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Fibromyalgia syndrome: metabolic and autophagic processes in intermittent cold stress mice

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Keywords
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Abstract

Fibromyalgia is characterized by widespread musculoskeletal pain, fatigue, and depression. The aim was to analyze potential mitochondrial dysfunction or autophagy in mice after exposure to intermittent cold stress (ICS). Muscle and liver specimens were obtained from 36 mice. Lactate dehydrogenase (LDH) activity was measured. Microtubule-associated protein light chain 3 (MAP1LC3B) and glycogen content were determined histologically; muscle ultrastructure by electron microscopy. Mitochondrial- and autophagy-related markers were analyzed by RT-qPCR and Western blotting. ATP level, cytotoxicity, and caspase 3 activity were measured in murine C2C12 myoblasts after ICS exposure. Coenzyme Q10B (COQ10B) transcript was up-regulated in limb muscle of ICS mice, whereas its protein content was stable. Cytochrome C oxidase 4 (COX4I1) and LDH activity increased in limb muscle of male ICS mice. Glycogen content was lower in muscle and liver tissue of male ICS mice. Electron micrographs of ICS mice specimens showed mitochondrial damage and autophagic vesicles. A significant up-regulation of autophagic transcripts of MAP1LC3B and BECLIN 1 (BECN1) was observed. Map1lc3b protein showed an aggregated distribution in ICS mice and SqSTM1/p62 (p62) protein level was stable. Furthermore, ATP level and caspase activity, detected as apoptotic marker, were significantly lowered after ICS exposure in differentiated C2C12 myoblasts. The present study shows that ICS mice are characterized by mitochondrial dysfunction, autophagic processes, and metabolic alterations. Further investigations could dissect autophagy process in the proposed model and link these mechanisms to potential therapeutic options for fibromyalgia.

Abbreviations

ACR, American college of rheumatology; ATP, adenosine triphosphate; ICS, intermittent cold stress; LDH, lactate dehydrogenase; NADH, nicotinamide adenine dinucleotide; ROS, reactive oxygen species; SEM, standard error of the mean; TEM, transmission electron microscopy.

Introduction

Fibromyalgia syndrome is characterized by widespread chronic pain, especially concerning skeletal muscles. It occurs among 1% of people aged between 50 and 70 years in the worldwide population with a higher incidence in women. As fibromyalgia's etiology and pathogenesis are still unknown (Bradley 2009; Solitar 2010), it represents a significant medical and socioeconomic burden, particularly because the pathognomonic chronic pain syndrome connected to fibromyalgia often leads to early retirement and psychological disorders, for example, sleep disturbance and depression (Jahan et al. 2012). Diagnosis is assured by the American College of Rheumatology (ACR) criteria (Wolfe et al. 1990). Treatment includes physio- and psychotherapy as well as pain therapy with paracetamol and gabapentin, as non-steroidal anti-inflammatory drugs and opioids are useless in therapy (Fibromyalgia: poorly understood; treatments are disappointing 2009; Solitar 2010; Arnold et al. 2012).

Despite central nervous disorders, (Mense 2000; Becker and Schweinhardt 2012) also dysfunctional mitochondria as well as morphological changes of muscle tissue could be related to the syndrome as shown in a small group of patients (Yunus et al. 1986; Pongratz and Spath 1998). In blood mononuclear cells and skin biopsies of fibromyalgia patients, a decrease of COQ10, an electron carrier in the mitochondrial respiratory chain (Turunen et al. 2004), and a reduction of mitochondrial membrane potential was observed (Cordero et al. 2010a,b). Both could lead to reduced activity of the essential enzymes ATP (adenosine triphosphate)- and citrate synthase that are necessary for generation of energy and disposal of oxygen radicals, in turn followed by an increase of lactate production and a fast fatigue of muscles (Fulle et al. 2000; Cordero et al. 2010a,b, 2011). Besides, promotion of autophagy was described in blood mononuclear cells of patients suffering from fibromyalgia; fibroblasts, deficient of COQ10, revealed high levels of autophagosomal/lysosomal markers (e.g., Cathepsin, Map1lc3b) (Rodriguez-Hernandez et al. 2009). Furthermore, Cordero et al. observed an up-regulation of Becn1 and Map1Lc3b, two essential autophagic genes, in blood mononuclear cells of affected patients (Cordero et al. 2010a,b). Autophagy is a crucial process involved in degradation of damaged cellular components and in balancing energy sources (Glick et al. 2010; Komatsu and Ichimura 2010). Hence, autophagy can be perceived as a necessary survival mechanism of the cell linked to mitochondria themselves (Hailey et al. 2010; Graef and Nunnari 2011).

Nowadays, several animal models for fibromyalgia syndrome were developed (Sluka 2009). Aside from the pharmacological influence on central nervous mediators (e. g. serotonin, noradrenalin) or intramuscular injection of acid, models of stress-induced hyperalgesia were established as adequate models for fibromyalgia (Desantana et al. 2013). One of these models is the intermittent cold stress (ICS) C57BL/6J mouse model (ICS model) characterized by a fibromyalgia-like long-lasting hyperalgesia and allodynia (Nishiyori and Ueda 2008). Analogous to the clinical situation with fibromyalgia patients, in this model, symptomatic pain is resolved by administration of both antidepressants and gabapentin, but not morphine (Nishiyori and Ueda 2008; Nishiyori et al. 2010, 2011). However, it has not yet been investigated whether ICS could also have any effect on morphologic or biochemical and molecular changes in skeletal muscle. Therefore, the aim of this work was to assess the impact of ICS on metabolism, mitochondrial function, morphology and autophagy in mice.

Materials and Methods

Intermittent cold stress (ICS) mouse model

The mouse model used in this study is described by Nishiyori et al. ICS mice are affected by long-lasting hyperalgesia and allodynia, which can be reverted by treatment with Gabapentin and anti-depressants but not with opioids (Nishiyori and Ueda 2008; Nishiyori et al. 2010, 2011). 36 C57BL/6J mice (6 weeks old), provided by Charles River (Paris, France), were kept in a room maintained at 22 ± 2°C and ad libitum feeding of a standard laboratory diet and tap water before use. About nine male and nine female mice were exposed to intermittent cold stress (ICS) model experiments, three mice per group were kept in a cold room at 4 ± 2°C at 4:30 PM on the first day (day 0), with ad libitum feeding and water. Mice were placed on a stainless steel mesh and covered with Plexiglas cage. At 10:00 AM the next morning, mice were transferred to the normal temperature room at 22 ± 2°C. After they were placed at the normal temperature for 30 min, mice were put in the cold room again for 30 min. These processes were repeated until 4:30 PM. Mice were then put in the cold room overnight. After the same treatments on the next day, mice were finally taken out from the cold room at 10:00 AM on day 3 and sacrificed by cervical dislocation (60 h ICS exposure). The remaining mice served as control animals and were constantly kept at room temperature. All animals received human care. The weight, measured shortly before sacrifice, was not affected by ICS, as shown in the Table S1; 10% weight loss was observed in male mice only. Tissue was snap frozen with liquid nitrogen and stored at −80°C. The study protocol complied with the institute's guidelines and was approved by the Government of Hessen, Germany (V 54–19 c 20 15 (1) MR 20/19 Nr. 8/2012).

Cell culture

Mouse skeletal muscle C2C12 myoblasts (ATCC) were passaged and proliferated in Dulbecco's modified Eagle's medium (DMEM, Gibco ThermoFisher Scientific, Dreieich, Germany) containing 4.5 g/L glucose and supplemented with 20% heat inactivated FBS (Gibco). At 80–90% confluence, myogenic differentiation and fusion was induced by a switch to DMEM supplemented with 2% horse serum (Gibco). Differentiation medium (DM) was changed twice per day. Proliferating and differentiating cultures were kept at 37°C and 5% CO_2 and all media were supplemented with 1% penicillin/streptomycin (PAA LABORATORIES GmbH, Germany). Proliferating and differentiated cells were cultured for 4 days in DM and then exposed to ICS. The plates containing both C2C12 cells were kept overnight at 4°C. At 10:00 AM the next morning, the plates were transferred to the normal temperature room at 22 ± 2°C. After they were placed at the normal temperature for 30 min, they were put in the cold room again for 30 min. These processes were repeated until 4:30 PM. This process was repeated for the next day. The ICS was stopped on the third day (60 h total exposure time) and the cells were further processed.

