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Transgenic overexpression of mitofilin attenuates diabetes mellitus-associated cardiac and mitochondria dysfunction

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Abstract

Mitofilin, also known as heart muscle protein, is an inner mitochondrial membrane structural protein that plays a central role in maintaining cristae morphology and structure. It is a critical component of the mitochondrial contact site and cristae organizing system (MICOS) complex which is important for mitochondrial architecture and cristae morphology. Our laboratory has previously reported alterations in mitochondrial morphology and proteomic make-up during type 1 diabetes mellitus, with mitofilin being significantly down-regulated in interfibrillar mitochondria (IFM). The goal of this study was to investigate whether overexpression of mitofilin can limit mitochondrial disruption associated with the diabetic heart through restoration of mitochondrial morphology and function. A transgenic mouse line overexpressing mitofilin was generated and mice injected intraperitoneally with streptozotocin using a multi low-dose approach. Five weeks following diabetes mellitus onset, cardiac contractile function was assessed. Restoration of ejection fraction and fractional shortening was observed in mitofilin diabetic mice as compared to wild-type controls ($P < 0.05$ for both). Decrements observed in electron transport chain (ETC) complexes I, III, IV and V activities, state 3 respiration, lipid peroxidation as well as mitochondria membrane potential in type 1 diabetic IFM were restored in mitofilin diabetic mice ($P < 0.05$ for all). Qualitative analyses of electron micrographs revealed restoration of mitochondrial cristae structure in mitofilin diabetic mice as compared to wild-type controls. Furthermore measurement of mitochondrial internal complexity using flow cytometry displayed significant reduction in internal complexity in diabetic IFM which was restored in mitofilin diabetic IFM ($P < 0.05$). Taken together these results suggest that transgenic overexpression of mitofilin preserves mitochondrial

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Conflict of Interest

There are no conflicts of interest to disclose.

structure, leading to restoration of mitochondrial function and attenuation of cardiac contractile dysfunction in the diabetic heart.

Keywords

mitofilin; diabetes mellitus; mitochondria; electron transport chain

1. Introduction

Mitochondria are ubiquitous, double membranous organelles with an outer membrane and inner membrane. The inner mitochondrial membrane (IMM) of the mitochondria is comprised of an inner boundary membrane which is in close proximity with the outer membrane and cristae membrane [1, 2]. The regions in between the inner boundary and cristae membranes are narrow tubular openings with a fixed diameter and length called crista junctions [1-3]. The cristae membrane is composed of large tubular invaginations that protrude into the matrix space and house the respiratory chain complexes, as well as the F_1F_0 -ATP synthase, rendering it indispensable for proper mitochondrial function [4-7]. Numerous human pathologies have been associated with abnormal mitochondrial structure [8, 9]. Thus, proper integrity of mitochondrial cristae morphology is crucial for mitochondria structure and function.

A number of mitochondrial proteins including optic atrophy 1 (OPA1), coiled-coil-helix coiled-coil-helix domain 3 (CHCHD3), coiled-coil-helix cristae morphology 1 (CHCM1), and ATP synthase subunits have been associated with the regulation of cristae morphology [10-13]. One of the recently identified proteins, mitofilin, has been reported to be requisite for the maintenance of proper cristae morphology. Mitofilin, also known as heart muscle protein [14] due to its high abundance in the heart, is an IMM structural protein specifically localized to the cristae junction [15]. Down-regulation of mitofilin in Hela cells results in abnormal mitochondrial morphology with concentric layers of inner membrane, reduced cell proliferation, increased apoptosis and elevated reactive oxygen species (ROS) production [16]. Moreover, mitofilin depletion studies in yeast cells and *C. elegans* results in curved and stacked mitochondrial cristae tubules, increased ROS production, as well as a reduction in cristae junctions which is associated with decreased mitochondrial DNA content [3, 17]. Recently, mitofilin was identified as a critical component of the mitochondrial contact site and cristae organizing system (MICOS) complex where it functions as a central organizer of mitochondrial architecture, cristae junctions and cristae morphology [18-23]. Thus, a number of studies substantiate the significant role of mitofilin in maintaining proper mitochondrial structure and function.

