light levels. The testing apparatus was cleaned with 70% ethanol (Panreac Química S.A.U.) between trials to eliminate any influence of animal odor on the exploratory behavior.

2.6. Pain assay

For the hot-plate test, a glass cylinder (16 cm high, 16 cm in diameter) was used to constrain the mice to the heated surface of the plate. The plate surface was maintained at 50–55 ± 0.5 °C and the latency to paw-licking was measured, with a cut-off of 30 s.

2.7. Fibroblast cultures

Control fibroblasts were human primary fibroblasts from healthy volunteers. Samples from patients and controls were obtained according to the Helsinki Declarations of 1964, as revised in 2001. Fibroblasts were cultured in DMEM media (4500 mg/L glucose, L-glutamine, piruvate), (Gibco, Invitrogen, Eugene, OR, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Invitrogen, Eugene, OR, USA) and antibiotics (Sigma Chemical Co., St. Louis, MO, USA). Cells were incubated at 37 °C in a 5% CO_2 atmosphere.

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2.8. Treatment

2 mM metformin (Sigma Aldrich) and/or 100 μM of H_2O_2 at 48 h were used for *in vitro* experiments.

2.9. Mitochondrial respiratory chain enzyme activities

Activities of NADH:coenzyme Q1 oxidoreductase (complex I), succinate deshydrogenase (complex II), ubiquinol:cytochrome c oxidoreductase (complex III), cytochrome c oxidase (complex IV), NADH: cytochrome c reductase (complex I + III), succinate:cytochrome c reductase (complex II + complex III) and citrate synthase (CS) were determined in sonicated-permeabilized fibroblasts using spectrophotometric methods. Results are expressed as Units/CS (mean ± SD). Proteins of fibroblast homogenates were analyzed by the Lowry procedure.

2.10. Western blotting

Whole cellular lysate from fibroblasts was prepared by gentle shaking with a buffer containing 0.9% NaCl, 20 mMTris-ClH, pH 7.6, 0.1% triton X-100, 1 mM phenylmethylsulfonylfluoride and 0.01% leupeptine. Electrophoresis was carried out in a 10–15% acrylamide SDS/PAGE. Proteins were transferred to Immobilon membranes (Amersham Pharmacia, Piscataway, NJ). Mouse anti-Complex I (8 and 39 kDa subunit), mouse anti-complex II (30 kDa subunit I), mouse anti-Complex III (Core 1 subunit), mouse anti-complex IV (COX II), AMPK-P, PGC-1 α, MnSOD, catalase and DNA repair enzyme 8-oxoguanine DNA glycolase-1 (OGG-1) antibodies were used to detect proteins by Western blotting. Proteins were electrophoresed, transferred to nitrocellulose membranes and, after blocking over night at 4 °C, incubated with the respective antibody solution, diluted at 1:1000. Membranes were then probed with their respective secondary antibody (1:2500). Immunolabeled proteins were detected by using a chemiluminescence method (Immun Star HRP substrate kit, Bio-Rad Laboratories Inc., Hercules, CA). Protein was determined by the Bradford method.

2.11. Measurement of CoQ levels

CoQ levels in cultured fibroblasts were performed using a method previously described by our group [8].

2.12. Antioxidant enzyme activity

Catalase activity was determined in cellular lysate by monitoring H_2O_2 decomposition at 240 nm [12]. SOD activity was determined on the basis of the inhibition of the formation of NADH—phenazine methosulfate-nitroblue tetrazolium formazan [13].

2.13. Quantification of mtDNA

Nucleic acids were extracted from fibroblasts by standard cellular lysis. The primers used were: mtF3212 (5′-CACCCAAGAACAGGGTTTGT-3′) and mtR3319 (5′-TGGCCATGGGTATGTTGTTAA-3′) for mtDNA, and, 18S rRNA gene 18S1546F (5′-TAGAGGGACAAGTGGCGTTC-3′) and 18S1650R (5′-CGCTGAGCCAGTCAGTGT3′) for nDNA for loading normalization. Arbitrary units were computed as the ratio between the optical density band corresponding to the mtDNA studied in the 20–30th cycle and that of the nDNA in the 15th amplification cycle. One unit was considered to be the ratio corresponding to the control patient. For imaging of mtDNA in living cells, control and FM fibroblasts cells were cultured in dishes with a glass bottom (MatTek Corporation, Ashland, MA) and stained with PicoGreen (3 μL/mL) for 1 h at 37 °C. TMRM (100 nM) staining was used to visualize mitochondria.

2.14. Mitochondrial ROS production

Mitochondrial ROS generation in BMCs and fibroblasts were assessed by MitoSOX™ Red, a red mitochondrial superoxide indicator. MitoSOX Red is a novel fluorogenic dye recently developed and validated for highly selective detection of superoxide in the mitochondria of live cells. MitoSOX™ Red reagent is live-cell permeant and is rapidly and selectively targeted to the mitochondria. Once in the mitochondria, MitoSOX™ Red reagent is oxidized by superoxide and exhibits red fluorescence.

2.14.1. Fluorescence microscopy

Cells grown on microscope slides in 6-well plates for 24 h were incubated with MitoSOX™ Red for 30 min at 37 °C, washed twice in PBS, fixed with 4% paraformaldehyde in PBS for 0.5–1 h at room temperature, and washed twice with PBS. After that, cells were incubated for 10 min at 37 °C with anti-cytochrome c antibody (Invitrogen, Barcelona, Spain) to label mitochondria. Slides were analyzed by immunofluorescence microscopy.

2.14.2. Flow cytometry

Approximately 1 × 106 cells were incubated with 1 μM MitoSOX™ Red for 30 min at 37 °C, washed twice with PBS, resuspended in 500 μL of PBS and analyzed by flow cytometry in an Epics XL cytometer, Beckman Coultier, Brea, California, USA (excitation at 510 nm and fluorescence detection at 580 nm).