Mitochondria and caspase activities assay

The mitochondrial activity was measured by the use of Mitochondrial ToxGlo Assay and Caspase Glo® 3/7 Assay (Promega Inc, Madison, WI). Mouse C2C12 myoblasts were seeded in three 96-well plates. One 96-well plate contained proliferating C2C12 cells as control. In the other two 96-well plates, the differentiation and fusion was induced as described above. At day four of differentiation, one of the two 96-well plates was exposed to ICS, the other one was maintained at 37°C and 5% CO_2. In addition, the control plate containing the proliferating cells was exposed to ICS. Cytotoxicity was measured by fluorescence with a filter with 490 nm excitation and 510–570 nm emission as suggested by manufacturer instructions. Afterwards, the ATP level was measured by luminescence. The fluorescence/luminescence was detected and acquired by Glomax (Promega) in 16 wells per plate. The data were processed and analyzed with Excel 97/2003.

Transmission electron microscopy

Transmission electron microscopy- directly after removal, gastrocnemius and soleus muscle were fixed in 2.5% glutaraldehyde, 1.25% (para)formaldehyde (PFA), 2 mmol/L picric acid (in 0.1 mol/L cacodylate buffer [pH 7.0]), afterwards postfixed in 1% Osmium tetroxide, dehydrated with ethanol/propylene oxide, and embedded in glycidether 100 (EPON 812, SERVA Electrophoresis GmbH, Heidelberg, Germany) at a longitudinal orientation. Ultrathin sections (60–80 nm) were mounted on copper grids, contrasted with 4% uranyl acetate and lead citrate and observed with a Zeiss EM 10 C TEM (Carl Zeiss AG, Oberkochen). The analysis was performed with Image SP System (SYSPROG, Minsk, Belarus).

Histological analysis

Formalin-fixed (4% formalin-solution buffered in PBS for 48 h) liver and tibialis anterior muscle specimens were paraffin-embedded and stained with periodic acid-Schiff reagent. Tissue sections were observed under a light microscope Leica DM5000 (Leica, Wetzlar, Germany) and analyzed by the software ImageJ version 1.48p (National Institutes of Health, Maryland).

Immunohistochemical studies

Antibody retrieval was performed in citrate buffer (10 mmol/L, pH6 96°C). Endogenous peroxidase was blocked with 3% H_2O_2 in methanol. After blocking with goat serum (VECTASTAIN® ABC KIT Elite PK-6101, Burlingame, CA), rabbit polyclonal primary antibody Map1Lc3b (ab51520 Abcam, Cambridge, UK, 1:10,000) was applied (2 h; 37°C). The secondary anti-rabbit polyclonal antibody (1:3000) was applied for 30 min prior to the ABComplex (Dako, Hamburg, Germany). As chromogenic substrate, diaminobenzidine was used; nuclei were counterstained with hematoxylin. Negative controls were performed by incubation with secondary antibody only. Slides were digitalized and analyzed, using Leica DM5000 light microscope and ImageJ version 1.48p software.

Extraction of whole cell and mitochondrial protein content

Aliquots of biceps femoris muscle were diluted in 400 μL lysis buffer (100 mmol/L NaCl, 20% (V/V) NP-40, 20 mmol/L Tris-HCl pH 7.4, 5 mmol/L $MgCl_2$, 100 mmol/L PMSF) and mechanically disrupted in MP FastPrep®-24 (M.P. Biomedicals, Irvine, CA). Hereafter, the samples were kept on ice and vortexed every ten minutes followed by the first centrifugation (30 min, 14,000*g*, 4°C). Supernatant was transferred into a new tube and 500 μL of lysis buffer was added. Vortexing and centrifugation (30 min, 14,000*g*, 4°C) were repeated and the supernatant, containing the whole cell protein lysate, was separated.

For isolation of intact mitochondria, quadriceps femoris muscle was directly processed after preparation,

using the protocol described by Gostimskaya and Galkin (2010). Protein content was determined photometrically using Pierce® BCA Protein Assay Kit (Thermo Scientific Inc., Waltham, MA).

Lactate dehydrogenase (LDH) activity in mitochondrial extracts

LDH activity was measured in mitochondrial extracts, using BioVision activity assay (BioVision Research Products, Mountain View, CA), according to the manufacturer protocol.

Western blotting

A quantity of 20 μg mitochondrial and whole cell lysates were loaded onto 12% Tris-Glycine polyacrilammide gels and run for electrophoresis for 90 min at constant 120V. The electroblotting had run for 30 min at 20V in semi-dry blotting cassette (Biozym). Coq10b (ab41997, 1:200), Cox4i1 (ab16056, 1:700), Map1lc3B (ab51520, 1:1000), SqSTM1/p62 (ab96706 1:300), GAPDH (ab9485, 1:1000) (Abcam) and goat anti-rabbit (A5420 Sigma-Aldrich, Munich, Germany, 1:3000) antibodies were used. As a chemiluminescent substrate, SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) was used. The luminescent reactivity was then measured using Fusion image capture and further densitometrically quantified by ImageJ version 1.48p software. GAPDH was used for quality and equal loading control.

RNA purification and reverse transcription

Total cellular RNA of gastrocnemius muscle was purified by QIAzol reagent together with RNeasy mini Kit (QIAGEN, Hilden, Germany). RNA concentration was determined photometrically. Reverse transcription was performed with 100–500 ng RNA, using the iScript cDNA Synthesis Kit (Bio-Rad, Munich, Germany), according to the manufacturer protocol.

Quantitative polymerase chain reaction

Quantitative PCR was performed on a Bio-Rad CFX96 Real Time System, using SsoFastEvaGreen Supermix (Bio-Rad Laboratories) and COQ10B (QT00162260), COX4i1 (QT00108626), MAP1LC3B (QT01750323), BECN1 (QT00139006), GAPDH (QT01658692) *Mus musculus* QuantiTect Primer Assays (QIAGEN). Gene expression was analyzed by CFX Manager 2.0 (Bio-Rad) and REST 2009. Results were normalized to GAPDH.

Statistical analysis

Statistical analysis was performed, using GraphPad Prism 5 (GraphPad Prism Software, Inc, CA), REST 2009 and EXCEL 2010 software. Significance was calculated as indicated, using the *t*-test. *P*-values <0.05 and <0.005 were considered statistically significant. Error bars indicate standard error of the mean (SEM) if not specified otherwise.

Results

Expression of mitochondrial enzymes

Alterations of COQ10 concentration commonly affect fibromyalgia patients (Cordero et al. 2009, 2010a,b). We determined the expression of COQ10B transcript in muscle specimens of ICS and control mice and found a significant up-regulation in male (1.8-fold) and female ICS mice (2.0-fold) ($*P < 0.05$ as significant) (Fig. 1A). Coq10b protein level was found to be stable in male and female ICS mice (Fig. 1B).

COX4i1, another essential mitochondrial enzyme regulating the respiratory chain, showed a significant higher expression (2.6-fold) of its transcript in limb muscle of male ICS mice only. In female mice no variation of COX4i1 expression could be detected (Fig. 1A). Western blot analysis of Cox4i1 showed no valuable modulation of its protein level after ICS exposure (Fig. 1B). Here, Cox4i1 is not considered as loading control protein due to its general high variability in all analyzed samples.

Glycogen content and LDH-activity in ICS mice

A slight down-regulation of glycogen content was detected in muscle and a significant down-regulation in liver tissue of male ICS mice. Both, muscle and liver tissue of female mice showed stable levels of glycogen after ICS (Fig. 2A, B and C). LDH activity, a gold standard in the measurement of cell stress, was quantified in mitochondrial extracts from muscle tissue. Its activity was increased in male and female ICS mice (Fig. 2D).

Electron microscopy: validation of mitochondrial alterations

To decipher, whether ICS could lead to ultra structural changes in muscle tissue of mice, electron microscopic analysis was performed.