Decrements in mitofilin content have been observed in many human diseases such as Down's syndrome [24, 25], Parkinson's disease [26, 27], Epilepsy [28, 29] and Neurodegeneration [30, 31]. Abnormal mitochondrial morphology, significant reduction in cristae density, as well as decrements in mitofilin content have been reported in the type 1 diabetic heart [32, 33]. Our laboratory has reported that following a type 1 diabetic insult, IFM exhibit greater dysfunction characterized by enhanced oxidative stress, changes in

mitochondrial morphology and function as well as altered mitochondrial proteomic signature which is not observed in type 1 diabetic subsarcolemmal mitochondria (SSM) [33, 34].

The IMM proteomic signature is significantly impacted following type 1 diabetic insult and mitofilin is one particular protein that shows significant decrease, specifically in the IFM. The impact of mitofilin loss in type 1 diabetic IFM was associated with changes in mitochondrial morphology and function. Mitofilin overexpression studies in yeast cells have revealed increased diameter as well as branching of cristae and cristae junctions [3]. Nevertheless, overexpression of mitofilin in the context of the diabetic heart have not been undertaken. To address this gap in knowledge, we generated a novel transgenic mouse model of mitofilin overexpression. The goal of the current study was to determine whether mitofilin overexpression provides cardioprotective benefits to the type 1 diabetic heart and if these effects are associated with improved mitochondrial structure and function.

2. Materials and Methods

2.1 Mitofilin Transgenic Mouse Development

The animal models used in this study conform to the NIH guidelines for the care and use of laboratory animals and were approved by the West Virginia University, School of Medicine Animal Care and Use Committee. Mitofilin transgenic mouse lines were generated by inserting a cDNA encoding the human mitofilin gene into the pCAGGS vector as previously described [35-38]. Briefly, the pCAGGS vector places the mitofilin gene (RG 201854, Origene) under the control of the human cytomegalovirus (CMV) immediate early enhancer and chicken β -actin (Ch. β -actin) promoter with first intron. The mitofilin cDNA was inserted into the XhoI cloning site of pCAGGS via sticky-end ligation (XhoI/XhoI) and blunt-end ligation of an SgfI/XbaI fragment of approximately 2227 bp (Figure 1A). The chimeric transgene was cut out of the plasmid by SspI and BamHI digestion, purified, and used to generate transgenic mice. The construct was given to the West Virginia University Transgenic Animal Core Facility where the pronucleus of fertilized eggs from superovulated FVB female mice crossed with FVB male mice, was injected with 1-2 μ l of purified DNA fragment at a concentration of 2 μ g/ml, and transferred into the oviducts of pseudopregnant CD-1 mice. All control and transgenic mice were generated using an FVB background, and experimental procedures were initiated on animals of approximately 5 weeks of age. Animals were maintained in individual microisolator cages within the West Virginia University Transgenic Barrier Facility and given food and water ad libitum.

2.2 Mitofilin transgenic mouse screening

Mouse litters were delivered after 19-20 days of gestation. To verify whether the chimeric transgene was present in the genome, DNA from 3-week-old mice was isolated from tail clips using a Qiagen DNeasy tissue kit (Qiagen, Valencia, CA). Transgene screening was performed by qPCR using a mitofilin probe (#hs00272794-m1; Applied Biosystems, Foster City, CA). Using this approach, only DNA that contains the exogenous mitofilin cDNA was detected. Briefly, isolated tail DNA, probe, and universal master mix were brought up to 25 μ l and qPCR was performed in a 96 well plate using an Applied Biosystems 7900HT Fast

Real-Time PCR system (Life Technologies, Grand Island, NY). A reaction time versus cycle number amplification plot was generated and transgene positive and negative animals were determined.