2.15. Oxygen consumption rate (OCR)

The oxygen consumption rate (OCR) was assessed in real-time using the 24 well Extracellular Flux Analyzer XF-24 (Seahorse Bioscience, North Billerica, MA, USA) according to the manufacturer's protocol, which allows measuring OCR changes after up to four sequential additions of compounds. Cells (5×10^4/well) were seeded for 16 h in the XF-24 plate before the experiment in a DMEM/10% serum medium and then incubated for 24 h with the different compounds studied. Before starting measurements, cells were placed in a running DMEM medium (supplemented with 25 mM glucose, 2 mM glutamine, 1 mM sodium Pyruvate, and without serum) and pre-incubated for 20 min at 37 °C in the absence of CO_2 in the XF Prep Station incubator (Seahorse Bioscience, Billerica MA, USA). Cells were transferred to an XF-24 Extracellular Flux Analyzer and after an OCR baseline measurement a profiling of mitochondrial function was performed by sequential injection of four compounds that affect bioenergetics, as follows: 55 μL of oligomycin (final concentration 2.5 μg/mL) at injection in port A, 61 μL of 2,4-dinitrophenol (2,4-DNP) (final concentration 1 mM) at injection in port B, and 68 μL of antimycin/rotenone (final concentration 10 μM/1 μM) at injection in port C. A minimum of five wells was utilized per condition in any given experiment. Data are expressed as pMol of O_2 consumed per minute normalized to 1000 cells (pMol O2/1000 cells/min).

2.16. Lipid peroxidation

Fibroblasts were cultured on coverslips and incubated with 1 μM C11-Bodipy (BODIPY® 581/591 C11) for 30 min at 37 °C. Coverslips were then rinsed with PBS and mounted onto slides as described above for analysis with a fluorescence microscope. Fluorescent intensity was measured using the Image J software (National Institutes of Health, Bethesda, Maryland, USA).

Lipid peroxidation in serum from mice was detected by measuring the concentration of TBARS in fluorescence at 532 nm (F7000, HITACHI), using a TBARS detection kit according to the manufacturer's instructions. Absorbance of was measured at 535 nm. TBARS concentrations of the samples were calculated using the extinction co-efficient of 156,000 M^{-1} cm^{-1}.

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2.17. PCR Amplification and mtDNA sequencing

The complete mtDNA was amplified from total DNA in 24 overlapping 800–1000-bp-long PCR fragments. Primers were carefully designed using the revised human mtDNA Cambridge reference sequence (www.mitomap.org/mitoseq.html).

The PCR fragments were sequenced in both strands in an ABI 3730 (Applied Biosystems; www.appliedbiosystems.com; Foster City, CA) sequencer using a BigDye v3.1 sequencing kit (Applied Biosystems; www.appliedbiosystems.com; Foster City, CA). Assembly and identification of variations in the mtDNA were carried out using the Staden package. For this purpose the revised human mtDNA Cambridge reference sequence (www.mitomap.org/mitoseq.html) was used. The whole process was carried out at Secugen (Madrid, Spain).

2.18. Analysis of apoptosis and viable cells

Viable cells were determined from their normal cell and nuclear morphology and exclusion of propidium iodide. In each case 10 random fields and more than 500 cells were counted.