Control mice showed a typical ultrastructure of gastrocnemius and soleus muscles, with a regular disposal of sarcomers and a consistent appearance of *Z* lines

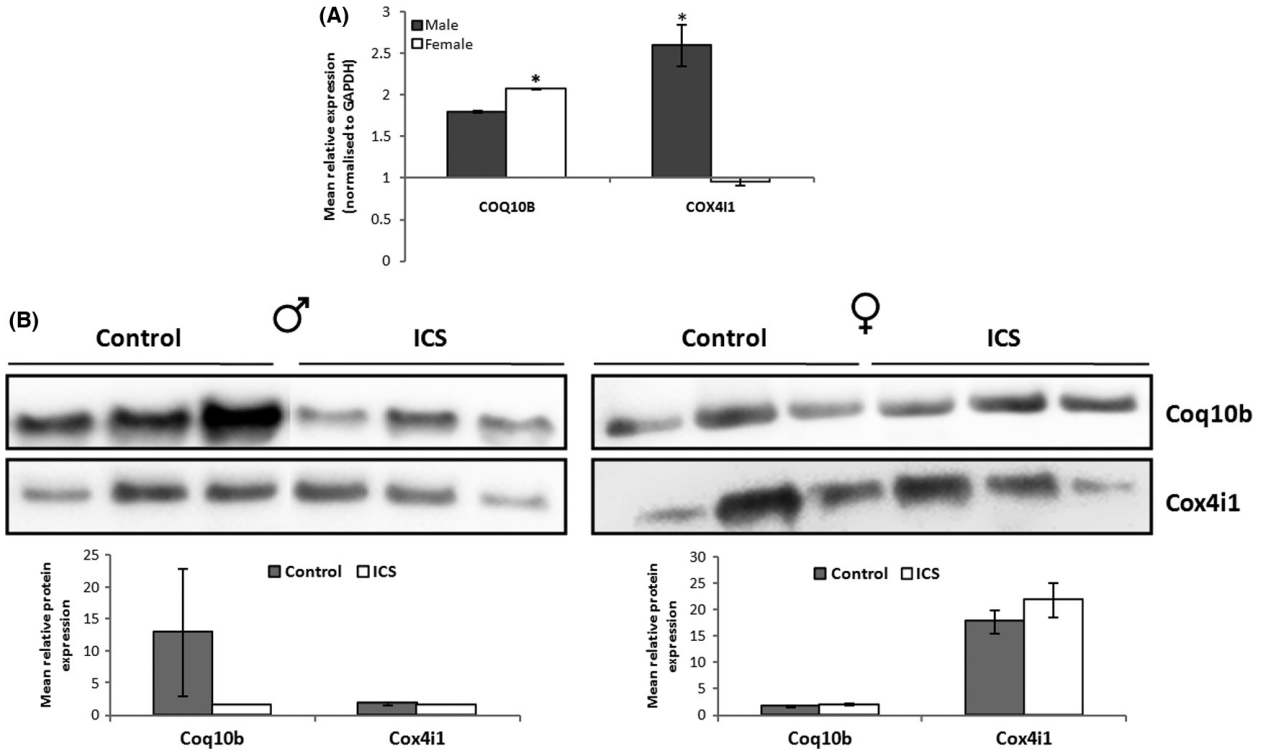

Figure 1. Expression of Coq10b and Cox4i1 in C57BL/6J mice. (A) RT-qPCR of COQ10B and COX4I1 in gastrocnemius muscle of intermittent cold stress (ICS) and control mice. Results are expressed relative to untreated controls set at 1.0 and normalized to GAPDH. Bars show mean ± SEM of three independent experiments performed in triplicates. $*P < 0.05$ versus control. (B) Western blot analysis of Coq10b and Cox4i1 in mitochondrial extracts from quadriceps femoris muscle of ICS and control mice. Shown is protein expression of three biological replicates. Densitometric protein level of ICS mice samples was statistically compared to the level of the control samples. Bars show densitometric mean values ± SEM (three independent experiments) of ICS mice probes normalized to untreated control.

composed of actin and myosin filaments. The typical arrangement of mitochondria between and on the peripheral side of muscle fibers could be clearly observed (Fig. 3). Moreover, mitochondria seemed consistently arranged and showed a sleek confined and intact outer membrane. Cristae, the interior structures of mitochondria, seemed intact and were clearly detectable.

In ICS mice, by contrast, muscle tissue was structurally altered and damaged. Mitochondria showed a smaller size and no clear arrangement anymore; instead they were disposed irregularly among the muscle fibers; subsarcolemmal accumulation of glycogen could be observed. Therefore, ICS treatment affects the morphology of mitochondria in ICS mice (Fig. 3A and B). Parts of the outer membranes of mitochondria seemed dissolved in ICS mice, and interior structures (cristae) looked damaged and distorted. Additionally, altered mitochondria seem to be surrounded by a double-layer membrane, thus indicating autophagosomes formation in muscles of ICS mice with possible autophagic/mitophagic process activation (Fig. 3A and B). These alterations were furthermore confirmed by higher resolution electron micrographs, as shown in Figure 3D. In the most severely damaged mitochondria, there is almost complete dissolution of the internal architecture (Fig. 3D). Finally, the quantification of the mitochondrial density showed that ICS treatment caused no significant change in the mitochondrial density by male (Fig. 3E) and female mice (Fig. 3F).

Autophagy markers involvement

To identify the relevance of autophagy in fibromyalgia mouse model (Nishiyori and Ueda 2008), we investigated the expression of two key players of the autophagic process, after exposure to ICS. Transcript level of MAP1LC3B and BECN1 were analyzed in muscle tissue of control and ICS mice. The expression of MAP1LC3B was significantly higher ($*P < 0.05$ as significant) in both male (1.6 fold) and female (1.5 fold) ICS mice (Fig. 4A). Western blot analyses showed a stable Map1lc3b protein level in male and female ICS mice (Fig. 4D). Furthermore, an induction of BECN1 transcript was detected in gastrocnemius muscle of male (2.3 fold) and female (1.2 fold) ICS mice (Fig. 4A). SqSTM1/p62 protein level, a gold standard marker for autophagy process, was stable in both male and female ICS mice (Fig. 4D).