2.3 Diabetes induction

Male mice were separated into four groups: 1) control; 2) diabetic; 3) mitofilin overexpression; and 4) mitofilin overexpression diabetic. Type 1 diabetes mellitus was induced in diabetic and mitofilin overexpression diabetic mice following the protocol of the Animal Models of Diabetic Complications Consortium utilizing multiple low-dose streptozotocin (STZ; Sigma, St. Louis, MO) injections as previously described by our laboratory [33, 34, 39-42]. Briefly, sodium citrate buffer (pH 4.5) with 50 mg/kg body weight STZ was administered to mice for 5 consecutive days via intraperitoneal injections after 6 hours of fasting. Vehicle control animals were injected with same volume per body weight sodium citrate buffer. Three days following the last injection, hyperglycemia was measured and confirmed (Contour Blood Glucose Test Strips, Bayer Healthcare, Mishawaka, IN). Mice with blood glucose levels greater than 250 mg/dL were considered diabetic. All of the mice injected with STZ became diabetic and no differences in degree of hyperglycemia was evident with mitofilin transgene presence (values in mg/dL; control diabetic 401 ± 81 , mitofilin diabetic 392 ± 91). After diabetic induction, animals were maintained for 5 weeks and then euthanized for further experimentation.

2.4 Cardiac contractile function

Transthoracic echocardiography was performed as previously described by our laboratory [39] and others [43]. Briefly, mice were anesthetized with inhalant isoflurane and transferred to dorsal recumbency. Using the Vevo 2100 Imaging System (Visual Sonics, Toronto, Canada) and a 32- to 55-MHz linear array transducer, micro-ultrasound images were acquired. M-mode images were captured via the parasternal short axis at midpapillary level with all images acquired at the highest possible frame rate (233-401 frames/s). Left ventricular M-mode images provided end-diastolic and end-systolic diameters and volumes, stroke volume, ejection fraction, fractional shortening, heart rate and cardiac output measurements. All echocardiographic measurements were performed in conjunction with the West Virginia University Animal Models of Imaging Core Facility.

2.5 Human patient population

The West Virginia University Institutional Review Board (IRB) and Institutional Biosafety Committee (IBC) approved all protocols. Individuals undergoing coronary artery bypass graft surgery or cardiac valve replacement at Ruby Memorial Hospital in Morgantown, West Virginia, consented to the release of their cardiac tissue to the West Virginia University School of Medicine. Consenting individuals were then characterized as non-type 1 diabetic and type 1 diabetic based upon previous diagnosis of diabetes mellitus.

2.6 Preparation of individual mitochondrial subpopulations

At 5 weeks post-hyperglycemia onset, control, diabetic, mitofilin overexpression, and mitofilin overexpression diabetic mice were euthanized and their hearts excised. For human

samples, right atrial appendages were removed from patients, pericardial fat was trimmed and the heart tissue was weighed. For both mouse and human samples, hearts were rinsed in PBS (pH 7.4) and SSM and IFM subpopulations were isolated as previously described following the methods of Palmer *et al.* [44] with minor modifications by our laboratory [33, 34, 38-42, 45, 46]. Mitochondrial pellets were resuspended in KME buffer (pH 7.4) for mitochondrial respiration analyses, flow cytometric analyses and enzymatic activity measurements. Protein concentrations were determined by the Bradford method using bovine serum albumin as a standard [47].

2.7 Mitochondrial respiration rates

State 3 and state 4 respiration rates were assessed in isolated mitochondrial subpopulations as previously described [48-50] with slight modifications [38, 45]. Briefly, isolated mitochondria were resuspended in KME buffer and protein content was determined by the Bradford method as above [47]. Mitochondria protein was added to respiration buffer and loaded into a respiration chamber which was connected to an oxygen probe (OX1LP-1mL Dissolved Oxygen Package, Qubit System, Kingston, ON, Canada). The substrates glutamate (5mM) plus malate (5mM) were used to initiate respiration, and measurements of state 3 (250mM ADP) and state 4 (ADP-limited) respiration were made. Values were expressed as nmol of oxygen consumed/min/mg protein.