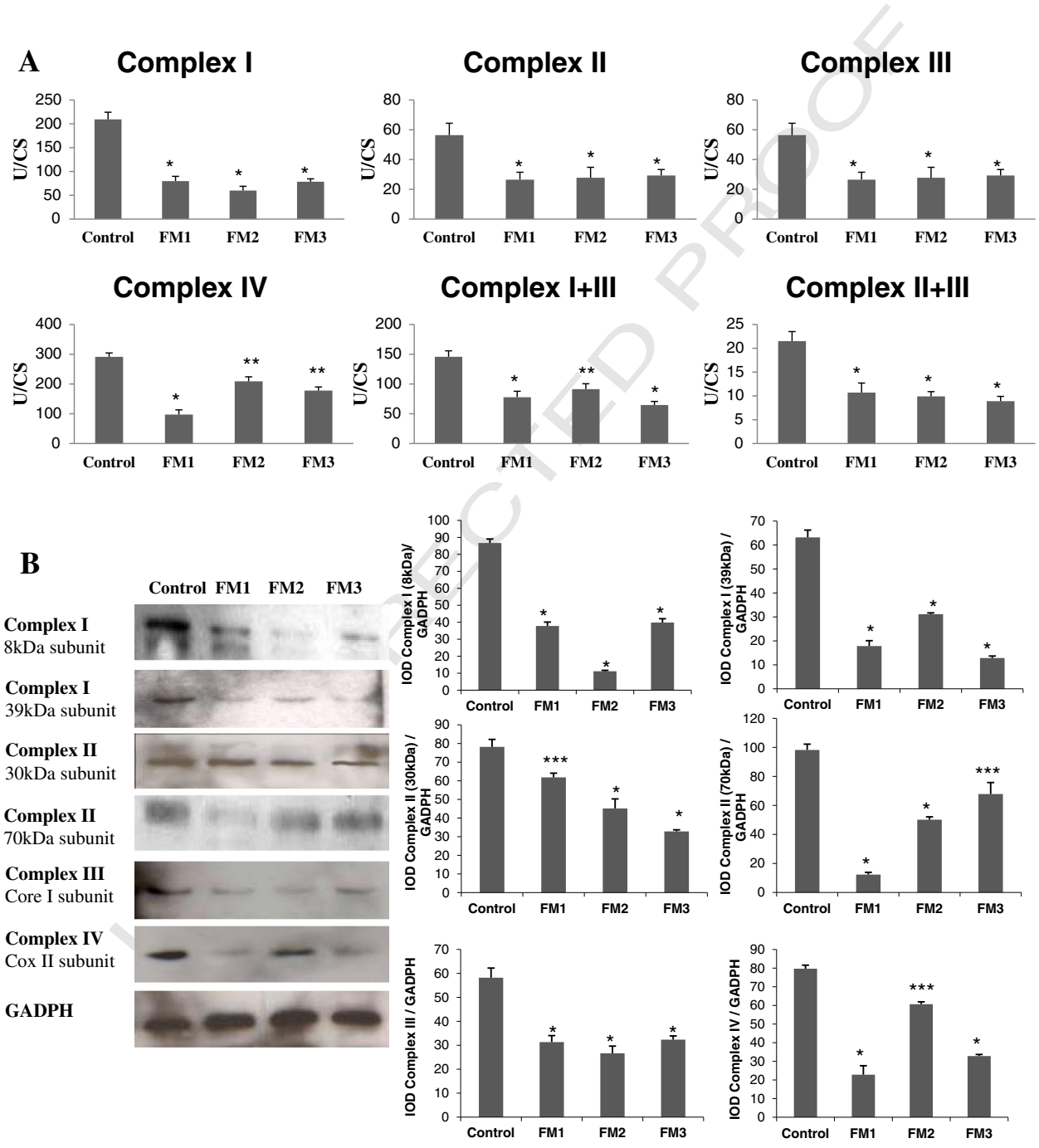

Fig. 1. Mitochondrial dysfunction in skin fibroblasts from FM patients. (A) Mitochondrial enzymatic activities were determined as described in Material and methods. Results (mean ± SD) are expressed in U/CS (units per citrate synthase). (B) Protein expression levels of mitochondrial subunits of complex I, II, III and complex IV. (C) Protein levels were determined by densitometric analysis (IOD, integrated optical intensity) of three different Western blots and normalized to GADPH signal, using fibroblasts from three representative FM patients, compared with a pool of fibroblasts from 5 healthy age- and sex-matched control subjects. $^{*}P < 0.001$; $^{**}P < 0.01$; $^{***}P < 0.05$ between control and FM patients.

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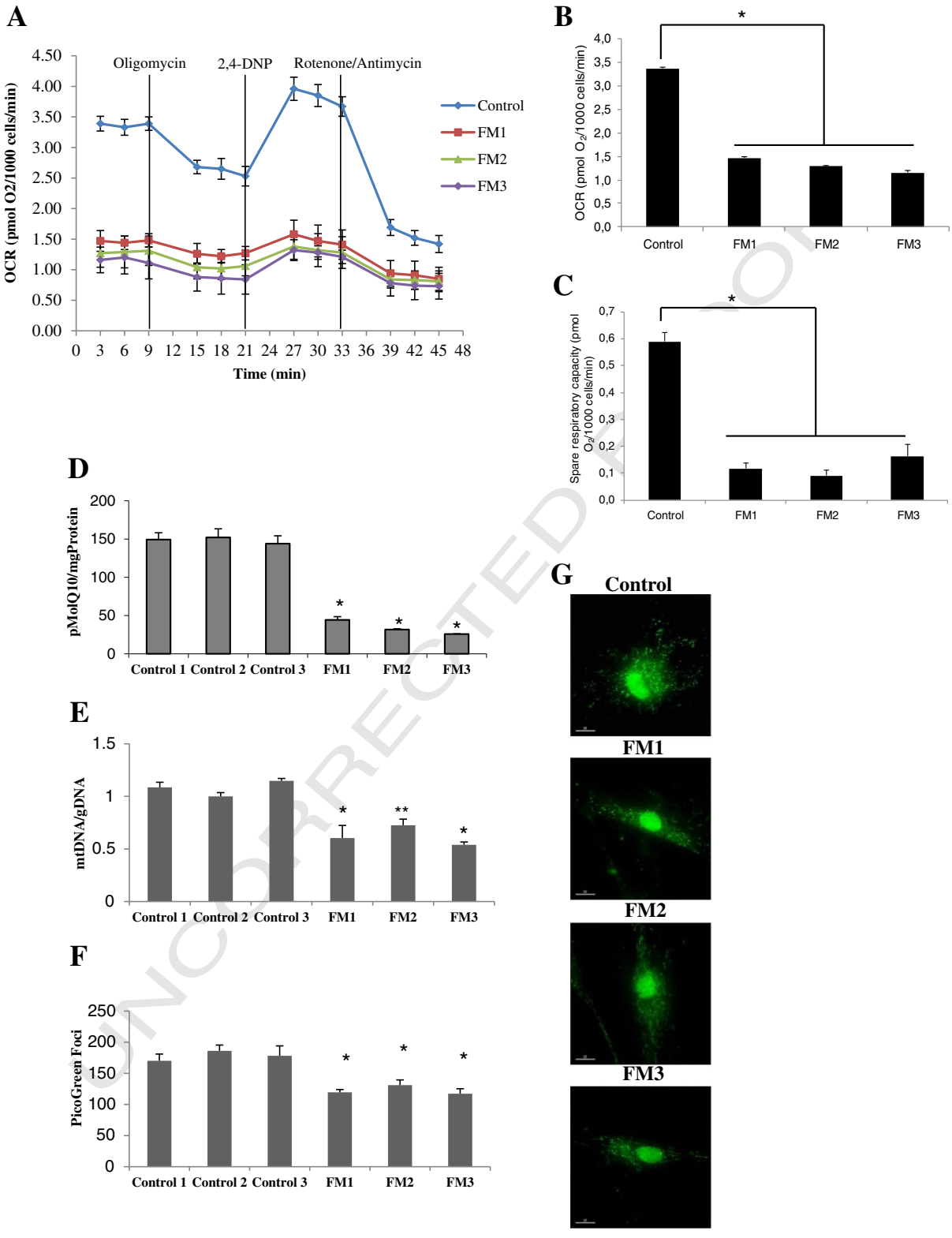

Fig. 2. Abnormalities in various aspects of bioenergetic function of mitochondria. Oxygen consumption rate (OCR) in cells from control and FM patients. (A) OCR was monitored using the Seahorse XF-24 Extracellular Flux Analyzer with the sequential injection of oligomycin (1 μg/mL), 2,4-DNP (100 μM), rotenone (1 μM) at the indicated time point (B) The basal OCR was markedly affected in cells from FM compared to control. (C) The spare respiratory capacity (SRC) of FM fibroblasts showed a significant decrease with respect to control fibroblasts. (D) CoQ_{10} levels in control and FM cells. (E) mtDNA copy number was measured by RT-PCR as described in Material and methods. (F and G) mtDNA imaging by PicoGreen staining and quantification of PicoGreen foci in control and FM fibroblasts. For the control cells, data are the means ± SD for experiments performed on two different control cell lines. Data represent the mean ± SD of three separate experiments. Bar = 15 μm.*$P < 0.001$; **$P < 0.01$ between control and FM patients.