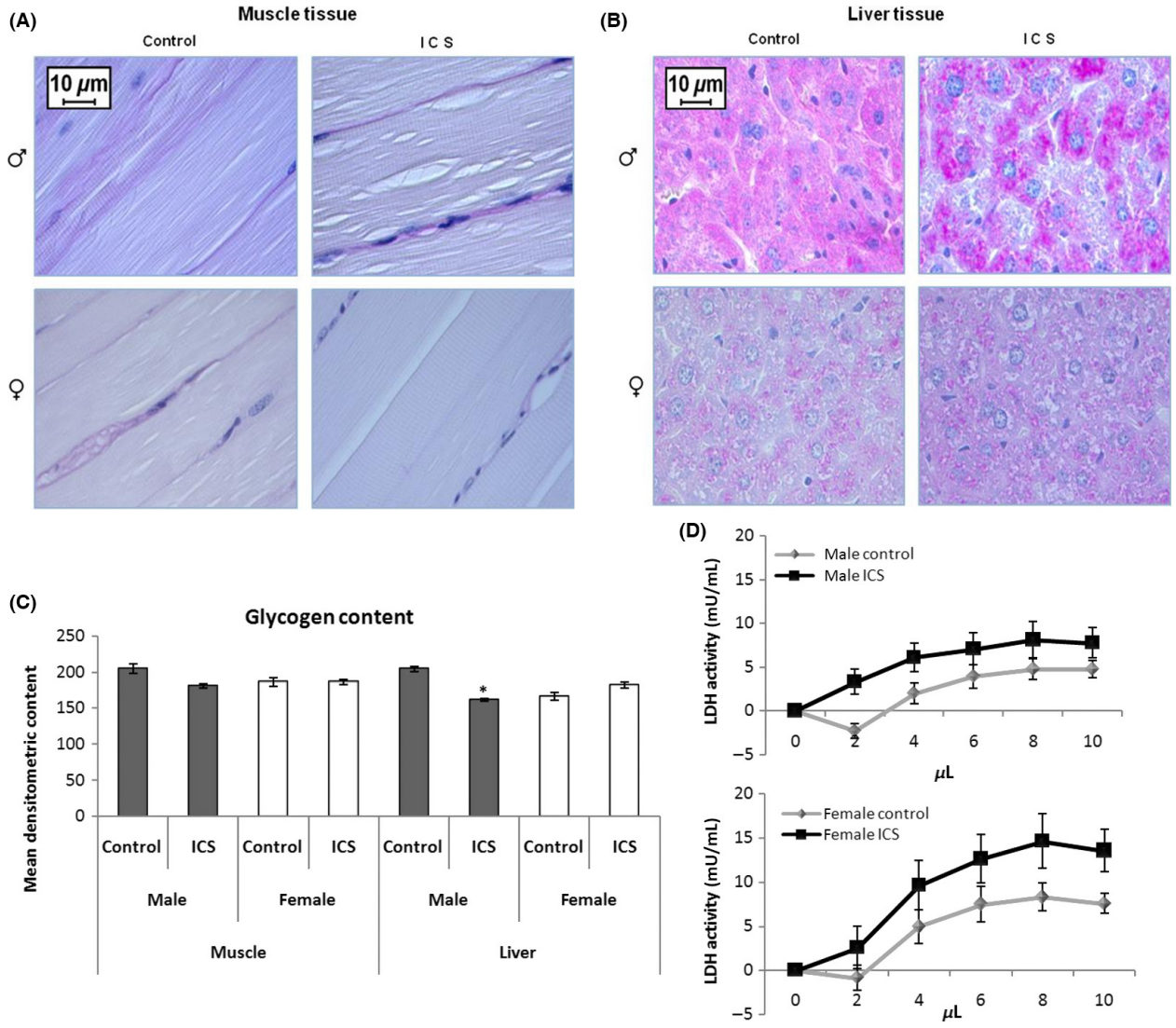

Figure 2. Glycogen content and lactate dehydrogenase (LDH) activity in C57BL/6J mice. PAS staining of tibialis anterior muscle (A) and liver tissue (B) of control and intermittent cold stress (ICS) mice. Original magnification: ×400. (C) Glycogen distribution in muscle and liver tissue. The intensity of PAS staining was quantified and graphically shown. Bars show mean ± SEM of three independent experiments performed in triplicate. $*P < 0.05$ versus control. (D) LDH activity (mU/mL) in male (upper panel) and female (lower panel) control and ICS C57BL/6J mice. Activity was tested at increasing mitochondrial extract amounts from quadriceps femoris muscle. Dots show mean ± SEM of three independent experiments performed in triplicate.

Immunohistochemistry of Map1lc3b protein, showed no variation between ICS and control male and female mice (Fig. 4B and C).

Morphological alterations, mitochondria and caspases 3/7 activity in mouse myoblasts

Undifferentiated and differentiated mouse C2C12 myoblasts/myotubes were exposed to ICS. Morphological changes were detected under phase contrast microscope. As shown in Figure 5A, exposure to ICS caused a visible alteration of cell morphology. Differentiated myotubes acquired a roundish morphology and some of them were pycnotic, whereas myotubes kept at 37°C did not show any change of their morphology. Undifferentiated C2C12 myoblasts did not show any alteration of their morphology after ICS exposure.

During differentiation and fusion of C2C12 myoblasts, an increment of the intracellular ATP has been observed

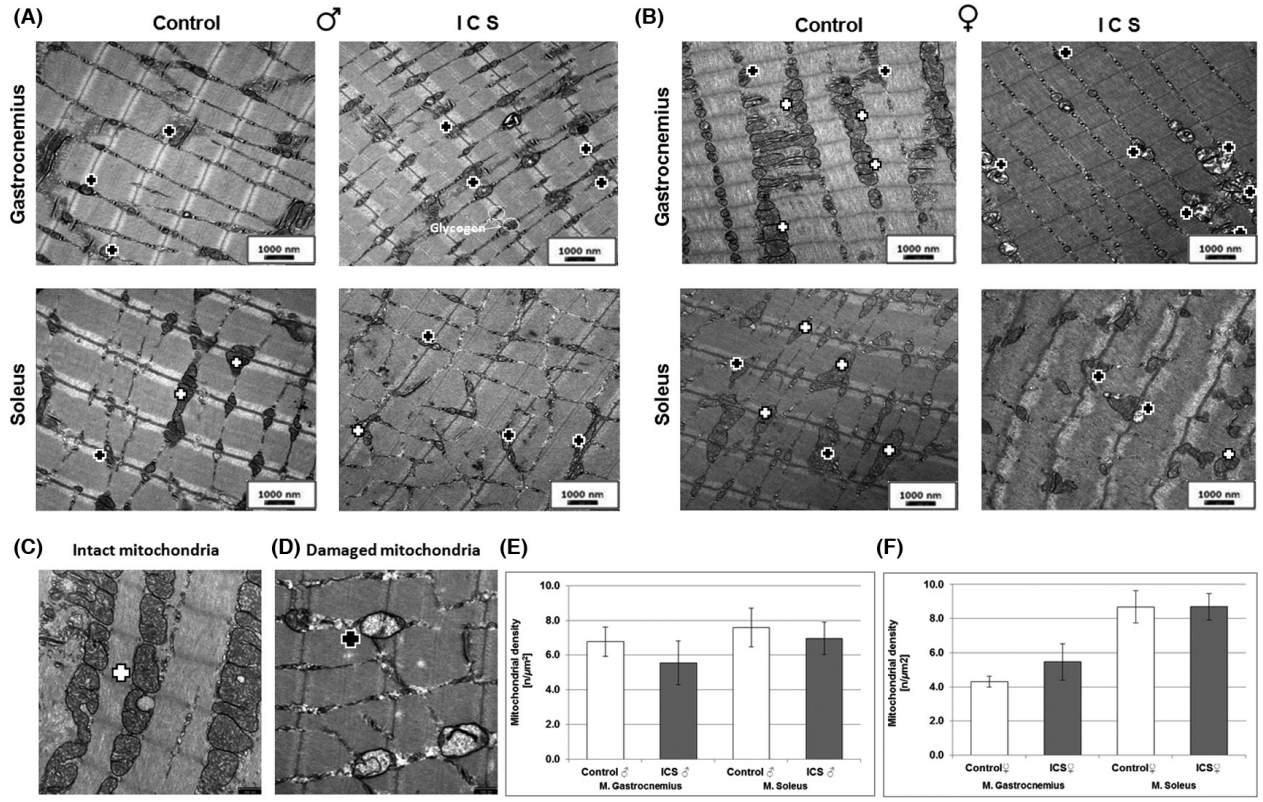

Figure 3. Electron microscopy of muscle tissue from intermittent cold stress (ICS) and control mice. Representative transmission electron microscopic (TEM) images of gastrocnemius (upper) and soleus (lower) of male (A) and female (B) mice. Representative example of intact (C) and damaged mitochondria (D) Scale bars represent 1000 nm and 500 nm. Mitochondrial density in number of mitochondria/μm^2, in muscle gastrocnemius and soleus of male (E) and female mice (F) $n = 5$. White-filled cross indicates intact mitochondria, whereas black-filled cross indicates damaged mitochondria. Original magnification: 10,000× (A and B); 20,000× (C and D).

(Siengdee et al. 2015). To determine whether ICS affects mitochondrial activity in C2C12 myotubes, as already shown by Roobol et al. (2009) in mammalian cells, ATP level was measured (Fig. 5B). ICS caused a significant reduction of the ATP level in differentiated myotubes. Thus, confirming the previous results attributing to ICS, the capability to cause a metabolic change in mouse skeletal muscle. Interestingly, the cytotoxicity level was strongly reduced by ICS, even in undifferentiated myoblasts. Furthermore, in order to exclude the involvement of apoptotic mechanism triggering cell death in C2C12 cells exposed to ICS, the activity of caspases 3/7 was detected. As shown in Figure 5C, the ICS caused a significant suppression of the caspases 3/7 activity in comparison with differentiated cells kept under normal condition. The activity of undifferentiated cells resulted even lower due to a less amount of cells (data not shown). Differentiated C2C12 cells exposed to ICS were processed for flow cytometry evidencing a sub-G1 population below 10% further confirming the absence of the ongoing cell death process (Fig. 5D).