2.8 ETC complex activities

ETC complexes I, III and IV activities were measured spectrophotometrically as previously described [34, 38, 46, 51]. Briefly, complex I activity was determined by measuring the oxidation of NADH at 340 nm. Complex III activity was determined by measuring the reduction of cytochrome *c* at 550 nm in the presence of reduced decylubiquinone. Complex IV activity was determined by measuring the oxidation of cytochrome *c* at 550 nm. Protein content was determined by the Bradford method as described above [47], and ETC activity values were expressed as nmol substrate consumed/min/mg protein. ATP synthase activity was measured as oligomycin-sensitive ATPase activity using an assay coupled with pyruvate kinase, which converts ADP to ATP and produces pyruvate from phosphoenolpyruvate as previously described [46, 52-54]. Final values were expressed as nmol of NADH oxidized/min/mg protein.

2.9 Western blot analysis

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was run on 4-12% gradient gels as previously described [42, 55] with equal amounts of protein loaded. Relative amounts of mitofilin, cytochrome *c* oxidase (COX IV), mitofusin 1 (Mfn1), mitofusin 2 (Mfn2), OPA1, CHCHD3, dynamin-related protein 1 (Drp1) and GAPDH were assessed using the following primary antibodies; anti-mitofilin rabbit antibody (product no. ab48139, Abcam, Cambridge, MA), anti-COX IV rabbit antibody-mitochondrial loading control (product no. ab16056, Abcam, Cambridge, MA), anti-Mfn1 rabbit antibody (product no. ab104585, Abcam, Cambridge, MA), anti-Mfn2 rabbit antibody (product no. ab50838, Abcam, Cambridge, MA), anti-OPA1 rabbit antibody (product no. ab42364, Abcam, Cambridge, MA), anti-CHCHD3 rabbit antibody (product no. ab98975, Abcam, Cambridge, MA), anti-Drp1 rabbit

antibody (product no. sc32898, Santa Cruz Biotechnology, Dallas, TX), and anti-GAPDH rabbit antibody (product no. ab8245, Abcam, Cambridge, MA). The secondary antibody used was goat anti-rabbit IgG horseradish peroxidase conjugate (product no. 10004301, Cayman Chemical Company, Ann Arbor, MI). Detection of signal was performed using a Pierce ECL Western blotting substrate detection system according to the manufacturer's directions (Thermo Fisher Scientific Inc., Rockford, IL). Quantification of chemiluminescent signals were assessed using a G:Box Bioimaging System (Syngene, Frederick, MD), and data were expressed as arbitrary optical density units. Densitometry was measured using Image J Software (National Institutes of Health, Bethesda, MD). Protein loading was further confirmed using Ponceau staining in addition to COX IV and GAPDH, where appropriate.

2.10 ETC Complex Protein Expression

To assess ETC complex abundances, blue native polyacrylamide gel electrophoresis (BN-PAGE) was performed as previously described [39, 45] with modifications according to the manufacturer's protocol (Invitrogen, Carlsbad, CA) using equal amounts of protein. Briefly, isolated mitochondria were solubilized with 1% digitonin on ice. After addition of Coomassie G-250, samples were run on 4–16% NativePAGE gels. Following BN-PAGE, gels were placed in a fixed solution containing 40% methanol and 10% acetic acid followed by microwaving for 45 seconds at 1,100 watts. Gels were then washed for 15 minutes at room temperature after which the solution was decanted. Destaining was accomplished by addition of 50 ml of an 8% acetic acid solution and microwaved a second time for 45 seconds at 1,100 watts. The gel was then shaken at room temperature until the desired background was obtained. To control for destaining time and enable comparison between gels, each band of interest was expressed per the molecular weight marker 480 kDa band. The gel was then scanned and densitometry was measured using Image J Software (National Institutes of Health, Bethesda, MD).

2.11 Mitochondrial DNA (mtDNA) content

Examination of mtDNA content was performed on whole heart lysate from control and mitofilin transgenic mice using a NovaQUANT™ mouse mtDNA to nuclear DNA ratio kit that compares the levels of nuclear DNA to mtDNA. As per the manufacturers protocol (Millipore, Billerica, MA), an RTPCR platform was utilized to compare several nuclear and mitochondrial genes and the mtDNA copy number was quantified per diploid nuclear genome. An Applied Biosystems 7900HT Fast Real-Time PCR system (Life Technologies, Grand Island, NY) was used for the analyses.