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2.19. Statistical analysis

Data in figures is given as mean ± SD. Data between different groups were analyzed statistically by using ANOVA on Ranks with Sigma Plot and Sigma Stat statistical software (SPSS for Windows, 19, 2010, SPSS Inc. Chicago, IL, USA). For cell-culture studies, Student's t test was used for data analyses. A value of $P < 0.05$ was considered significant.

3. Results

3.1. Mitochondrial metabolism

As AMPK signaling has been previously reported to be altered in BMCs from FM patients [9], we have next studied the role of AMPK in FM pathophysiology using isolated fibroblasts from three representative FM

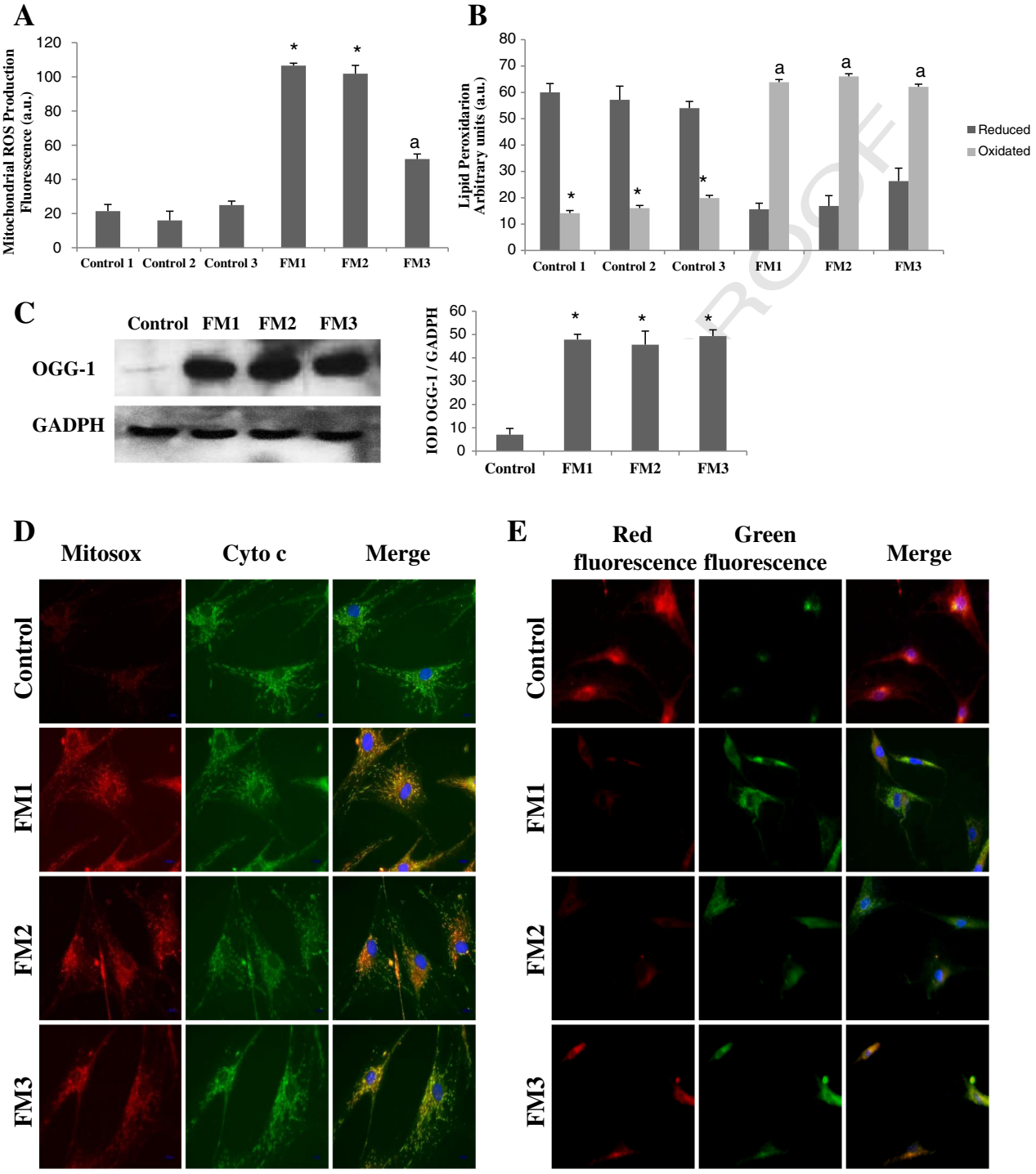

Fig. 3. Oxidative stress and oxidative damage levels in fibroblasts from FM patients. (A) Mitochondrial ROS production was analyzed in BMCs from control and FM patients by flow cytometry as described in Material and Methods. (B) Quantification of lipid peroxidation in control and FM fibroblasts. Data represent the oxidized lipid/reduced lipid ratio. Data represent the mean ± SD of three separate experiments.*$P < 0.001$, [a]$P < 0.01$ between control and FM patients. (C) Protein expression levels of 8-oxoguanine glycosylase (OGG-1, a DNA glycosylase enzyme responsible for the excision of 7,8-dihydro-8-oxoguanine (8-oxoG)). (D) Mitochondrial ROS generation in fibroblasts cultured for 72 h in normal growth medium prior to analysis. MitoSOX Red staining revealed increased superoxide anion. MitoSOX Red colocalized with subunit II of cytochrome c oxidase (COX II) in merged images, indicating that superoxide anion production was mainly in mitochondria. (E) Lipid peroxidation in control and FM fibroblasts using C11-Bodipy staining. Red fluorescence represents non-oxidized lipids, and green fluorescence represents oxidized lipids. Scale bar 30 μm.