Discussion

Fibromyalgia syndrome represents a complex cluster of symptoms of unknown etiology that include trials of pain processing within central and peripheral nervous system as well as endocrine and muscular system. While animal models associated with neuropathy or inflammation have been established and used in drug discovery research (Negus et al. 2006), only a few have been developed for examining fibromyalgia. The repeated cold stress model was first introduced to study autonomic imbalance in response to sudden changes in environmental temperature (Kita et al. 1975; Ohara et al. 1991). Nishiyori and Ueda (2008) revised this model as an animal model for fibromyalgia syndrome. This mouse model is characterized by fibromyalgia-like symptoms such as hyperalgesia and allodynia (Nishiyori and Ueda 2008). Considerably, Nishiyori et al. could show a similar response of mice under ICS toward analgesics when compared with human patients suffering from fibromyalgia. It was observed that the effects of ICS could be neutralized by intrathecal

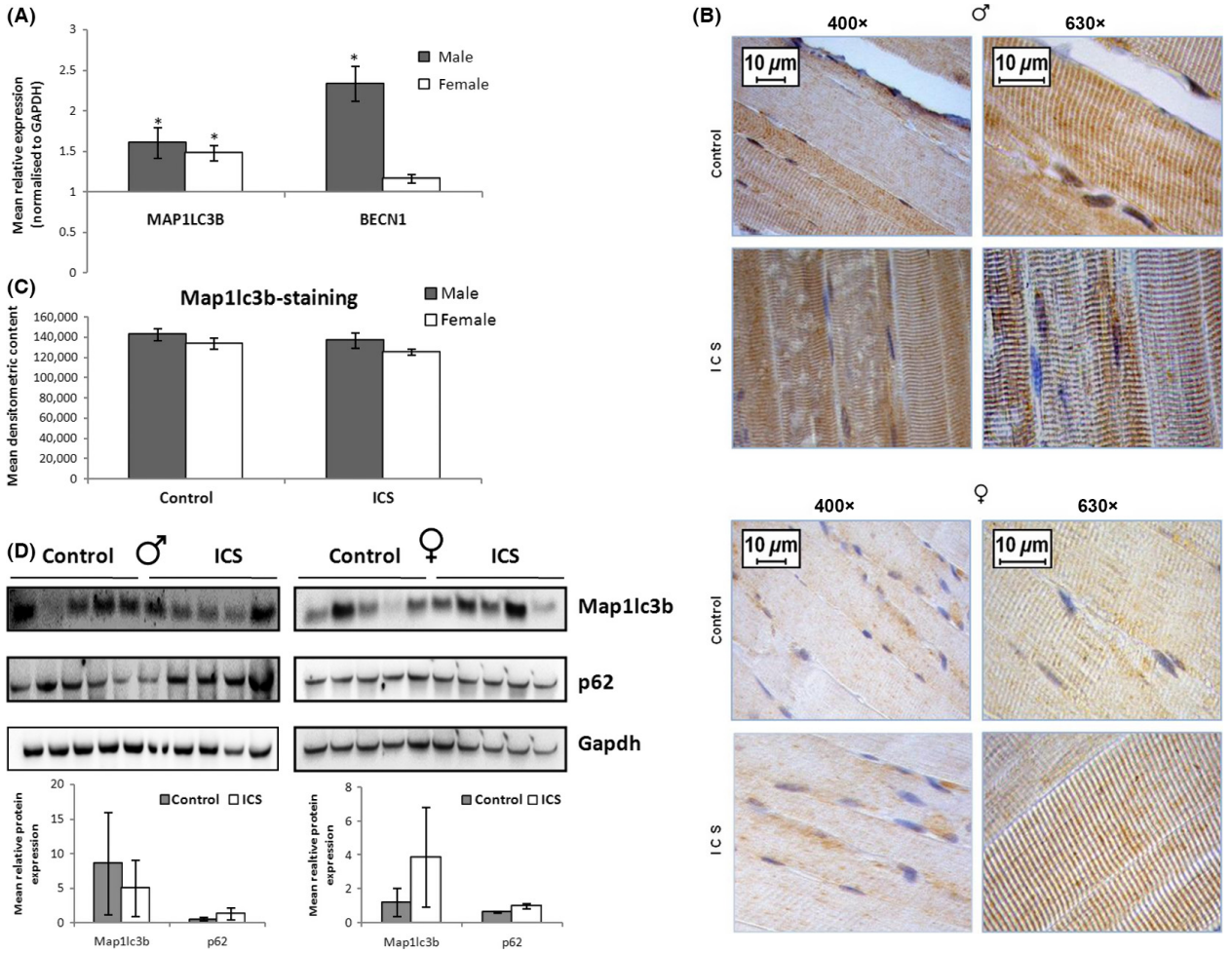

Figure 4. Expression of autophagic markers. (A) RT-qPCR of Map1lc3b and Becn1 expression in gastrocnemius muscle of intermittent cold stress (ICS) mice. Results are expressed relative to control mice set at 1.0 and normalized to GAPDH. Mean ± SEM of three independent experiments performed in triplicate are shown. $*P < 0.05$ versus control. (B) Distribution of Map1Lc3b in tibialis anterior muscle of ICS and control mice. Aggregated spots are clearly visible in both male and female mice after ICS. Nuclei were counterstained with hematoxylin. Magnifications: ×400 and ×630. (C) Quantification of Map1lc3b staining was performed showing no significant difference between control and ICS mice. Bars show mean ± SEM of three independent experiments performed in triplicate. (D) Western blot analysis of Map1lc3b and SqSTM1/p62 in whole cell lysates from biceps femoris muscle of control and ICS mice. Protein expression of five biological replicates for control and ICS mice is shown. Gapdh served as loading control. Densitometric protein level of ICS mice samples was statistically compared to the level of the control samples. Gapdh was used as equal loading control. Bars show densitometric mean values ± SEM (three independent experiments) of treated samples normalized to untreated control.

application of gabapentin, but not by administering opioids (Nishiyori et al. 2010). Recently, Nishiyori et al. (2011) demonstrated that antidepressants, for example, milnacipran, amitriptyline, mianserin, or paroxetine, were also able to neutralize the ICS effect. Furthermore, the stimulation of the muscarinic cholinergic system with pilocarpine (Donepezil) was able to reverse the effect of intermittent cold stress in mice (Mukae et al. 2015). Despite of these similarities between the mouse model and the human symptoms, there is no data available concerning the impact of ICS on molecular and metabolic characteristics such as alterations in muscle tissue and/or mitochondrial metabolism. Therefore this study investigated such alterations for the first time.