2.12 Mitochondrial size, internal complexity and membrane potential

Mitochondrial size, internal complexity and membrane potential (Ψ_m) were measured as previously described by our laboratory with modifications [34, 42, 46]. Briefly, flow cytometric analyses were performed using a FACS Calibur equipped with a 15-MW 488-nm argon laser and 633-nm red diode laser (Becton Dickinson, San Jose, CA). Each individual parameter (gating, size, and complexity) was performed using specific light sources (laser and photomultiplier tube) and specific detectors. MitoTracker deep red 633 (Invitrogen, Carlsbad, CA), which moves into intact mitochondria due to membrane potential, was used

to selectively stain intact mitochondria (emission wavelength: 633 nm, fluorescent 633 red diode laser) and exclude debris, enabling accurate gating of intact mitochondria. Freshly isolated mitochondria were incubated with the MitoTracker dye and subsequently assessed for size and internal granularity. Forward scatter (FSC) and side scatter (SSC) detectors were used to examine approximate size (FSC; absolute particle size) and approximate internal complexity (SSC; refracted and reflected light which is proportional to granularity of the object) in isolated mitochondria. Internal complexity or granularity of isolated mitochondria was expressed with respect to size (arbitrary unit) of the mitochondria as FSC/SSC ratio. The ratiometric dye 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol carbocyanine iodide (JC-1; Molecular Probes, Carlsbad, CA), was used to assess mitochondrial membrane potential. Changes in membrane potential were recorded as the ratio between the color shifts from green to orange. All flow cytometric measurements were performed in conjunction with the West Virginia University Flow Cytometry Core Facility.

2.13 Lipid peroxidation

Lipid peroxidation by-products malondialdehyde (MDA) and 4-hydroxyalkenal (4-HAE), stable end products formed from the oxidation of polyunsaturated fatty acids and esters, were assessed as previously described [34, 46]. Absorbance was measured on a Molecular Devices Flex Station 3 spectrophotometer (Molecular Devices, Sunnyvale, CA), and protein content was assessed as described above [47] with final values expressed per milligram of protein.

2.14 Electron microscopy

A section of left ventricle was cut and fixed for electron microscopy images. Briefly, specimens were washed in 0.2M phosphate buffer, post-fixed by incubation for 2 hours with 2% osmium tetroxide (Electron Microscopy Science, Hatfield, PA), dehydrated in a graded series of ethanol solutions (from 50% to 100%) and propylene oxide, and embedded in Epon resin (SPI Supplies, Westchester, PA). Embedded samples were allowed to polymerize for 48 hours at 62°C. Ultrathin sections (50nm) were cut from the resulting blocks with a Leica Ultracut UCT ultramicrotome (Leica Biosystems, Buffalo Grove, IL) and then captured on 200 mesh copper electron microscopy grids. The sections were observed at 120kV with a Zeiss Libra 120 electron microscope (Carl Zeiss NTS, LLC, Peabody, MA, USA) connected to a Gatan Orius SC 1000 CCD digital camera driven by Digital Micrograph software (Gatan, Pleasanton, CA) for image acquisition and analysis. All electron microscopy imaging was performed in conjunction with the West Virginia University Tissue Processing and Analysis Core Facility.

2.15 Statistics

Means $\pm$ SE were calculated for all data sets. Data were analyzed using a one way ANOVA (GraphPad software, La Jolla, CA). A Tukey comparison of all groups was used as the post-hoc test to determine the significant differences among means. A Student's *t*-test was utilized when evaluating mitofilin content in mouse and human mitochondria. $P < 0.05$ was considered significant.

3. Results

3.1 Mitofilin transgenic mouse characterization

Two mitofilin transgenic mouse lines were created, mitofilin Tg Line 1 and mitofilin Tg Line 2. DNA from mitofilin Tg Line 1 displayed average CT values of 26.5, while mitofilin Tg Line 2 displayed average CT values of approximately 21, indicating that Line 2 possessed greater mitofilin cDNA content. In contrast, DNA from transgenic negative mice of all lines displayed CT values of approximately 34-36 which is similar to the CT value of water. As a result, we chose to perform all experimentation using mitofilin Tg Line 2 and its associated littermate controls.