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patients. FM fibroblasts displayed a significant reduction in the activities of mitochondrial respiratory enzymes compared to control fibroblasts (Fig. 1A). Mitochondrial protein expression levels correlated with the depressed activities found in respiratory enzymes (Fig. 1B). Next, we investigated mitochondrial function by measuring the OCR values in control and FM fibroblasts, exposed sequentially to each of four modulators of oxidative phosphorylation (OXPHOS): oligomycin (an inhibitor of F1Fo-ATPase or complex V), 2,4-DNP (uncoupling of the OXPHOS electron transport chain) and antimycin/rotenone (complex I and III inhibitors respectively) (Fig. 2A). The basal OCR was markedly affected in fibroblasts from FM patients compared to controls (Fig. 2B). The spare respiratory capacity (SRC) of cells was obtained by calculating the mean of OCR values after injection of 2,4-DNP minus the basal respiration and could be used as an indicator of how close a cell is operating to its bioenergetic limit. Fibroblasts from FM patients showed a significant decrease of SRC compared to control cells (Fig. 2C). Furthermore, similarly to what was previously found in BMCs [8], FM fibroblasts also showed decreased CoQ_{10} levels when compared to controls (Fig. 2D). CoQ_{10} content of fibroblasts from patient 1 was reduced by 70%, from patient 2 by 78% and from patient 3 by 82%. FM fibroblasts also had a smaller number of mitochondria; we measured mtDNA content and compared it with control values. Results showed that mtDNA content was 30–50% lower in fibroblasts from FM (Fig. 2E). This finding was further confirmed by visualizing the number of mtDNA nucleoids per cell using PicoGreen staining and fluorescence microscopy. Mitochondrial nucleoids were significantly reduced in FM fibroblasts (Figs. 2F and G).

Since mitochondrial respiratory chain defects are usually associated with mtDNA mutations or deletions, we next sequenced the complete mtDNA from FM patients. Sequence analysis did not show any important alterations as mutations or deletions which could justify the mitochondrial defects. We only found mitochondrial polymorphisms which are also observed in control fibroblasts (Table S1).

3.2. AMPK is implicated in oxidative stress response in FM

Mitochondrial superoxide production was significantly increased in FM fibroblasts compared to controls ($P < 0.001$), accompanied by high levels of lipid peroxidation (Figs. 3A, B, D and E). To confirm these results, the expression of an additional oxidative stress marker such as 8-oxoguanine glycosylase (OGG1) was also determined. FM fibroblasts showed high levels of OGG1 (Fig. 3C).

As AMPK induces PGC-1α phosphorylation which leads to increased antioxidant enzymes expression levels and mitochondrial biogenesis, we analyzed AMPK protein expression levels and activation in FM fibroblasts. Results showed low expression levels of active phosphorylated AMPK, PGC-1α and MnSOD (Fig. 4A), suggesting that AMPK-dependent activation of PGC-1α was indeed impaired in FM fibroblasts. As reduced antioxidant enzyme levels have been previously described in FM [5,6,11], we next investigated the response to moderate oxidative stress induced by exogenous addition of H_2O_2 in FM fibroblasts. Incubation of FM fibroblasts with H_2O_2 failed to activate AMPK and PGC-1α and to increase MnSOD expression levels (Fig. 4A). As a consequence of an impaired defensive response to oxidative stress, cell death increased in FM fibroblasts treated with H_2O_2 (Fig. 4B).

Under oxidative stress condition, AMPK was found to lead to an increase in the NADPH generation [14]. However, as FM fibroblasts had reduced activity of phosphorylated AMPK, we found low levels of NADPH. Interestingly, metformin, an AMP mimetic that directly activates AMPK, induced an increase of NADPH levels and the activity of SOD and catalase (Figs. 5A–C).

FM fibroblasts under moderate oxidative stress conditions mediated by H_2O_2 treatment or induction of AMPK by metformin showed PGC-1alpha activation (Fig. 5D) which increased protection against H_2O_2 exposure and reduced cell death (Fig. 5E). These results suggest that the induction of AMPK phosphorylation could to be an interesting