Oxidative stress has been proposed as a crucial factor in the pathogenesis of fibromyalgia (Bagis et al. 2005; Ozgocmen et al. 2006). CoQ10, an essential enzyme in oxidative metabolism, acts as an essential electron carrier between complexes I, II, and III of the mitochondrial respiratory chain (Rauchova et al. 1992). Its expression can be used to monitor mitochondrial function (Haas et al. 2008). A possible CoQ10 deficiency could lead to a

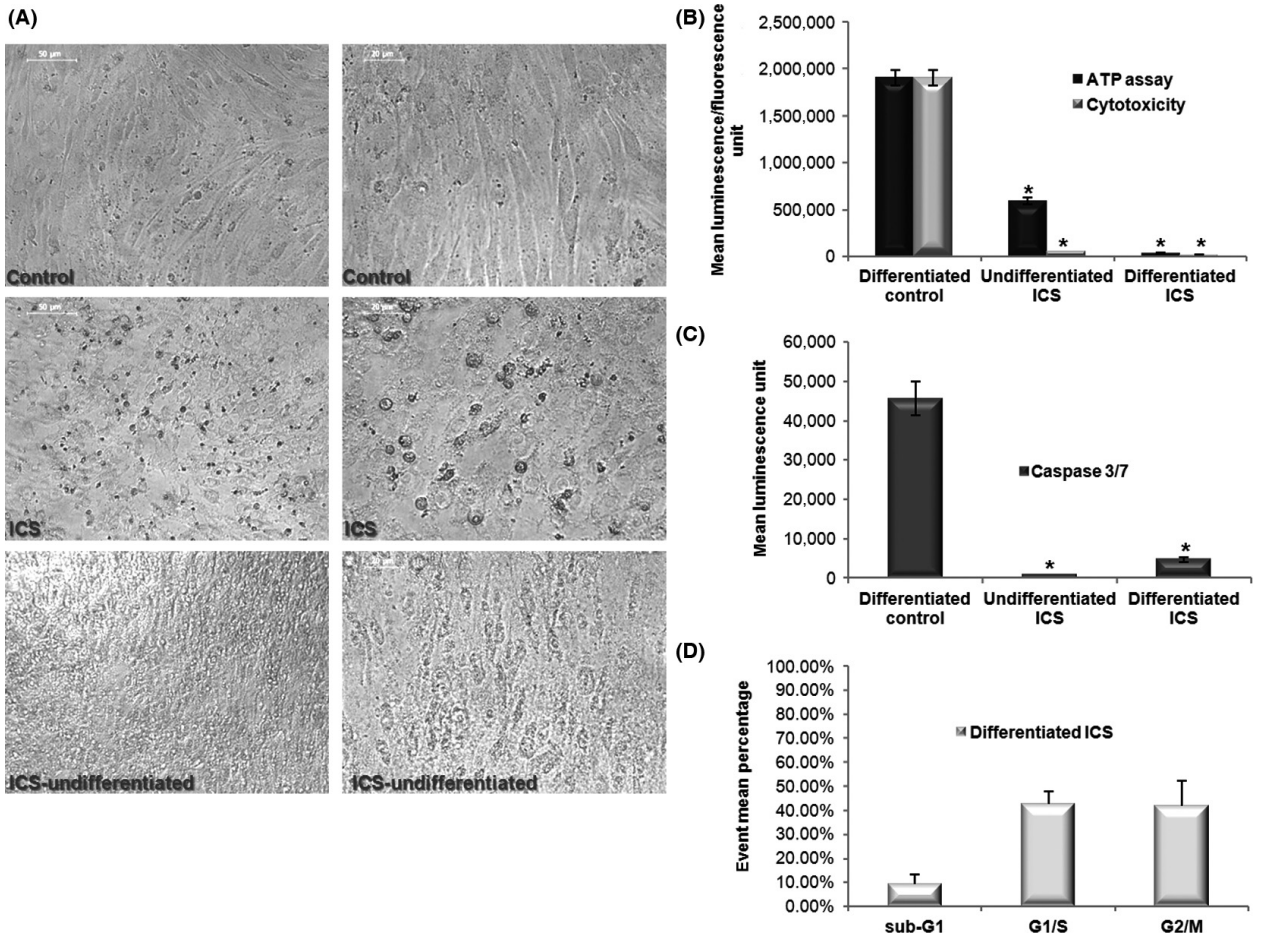

Figure 5. Morphological changes, adenosine triphosphate (ATP) level cytotoxicity and caspases activity detection in mouse myoblasts. Contrast phase micrographs showing the morphological alterations after intermittent cold stress (ICS) exposure in differentiated C2C12 myotubes (A). Control shows differentiated myotubes kept at 37°C; cells show the normal physiologic morphology. ICS exposed myoblasts (middle panels) are strongly altered in morphology. Undifferentiated myoblasts after ICS exposure show no visible alterations (lower panels). Magnification is 200× and 400× and the scale bars represent 50 μmol/L and 20 μmol/L. (B) Graph of ATP level and cytotoxicity. ICS exposure caused a significant reduction of ATP level in differentiated C2C12 myotubes. Cytotoxicity was lowered in undifferentiated cells exposed to ICS also. (C) Caspases 3/7 activity measured after ICS. The activity was significantly lower than in control cells. Shown are means of measurements performed in 16 wells each plate ±SEM. *$P < 0.005$ regarded as significant.

decrease of mitochondrial respiratory complex activity and consecutively to a reduction of mitochondrial membrane potential. Thus, CoQ10 deficiency leads to an increased production of reactive oxygen species (ROS) which damages mitochondria and can result in mitophagy finally (Quinzii et al. 2008; Rodriguez-Hernandez et al. 2009). Additionally, Cordero et al. observed decreased CoQ10 levels and increased production of ROS in blood mononuclear cells of fibromyalgia patients when compared to unaffected individuals (Cordero et al. 2010a,b). However, the present study showed a stable Coq10b protein level and an up-regulation of its transcript in limb muscles of male and female ICS mice. No alterations related to Coq10b could be linked to the unchanged mitochondrial density observed in mice after ICS exposure. Thus, could confer to the muscles the capability to overcome the stress by recycling the mitochondria and their related enzymes, as previously shown in rats (Kon et al. 2007).

Another essential enzyme, especially for the unimpaired function of the respiratory chain in mitochondria, is Cox4i1(Tan et al. 2004). In male ICS mice, a significant increase of COX4I1 transcript could be detected, whereas in female mice no differences were shown. Protein level of Cox4i1 was stable in control and ICS mice.

Recently, biochemical abnormalities and distinct changes concerning glucose metabolism in fibromyalgia patients have been shown (Eisinger et al. 1994). Our

study could show that activity of LDH in mitochondrial extracts of peripheral skeletal muscles increased in male and female ICS mice. LDH is essential for energy metabolism of the cell as it catalyses the conversion of pyruvate to lactate. It is also necessary for the provision of NADH, an essential energy source for cellular metabolism.

Previously published observations showed deposition and accumulation of glycogen in patients affected by fibromyalgia (Yunus et al. 1986; Park et al. 2000; Sprott et al. 2004). In the present study, glycogen content lowered in muscle and liver specimen of male ICS mice, whereas it was stable in female ICS mice.

Structural changes in muscle tissue of patients with fibromyalgia are still discussed controversially. In terms of light microscopy, morphological disorders and lesions in muscle biopsies of patients affected by fibromyalgia are rather minor and not relevant (Le Goff 2006). In line with this, no significant changes in sarcomer length and structure were observed in ICS mice (Fig. S1). Electron micrographs showed small mitochondria with an enlarged and affected structure; the number of mitochondria was not affected. Additionally, a nonlinear distribution of mitochondria and a subsarcolemmal accumulation of glycogen could be observed together with an ultrastructural damage of muscle fibers. These observations show a similarity to the previously published studies of *Cordero* et al. (Yunus et al. 1986; Park et al. 2000; Sprott et al. 2004) showing these compatible abnormalities in blood mononuclear cells of fibromyalgia patients and autophagosomal vesicles engulf damaged mitochondria also (Cordero et al. 2010a,b).

The present study broadly analyzed autophagic processes by the observation of changes in gene expression and protein level of autophagic markers Map1Lc3b, Beclin 1 and p62. Map1Lc3b plays a critical role in the formation of autophagosomes (Kirisako et al. 1999). It is associated with the autophagosome membranes after processing and is involved in both nucleation of membranes and in selecting cargo for degradation through binding with adaptor molecules, like p62 (Kabeya et al. 2000). Beclin 1 is a regulatory protein interacting with other several members of ATG family group during autophagosome formation (Diaz-Troya et al. 2008; Kundu et al. 2008).

Autophagy is a regulated pathway that refers to any process of degradation of cytosolic components by the lysosome and exerts a key role for cell fate (Marino and Lopez-Otin 2004; Baehrecke 2005; Codogno and Meijer 2005; Glick et al. 2010). Misfolded or damaged proteins, organelles, and intracellular pathogens are sequestered into double-membrane vesicles, the so-called autophagosomes, which subsequently fuse with lysosomes to become autolysosomes for the final degradation. Induction of autophagy can be caused by many infringements, for example, mitochondrial dysfunction, pathogen infections, and intrinsic cellular signals (Scherz-Shouval et al. 2007). The selective degradation of mitochondria mediated by autophagy is called mitophagy (Lemasters 2005; Tolkovsky 2009; Gomes and Scorrano 2012).