To verify increased protein expression in our transgenic mice, mitofilin protein content was determined in both whole heart homogenate and isolated mitochondrial subpopulations. Transgenic SSM and IFM subpopulations displayed significantly higher levels of mitofilin protein content when compared to littermate controls which was not significantly impacted by diabetic insult (Figure 1B-C). In addition, mitofilin protein levels were significantly higher in transgenic whole heart homogenate as compared to littermate controls (Figure 1D). Evaluation of mitofilin protein content in control and diabetic mitochondria revealed significant decrements in type 1 diabetic IFM with no significant decrease in diabetic SSM (Figures 2A-D; $P < 0.05$). Because down-regulation of mitofilin has been associated with loss of mtDNA content, we determined whether mtDNA levels were influenced by overexpression in our transgenic mouse model [3]. Examination of mtDNA content in mitofilin transgenic and littermate controls revealed no significant differences (Littermate Control 5319.83 ± 2265.05 copies vs. Mitofilin Transgenic 4866.88 ± 1995.13 copies). To determine whether type 1 diabetic patient heart mitochondria displayed similar mitofilin profiles as a result of the pathology, we examined mitofilin protein contents in non-diabetic and type 1 diabetic human patient atrial tissue. Our data reveal significant decreases in IFM mitofilin protein contents in diabetic patient atrial tissue as compared to non-diabetic patients with no significant differences in SSM between diabetic and non-diabetic patients (Figures 2E-H).

3.2 Body weight, heart weight and cardiac contractile function

Significant decreases in heart and body weights were observed in diabetic and mitofilin diabetic animals when compared with their respective controls (Table 1; $P < 0.05$). However, there was no significant differences observed in heart weight to body weight ratios. Cardiac contractile dysfunction was observed in diabetic hearts when compared with control hearts. Ejection fraction, fractional shortening and cardiac output were significantly decreased in the type 1 diabetic heart relative to control (Table 1; $P < 0.05$). Overexpression of mitofilin in the presence of diabetes mellitus restored ejection fraction and fractional shortening (Table 1; $P < 0.05$). Further, no significant differences were observed in stroke volume, or volumes at systole and diastole between any groups. Finally, no changes in diameter at systole and diastole were observed.

3.3 Mitochondrial functional assessment

Assessment of ETC complexes I, III, IV and V activities revealed significant decrements in diabetic IFM as compared with control IFM (Table 2; $P < 0.05$), with no significant differences between control and diabetic SSM (Table 2). Overexpression of mitofilin preserved ETC complexes I, III, IV and V activities in mitofilin diabetic IFM as compared to diabetic IFM (Table 2; $P < 0.05$). A significant increase in ATP synthase activity was also observed in the mitofilin SSM subpopulation as compared to control SSM (Table 2; $P < 0.05$). Using complex I substrates glutamate/malate, state 3 and state 4 respiration rates were significantly decreased in diabetic IFM compared to control IFM (Table 2; $P < 0.05$), with no significant differences observed between control and diabetic SSM (Table 2). Overexpression of mitofilin in the diabetic heart restored state 3 respiration rates in the IFM (Table 2; $P < 0.05$) with no significant effects observed in the SSM (Table 2).

3.4 ETC Complex Protein Expression

To gain insight into whether the observed decreases in ETC complex activities were the result of down-regulation of individual complex contents, we employed a BN-PAGE approach which enabled evaluation of the expression of each ETC complex as a whole. Our data indicated no significant differences in complexes I, III, IV, and V contents between the four treatment groups in SSM and IFM (Figure 3A-I).

3.5 Mitochondrial membrane potential (Ψ_m)

No significant differences in SSM Ψ_m were observed among the four groups, (Figure 4A), however, there was a significant reduction of Ψ_m in the diabetic IFM subpopulation compared with control which was restored with mitofilin overexpression (Figure 4B; $P < 0.05$).