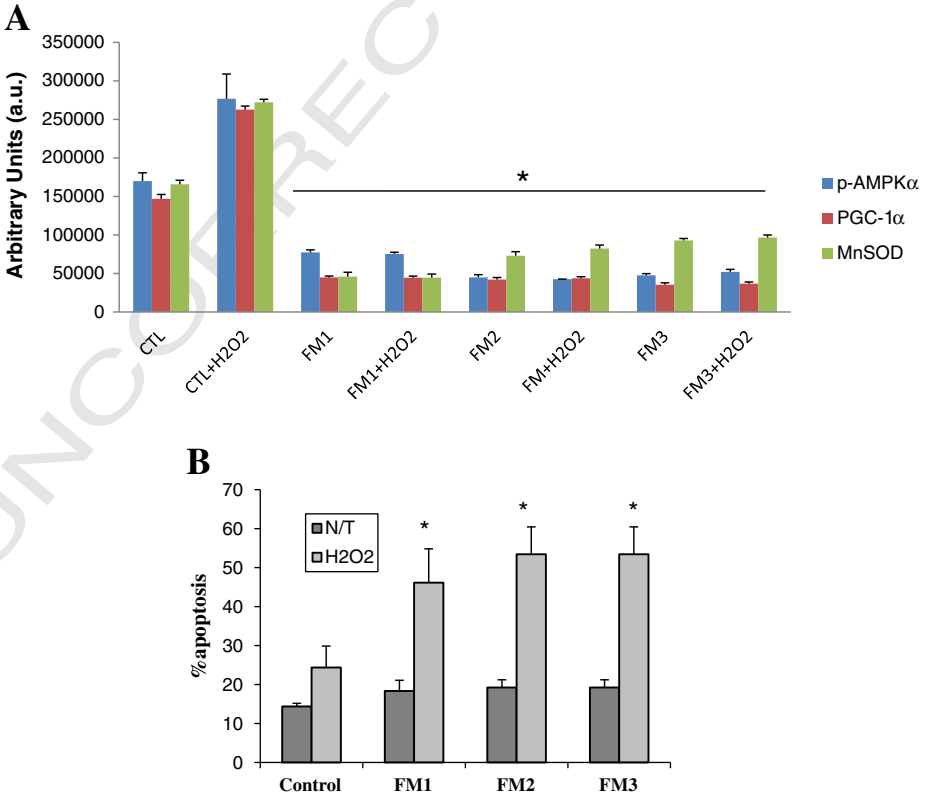

Fig. 4. Comparison of oxidative stress levels and metabolic response to H_2O_2 treatment of skin fibroblasts between FM patients and healthy subjects. (A) Protein expression levels of phosphorylated AMPK, PGC-1α, and MnSOD after incubation with 100 mM H_2O_2 for 48 h. Protein levels were determined by densitometric analysis (IOD, integrated optical intensity) of three different Western blots and normalized to GADPH signal. *$P < 0.001$ between control and FM patients. (B) Percentage of apoptosis in control and FM fibroblasts after incubation with 100 mM H_2O_2 for at 48 h. *$P < 0.001$ between control and FM patients.

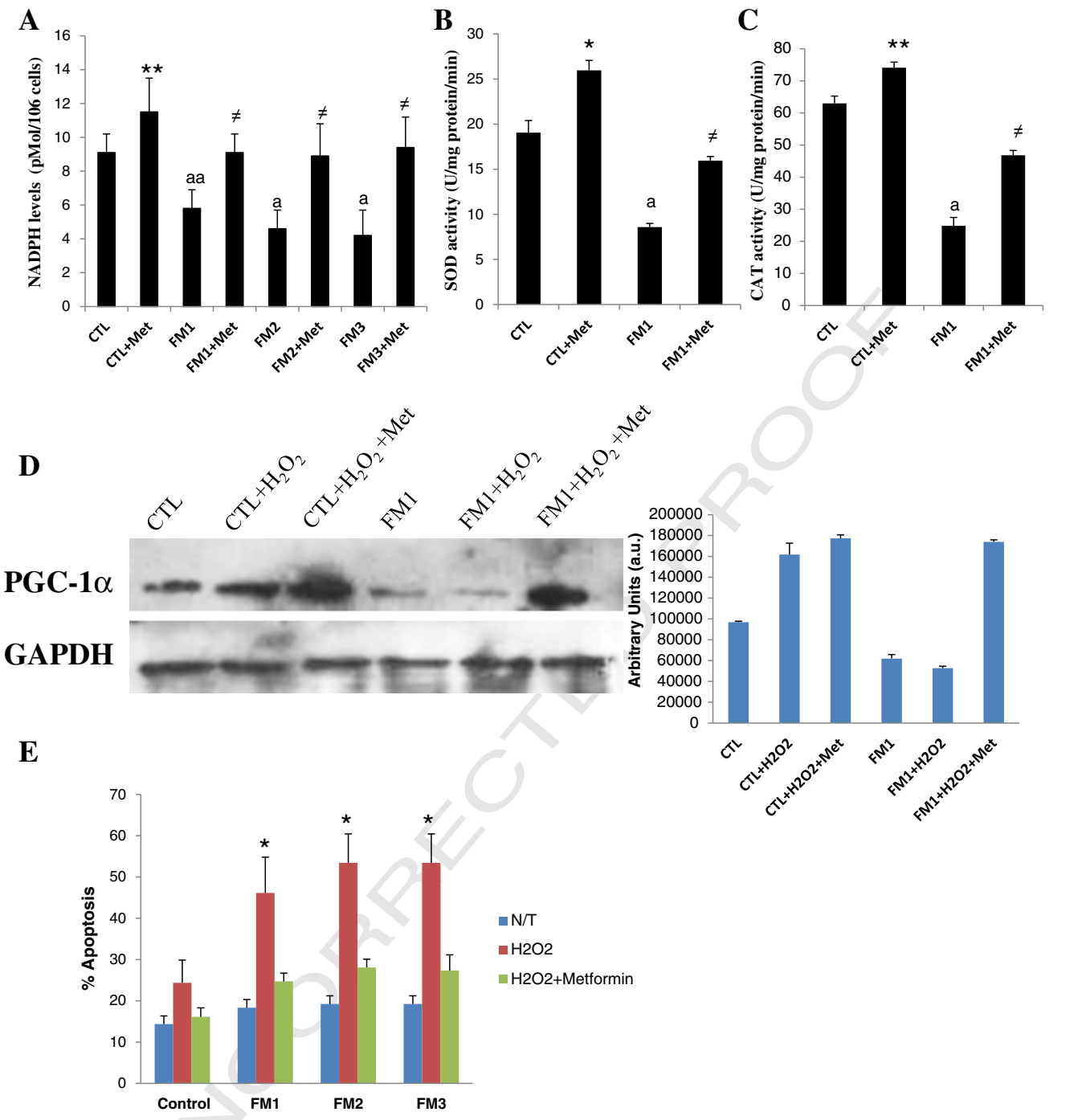

Fig. 5. Effects of metformin treatment on antioxidant defense and oxidative stress response of fibroblasts from FM patients. (A, B and C) NADPH levels and antioxidant enzymes SOD and catalase (CAT) activities in FM fibroblasts treated with metformin (Met). $^{*}P < 0.001$ and $^{**}P < 0.05$ between control and control with Met. $^{a}P < 0.001$ and $^{aa}P < 0.005$ between FM and control. $^{\neq}P < 0.001$ between FM and FM with Met. (D) Levels of phosphorylated PGC-α in FM fibroblasts after 100 mM H_2O_2 and 2 mM Met treatments for 48 h (representative subset is shown). (E) Percentage of apoptosis in control and FM fibroblasts after incubation with 100 mM H_2O_2 and 2 mM Met for 48 h. $^{*}P < 0.001$ between control and FM patients and between H_2O_2 and H_2O_2 + Met.

therapeutic approach in FM. Given that it has been speculated that the beneficial effects of caloric restriction (CR) could be mediated by AMPK [3], CR could be a promising method to alleviate oxidative damage in FM. Taken into account the possible role of AMPK in FM pathophysiology and the results with metformin treatment, we next studied the implication of AMPK in the protective effect of CR on FM fibroblasts.