Clear evidence of autophagic process in response to ICS were not only shown at morphological but also at molecular level. A distinct increase of MAP1LC3B and BECN1 transcript could be detected in male and female ICS mice. Map1lc3b protein showed a rather stable level in ICS mice. However, its distribution showed a spot aggregation that is typical of autophagosome formation. The P62 protein level was stable in ICS mice.

Dysregulated autophagy and mitochondrial dysfunction could represent key aspects of the pathophysiology of fibromyalgia syndrome. The present study could show a broad spectrum of mechanisms acting in ICS mice, which can be attributed to fibromyalgia syndrome. In particular, evidence of perturbation of mitochondria and muscle tissue could suggest that the harm provoked by ICS is overcome by the energy flux obtained by the anaerobic way of energy metabolism. In fact, the possible absence of functional mitochondria caused by metabolic and autophagic dysregulation forces the muscle cells to gain energy without the involvement of citrate cycle, which paths in intact mitochondria only. At this stage, the activity of LDH increases in order to convert pyruvate into lactate, to gain additional energy metabolites. These processes are supported by the reduction of glycogen deposits in male mice, as the cell activates all resources to obtain energy. In female mice, the stable glycogen levels could be declared by hormone differences regarding gender in accordance with progesterone activity which confers to female mice a diminished general metabolism (Frye 2011). Additionally, a strong reduction of ATP level, as determined in differentiated C2C12 myotubes, confirmed the reduction of mitochondrial activity; thus proving that ICS determine a strong reduction of mitochondrial activity and autophagy activation leading to activate alternative metabolic processes to rescue energy sources like NADH. Once again, it could be shown that ICS exposure provokes a disarrangement of cellular morphology in myotubes as well as in mice limb muscles. Additionally, it could be excluded that ICS triggers simply apoptosis due to the low caspases 3/7 activity showed by differentiated and undifferentiated myoblasts.

Up to now, it has been shown that induction of mito/autophagy does not seem to be correlated with the modulation of its related factors protein level, as confirmed also in our study. Moreover, autophagy proteins are finely regulated by posttranscriptional modifications, for example, phosphorylation and lipidation, promoting their

activation (Park et al. 2016). Furthermore, the mitochondrial metabolic process and autophagy are closely connected and monitored by circadian cycle (Wende et al. 2016).

Concerning the stable expression of CoQ10b, it could represent a steady state that could confer the cells the capability to overcome the stress induced by ICS exposure, as already shown in rats with a diet implemented with CoQ10 under sport-derived muscular injury (Kon et al. 2007).

The study shown here clarify that ICS induce a metabolic alteration characterized by reduction of ATP level, as confirmed in C2C12 cells and the previously shown effect on LDH and Glycogen content of mouse limb muscles. The alterations are accompanied by morphological alterations of C2C12 cells, with neither an induction of caspases nor an accumulation of dead cells, and ultrastructural alterations of subcellular organelles.

The present study shows novel aspects of fibromyalgia pathophysiology related to metabolic and molecular changes as a response to ICS, elucidating the prominent role of mitochondria and autophagy in response mechanisms activated by ICS. Some pathologic features described in the ICS mouse model have found similarities in the human presentation of fibromyalgia syndrome. New aspects of this disease, here shown, could be analyzed in patients and considered for further therapy options, especially with regard to vitamin or antioxidant supplementation. Detection of autophagy factors in patients could represent a novel marker for the diagnosis of fibromyalgia syndrome.

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Author Contributions

Participated in research design: Oezel, Önel, Ocker and Di Fazio. Conducted experiments: Oezel, Then, Merkel, Jabari and Bonaterra. Contributed new reagents or analytic tools: Wissniowski. Performed data analysis: Oezel, Bonaterra, Wissniowski, Ocker, Kinscherf and Di Fazio. Wrote or contributed to the writing of the manuscript: Oezel, Bonaterra, Wissniowski, Önel, Ocker, Thieme, Kinscherf, and Di Fazio.

Disclosures

None declared.

References

Arnold B, Hauser W, Arnold M, Bernateck M, Bernardy K, Bruckle W, et al. (2012). Multicomponent therapy of fibromyalgia syndrome: systematic review, meta-analysis and guideline. Schmerz 26: 287–290.

Baehrecke EH (2005). Autophagy: dual roles in life and death? Nat Rev Mol Cell Biol 6: 505–510.

Bagis S, Tamer L, Sahin G, Bilgin R, Guler H, Ercan B, et al. (2005). Free radicals and antioxidants in primary fibromyalgia: an oxidative stress disorder? Rheumatol Int 25: 188–190.

Becker S, Schweinhardt P (2012). Dysfunctional neurotransmitter systems in fibromyalgia, their role in central stress circuitry and pharmacological actions on these systems. Pain Res Treat 2012: 741746.

Bradley LA (2009). Pathophysiology of fibromyalgia. Am J Med 122(12 Suppl): S22.

Codogno P, Meijer AJ (2005). Autophagy and signaling: their role in cell survival and cell death. Cell Death Differ 12(Suppl 2): 1509–1518.

Cordero MD, Moreno-Fernandez AM, deMiguel M, Bonal P, Campa F, Jimenez-Jimenez LM, et al. (2009). Coenzyme Q10 distribution in blood is altered in patients with fibromyalgia. Clin Biochem 42: 732–735.

Cordero MD, De Miguel M, Moreno Fernandez AM, Carmona Lopez IM, Garrido Maraver J, Cotan D, et al. (2010a). Mitochondrial dysfunction and mitophagy activation in blood mononuclear cells of fibromyalgia patients: implications in the pathogenesis of the disease. Arthritis Res Ther 12: R17.

Cordero MD, Moreno-Fernandez AM, Carmona-Lopez MI, Sanchez-Alcazar JA, Rodriguez AF, Navas P, et al. (2010b). Mitochondrial dysfunction in skin biopsies and blood mononuclear cells from two cases of fibromyalgia patients. Clin Biochem 43: 1174–1176.

Cordero MD, Alcocer-Gomez E, de Miguel M, Cano-Garcia FJ, Luque CM, Fernandez-Riejo P, et al. (2011). Coenzyme Q (10): a novel therapeutic approach for Fibromyalgia? case series with 5 patients. Mitochondrion 11: 623–625.

Desantana JM, da Cruz KM, Sluka KA (2013). Animal models of fibromyalgia. Arthritis Res Ther 15: 222.

Diaz-Troya S, Perez-Perez ME, Florencio FJ, Crespo JL (2008). The role of TOR in autophagy regulation from yeast to plants and mammals. Autophagy 4: 851–865.

Eisinger J, Plantamura A, Ayavou T (1994). Glycolysis abnormalities in fibromyalgia. J Am Coll Nutr 13: 144–148.

Fibromyalgia: poorly understood; treatments are disappointing (2009). Prescrire Int 18: 169–173.

Frye CA (2011). Progesterone attenuates depressive behavior of younger and older adult C57/BL6, wildtype, and progesterone

receptor knockout mice. Pharmacol Biochem Behav 99: 525–531.

Fulle S, Mecocci P, Fano G, Vecchiet I, Vecchini A, Racciotti D, et al. (2000). Specific oxidative alterations in vastus lateralis muscle of patients with the diagnosis of chronic fatigue syndrome. Free Radic Biol Med 29: 1252–1259.

Glick D, Barth S, Macleod KF (2010). Autophagy: cellular and molecular mechanisms. J Pathol 221: 3–12.

Gomes LC, Scorrano L (2013). Mitochondrial morphology in mitophagy and macroautophagy. Biochim Biophys Acta 1833: 205–12.

Gostimskaya I, Galkin A (2010). Preparation of highly coupled rat heart mitochondria. J Vis Exp 43: e2202, doi:10.3791/2202

Graef M, Nunnari J (2011). Mitochondria regulate autophagy by conserved signalling pathways. EMBO J 30: 2101–2114.