Thus, we performed an experiment with a mouse model of CR. Several mice were fed with a normal diet and with CR for one month. Mice submitted to the CR diet for one month developed a marked analgesia when compared with *ad libitum* (AL) fed mice (Fig. 6A) accompanied by AMPK phosphorylation (Fig. 6B) and reduced levels of serum oxidative stress (Fig. 6C). To determine the potential effect of improvement of AMPK by CR, fibroblasts from FM patients were cultured with serum from AL and CR mice, and cell growth, ATP and mitochondrial mass were assessed. Serum from CR mice improved cell growth in controls and FM fibroblasts (Fig. 6D), accompanied by an increase in ATP levels and mitochondrial mass (determined by increased citrate synthase activity) and cell morphology normalization (Figs. 6E–G).

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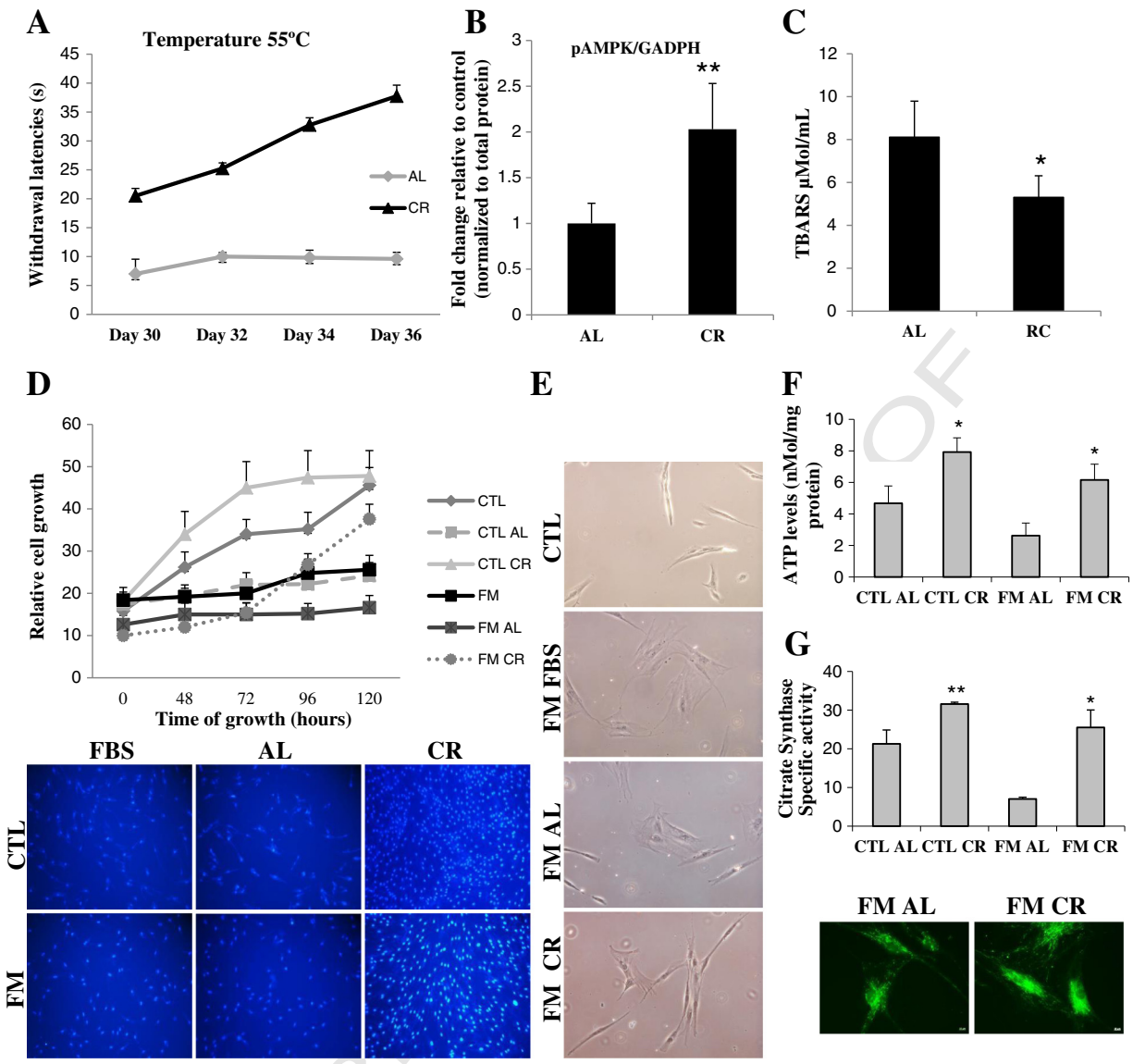

Fig. 6. Effects of caloric restriction on bioenergetics function of mice and skin fibroblasts from FM patients. (A) Evolution of pain sensitivity in *ad libitum* (AL) and caloric restriction (CR) mice evaluated in the hot plate test at 55 °C. (B) Phosphorylation of AMPK after CR. (C) Oxidative stress in serum evaluated by TBARS levels. (D) Cell growth with AL and CR serum determined in healthy and FM fibroblasts. (E) Morphological changes of fibroblasts incubated with FBS or serum from AL or CR mice. (F) ATP levels in control and FM fibroblasts. (G) Mitochondrial mass determined by measuring citrate synthase levels and cytochrome c levels by immnunofluorescence in control and FM fibroblasts. Data represents the means ± SD of three separate experiments. $^{*}P < 0.001$ AL or CR; $^{**}P < 0.01$ between AL or CR in control fibroblasts.