Haas RH, Parikh S, Falk MJ, Saneto RP, Wolf NI, Darin N, et al. (2008). The in-depth evaluation of suspected mitochondrial disease. Mol Genet Metab 94: 16–37.

Hailey DW, Rambold AS, Satpute-Krishnan P, Mitra K, Sougrat R, Kim PK, et al. (2010). Mitochondria supply membranes for autophagosome biogenesis during starvation. Cell 141: 656–667.

Jahan F, Nanji K, Qidwai W, Qasim R (2012). Fibromyalgia syndrome: an overview of pathophysiology, diagnosis and management. Oman Med J 27: 192–195.

Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, et al. (2000). LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. EMBO J 19: 5720–5728.

Kirisako T, Baba M, Ishihara N, Miyazawa K, Ohsumi M, Yoshimori T, et al. (1999). Formation process of autophagosome is traced with Apg8/Aut7p in yeast. J Cell Biol 147: 435–446.

Kita T, Hata T, Yoneda R, Okage T (1975). Stress state caused by alteration of rhythms in environmental temperature, and the functional changes in mice and rats. Nihon Yakurigaku Zasshi 71: 195–210.

Komatsu M, Ichimura Y (2010). Selective autophagy regulates various cellular functions. Genes Cells 15: 923–933.

Kon M, Kimura F, Akimoto T, Tanabe K, Murase Y, Ikemune S, et al. (2007). Effect of Coenzyme Q10 supplementation on exercise-induced muscular injury of rats. Exerc Immunol Rev 13: 76–88.

Kundu M, Lindsten T, Yang CY, Wu J, Zhao F, Zhang J, et al. (2008). Ulk1 plays a critical role in the autophagic clearance of mitochondria and ribosomes during reticulocyte maturation. Blood 112: 1493–1502.

Le Goff P (2006). Is fibromyalgia a muscle disorder? Joint Bone Spine 73: 239–242.

Lemasters JJ (2005). Selective mitochondrial autophagy, or mitophagy, as a targeted defense against oxidative stress, mitochondrial dysfunction, and aging. Rejuvenation Res 8: 3–5.

Marino G, Lopez-Otin C (2004). Autophagy: molecular mechanisms, physiological functions and relevance in human pathology. Cell Mol Life Sci 61: 1439–1454.

Mense S (2000). Neurobiological concepts of fibromyalgia–the possible role of descending spinal tracts. Scand J Rheumatol Suppl 113: 24–29.

Mukae T, Uchida H, Ueda H (2015). Donepezil reverses intermittent stress-induced generalized chronic pain syndrome in mice. J Pharmacol Exp Ther 353: 471–479.

Negus SS, Vanderah TW, Brandt MR, Bilsky EJ, Becerra L, Borsook D (2006). Preclinical assessment of candidate analgesic drugs: recent advances and future challenges. J Pharmacol Exp Ther 319: 507–514.

Nishiyori M, Ueda H (2008). Prolonged gabapentin analgesia in an experimental mouse model of fibromyalgia. Mol Pain 4: 52.

Nishiyori M, Nagai J, Nakazawa T, Ueda H (2010). Absence of morphine analgesia and its underlying descending serotonergic activation in an experimental mouse model of fibromyalgia. Neurosci Lett 472: 184–187.

Nishiyori M, Uchida H, Nagai J, Araki K, Mukae T, Kishioka S, et al. (2011). Permanent relief from intermittent cold stress-induced fibromyalgia-like abnormal pain by repeated intrathecal administration of antidepressants. Mol Pain 7: 69.

Ohara H, Kawamura M, Namimatsu A, Miura T, Yoneda R, Hata T (1991). Mechanism of hyperalgesia in SART stressed (repeated cold stress) mice: antinociceptive effect of neurotropin. Jpn J Pharmacol 57: 243–250.

Ozgocmen S, Ozyurt H, Sogut S, Akyol O (2006). Current concepts in the pathophysiology of fibromyalgia: the potential role of oxidative stress and nitric oxide. Rheumatol Int 26: 585–597.

Park JH, Niermann KJ, Olsen N (2000). Evidence for metabolic abnormalities in the muscles of patients with fibromyalgia. Curr Rheumatol Rep 2: 131–140.

Park JM, Jung CH, Seo M, Otto NM, Grunwald D, Kim KH, et al. (2016). The ULK1 complex mediates MTORC1 signaling to the autophagy initiation machinery via binding and phosphorylating ATG14. Autophagy 12: 547–564.

Pongratz DE, Spath M (1998). Morphologic aspects of fibromyalgia. Z Rheumatol 57(Suppl 2): 47–51.

Quinzii CM, Lopez LC, Von-Moltke J, Naini A, Krishna S, Schuelke M, et al. (2008). Respiratory chain dysfunction and oxidative stress correlate with severity of primary CoQ10 deficiency. FASEB J 22: 1874–1885.

Rauchova H, Battino M, Fato R, Lenaz G, Drahota Z (1992). Coenzyme Q-pool function in glycerol-3-phosphate oxidation

in hamster brown adipose tissue mitochondria. J Bioenerg Biomembr 24: 235–241.

Rodriguez-Hernandez A, Cordero MD, Salviati L, Artuch R, Pineda M, Briones P, et al. (2009). Coenzyme Q deficiency triggers mitochondria degradation by mitophagy. Autophagy 5: 19–32.

Roobol A, Carden MJ, Newsam RJ, Smales CM (2009). Biochemical insights into the mechanisms central to the response of mammalian cells to cold stress and subsequent rewarming. FEBS J 276: 286–302.

Scherz-Shouval R, Shvets E, Fass E, Shorer H, Gil L, Elazar Z (2007). Reactive oxygen species are essential for autophagy and specifically regulate the activity of Atg4. EMBO J 26: 1749–1760.

Siengdee P, Trakooljul N, Murani E, Schwerin M, Wimmers K, Ponsuksili S (2015). MicroRNAs regulate cellular ATP levels by targeting mitochondrial energy metabolism genes during C2C12 myoblast differentiation. PLoS ONE 10: e0127850.

Sluka KA (2009). Is it possible to develop an animal model of fibromyalgia? Pain 146: 3–4.

Solitar BM (2010). Fibromyalgia: knowns, unknowns, and current treatment. Bull NYU Hosp Jt Dis 68: 157–161.

Sprott H, Salemi S, Gay RE, Bradley LA, Alarcon GS, Oh SJ, et al. (2004). Increased DNA fragmentation and ultrastructural changes in fibromyalgic muscle fibres. Ann Rheum Dis 63: 245–251.

Tan ML, Balabin I, Onuchic JN (2004). Dynamics of electron transfer pathways in cytochrome C oxidase. Biophys J 86: 1813–1819.

Tolkovsky AM (2009). Mitophagy. Biochim Biophys Acta 1793: 1508–1515.

Turunen M, Olsson J, Dallner G (2004). Metabolism and function of coenzyme Q. Biochim Biophys Acta 1660: 171–199.

Wende AR, Young ME, Chatham J, Zhang J, Rajasekaran NS, Darley-Usmar VM (2016). Redox biology and the interface between bioenergetics, autophagy and circadian control of metabolism. Free Radic Biol Med doi:10.1016/j.freeradbiomed.2016.05.022.

Wolfe F, Smythe HA, Yunus MB, Bennett RM, Bombardier C, Goldenberg DL, et al. (1990). The American college of rheumatology 1990 criteria for the classification of fibromyalgia. Report of the multicenter criteria committee. Arthritis Rheum 33: 160–172.

Yunus MB, Kalyan-Raman UP, Kalyan-Raman K, Masi AT (1986). Pathologic changes in muscle in primary fibromyalgia syndrome. Am J Med 81: 38–42.

Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Sarcomer length in C57BL/6J mice. (A) Sarcomer length was determined in the left tibialis anterior muscle of control and ICS mice showing no variation. (B) POL- filtered micrographs showing HE- staining of male and female mice sarcomers structure. Magnification is ×630.

Table S1. Mouse weight after ICS. Female and male mice weight was measured before sacrifice.

Data S1. Supplementary methods, results and figure legends.