4. Discussion

Despite decades of intense research, the basic pathophysiological mechanisms of FM still remain elusive. Several important pathophysiological processes in FM onset and development have been described: oxidative stress, mitochondrial dysfunction, bioenergetic alterations and inflammation processes are only some of the most important mechanisms that have been postulated [5–11]. AMPK has been reported to play a master regulatory role in all these cellular processes and its dysregulation has been described in several other diseases [15]. Recently, we have reported alterations in AMPK signaling in BMCs from FM patients. However, the role of AMPK in FM remains unknown. In this study, we found a marked mitochondrial dysfunction in fibroblasts derived from 3 FM patients. It is interesting to remark that until now, all the studies in FM have explored the pathophysiological processes only in biological samples isolated directly from patients, *e.g.* BMCs, platelets, serum, plasma, saliva, muscle. In this work we have used human dermal fibroblasts that have a long track record of utility in mitochondrial disease biochemistry and molecular studies [1]. Skin fibroblasts represent a useful biological model in which defined mutations and the cumulative cellular damage can be examined. We found reduced mitochondrial chain enzimatic activities and proteins, CoQ_{10} levels, mitochondrial mass and ATP levels, accompanied by increased oxidative damage. We found no specific mutation after mtDNA sequencing; however, we cannot rule out the presence of mutations in nDNA or potential mtDNA mutations in other patients not included in this study.

Moreover, we observed reduced levels of phosphorylated PGC-1α accompanied by low levels of antioxidant MnSOD and impaired oxidative stress response which are protective mechanisms controlled by AMPK. Furthermore, reduced levels of active phosphorylated AMPK were observed in FM fibroblasts. These data are interesting because AMPK has been involved in the control of peripheral sensitization of nociceptors, providing evidence of AMPK activation as a novel treatment avenue for acute and chronic pain states [16]. In addition, the exposition of fibroblasts to moderate oxidative stress, as induced by exogenously added H_2O_2, fails to up-regulate AMPK, PGC-1α and

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antioxidant enzymes. Concerning this, AMPK has been deeply involved in the regulation of oxidative stress and mitochondrial dysfunction [17–19]. In this sense, AMPK phosphorylation by metformin treatment induced activation of PGC-1α accompanied by increased antioxidant enzyme activities and, as a consequence, protection of FM fibroblasts against stress exposure. PGC-1α is a key player in the ROS-induced mitochondrial biogenesis, along with the NRF-1 and the mitochondrial transcription factor Tfam [20]. According to our data, metformin could induce PGC-1α activation by AMPK phosphorylation. Furthermore, PGC-1α has a key role in the antioxidant enzymes biosynthesis, and its genetic deletion has shown an inhibitory effect in SOD2 and catalase expression levels [4,20]. Furthermore, it has been shown that PGC-1α induction by phosphorylation of AMPK increases SOD2 and catalase expression levels [21]. Our data show that PGC-1α activation by metformin induces increased mitochondrial biogenesis and antioxidant enzymes expression levels, and, as a consequence, a more physiological response to oxidative stress. A chronic exposure to oxidative stress and dysregulation of the stress response are accepted causative factors involved in the pathophysiology of FM [11,22–24]. Our results could represent the basis for a valuable new therapeutic target/strategy. We found in FM fibroblasts: (i) a lack of AMPK phosphorylation and (ii) restoration of its phosphorylation by AMPK activators, such as metformin. These findings suggest that AMPK plays a central role in FM pathophysiology and stress response. Identification of AMPK as a regulating factor in FM would have implications for patient management and treatment. We can hypothesize that the loss of sensitivity of AMPK activation is responsible for increased oxidative stress and impaired bioenergetics in FM patients. Furthermore, other metabolic events have been related with AMPK down-regulation. Reduced AMPK activity has been found in obesity or metabolic syndrome [3], both reported to be implicated in FM [25,26]. AMPK dysfunction seems to explain many of the pathophysiological alterations found in FM. In this sense, activation of AMPK with other activators having similar effects to metformin must induce similar beneficial effects. To investigate whether AMPK could be responsible for the ability of CR to improve the cells of FM patients, we used an *in vitro* cell culture model that recapitulates key *in vivo* proliferative and phenotypic features of CR [27]. In this model, cells from patients were cultured in the presence of serum from caloric restricted mice resulting in an important improvement in FM fibroblasts alterations. Future research should be focused on studying the significance of AMPK in FM etiology and as a therapeutic target. Furthermore, an important challenge in FM is the moderate effectiveness of pharmacological therapies; in this sense, AMPK activators, such as AICAR, metformin, CoQ_{10}, resveratrol, CR or physical activity, can provide new therapeutic opportunities [3]. As not all patients have a mitochondrial dysfunction, our results could help to characterize a subgroup of patients in which mitochondrial target treatment could be the most appropriate strategy. In this sense, mitochondrial protector drugs or mitochondrial biogenesis activators may also be considered as new possible therapeutic approach in FM. Nevertheless, more research is needed in order to establish a possible primary causation link between AMPK and FM.

The results described in this article could serve as a new way of designing experiments to better understand the influence of oxidative stress on the development of FM and generate new therapeutic strategies.

Abbreviations

AL	*ad libitum*
CAT	catalase
CoQ_{10}	Coenzyme Q_{10}
CR	caloric restriction
FBS	fetal bovine serum
FM	Fibromyalgia
HPLC	high-performance liquid chromatography
Met	metformin
OCR	oxygen consumption rate
OGG1	8-oxoguanine glycosylase
PGC-1α	peroxisomal proliferator activator receptor γ co-activator 1α
NRF1	nuclear respiratory factor-1
ROS	reactive oxygen species
SOD	superoxide dismutase
TBARS	thiobarbituric acid reactive substances

Author disclosure statement

All the Authors declare that no conflict of interest exists for any of them.

Author contributions

E.A.G.. and M.D.C. conceived of the study and wrote the manuscript. A.M.C., E.A.G., F.M.A., and DC. performed mouse experiments. E.A.G., J.G.M., J.M.A.S., F.G. P.B. and J.A.S.A. performed cell culture experiments. J.A.S.A. conducted patient evaluations and skin biopsies isolation. All authors analyzed and discussed the data and commented on the manuscript.

Acknowledgments

This work has been supported by Federación Andaluza de Fibromialgia y Fatiga Crónica (ALBA Andalucía) and Grupo de Investigacion Junta de Andalucia CTS113. Authors are indebted with Ms Monica Glebocki for extensive editing of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2015.03.005.

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