3.6 Oxidative stress

Lipid peroxidation by-products 4-HAE and MDA were significantly increased in diabetic IFM compared to control IFM (Figure 5B; $P < 0.05$), with no significant differences observed in diabetic SSM as compared to control SSM (Figure 5A). Mitofilin overexpression significantly decreased the accumulation of lipid peroxidation by-products in diabetic IFM, restoring them back to that of control IFM levels (Figure 5B; $P < 0.05$), suggesting an ability of mitofilin to attenuate ROS-induced damage to lipids.

3.7 Electron microscopy

Qualitative analysis of mitochondrial morphology was performed by electron microscopy. Visualization of IFM structure was assessed in control, diabetic, mitofilin control and mitofilin diabetic heart. Morphologically altered IFM with damaged cristae structures were observed in the diabetic heart when compared to the control heart (Figures 6A-B). Overexpression of mitofilin restored mitochondrial morphology and cristae structure in the mitofilin diabetic IFM (Figure 6D), suggesting a potential role for mitofilin in the maintenance of cristae morphology following type 1 diabetic insult.

3.8 Mitochondrial internal complexity

Because of the improved mitochondrial morphology and cristae structure observed within the electron micrographs of mitofilin diabetic IFM, we determined whether internal complexity of the mitochondria were affected by mitofilin expression using flow cytometry. FSC and SSC were used to estimate size and internal complexity of mitochondria respectively (Figures 7A-B). The ratio of SSC to FSC was calculated in an effort to determine changes in internal complexity with respect to size. Using this approach, diabetic IFM showed significantly decreased internal complexity when compared with control (Figure 7D; $P<0.05$) with no changes observed in the SSM (Figure 7C). Overexpression of mitofilin significantly restored mitochondrial internal complexity in mitofilin diabetic IFM (Figure 7D; $P<0.05$). Restoration of mitochondrial morphology and cristae structure with mitofilin overexpression could potentially account for the improvement in mitochondrial internal complexity in mitofilin diabetic IFM.

3.9 Mitochondrial dynamics

Because type 1 diabetes mellitus and mitofilin have been associated with mitochondrial dynamics and cristae morphological changes, we examined several ancillary proteins involved in the regulation of these processes. Western blot analyses indicated no significant differences in any of the proteins that we examined (Mfn1, Mfn2, OPA1, CHCHD3, and Drp1) (Figure 8) which are associated with mitochondrial dynamics, suggesting that mitofilin overexpression did not alter the levels of these proteins.

4. Discussion/Conclusion

A number of studies corroborate the notion that mitochondrial dysfunction plays a critical role in the pathogenesis of the diabetic heart. Previous studies from our laboratory suggest that during type 1 diabetic insult mitochondria are spatially impacted with those situated in between the myofibrils (IFM) affected to a greater extent [33, 34, 41, 42]. In particular, the IMM are disturbed as evidenced by decreased ETC complex activities, ATP synthase activity, protein import, cardiolipin content and cardiolipin synthase activity correlating with the manifestation of mitochondrial dysfunction [33, 34, 40-42]. Further, mitochondrial morphology including changes in size and internal complexity of IFM are also observed. Proteomic alterations during type 1 diabetic insult, which are disproportionately realized in the IMM of the IFM subpopulation, have been previously reported [33]. These alterations include a decrease in mitofilin, a mitochondrial structural protein. Among mitofilin's primary functions is the maintenance of cristae junctions, branching of cristae and preservation of IMM morphology [3, 17]. Preservation of cristae structure is essential for proper mitochondrial function and health due to the presence of ETC complexes vital for ATP production which are contained in the IMM [5, 6]. The goal of this study was to determine whether preservation of mitofilin provides cardioprotective benefits to the type 1 diabetic heart and whether the beneficial effects are associated with improved mitochondrial cristae structure. Overexpression of mitofilin in the SSM and IFM of transgenic mice revealed significantly higher expression of the protein in IFM as compared to SSM. These findings are in agreement with Ferreira *et al.* who showed a 37 fold increase of mitofilin in cardiac tissue IFM when compared with SSM [56].

Proteomic analyses from our laboratory reveal a significant decrease in mitofilin content in IFM during type 1 diabetic insult [33]. Further, morphological alterations are observed primarily in the IFM subpopulation [34, 57]. Thus, it is plausible that the decreased mitofilin content observed in the IFM, resulting from type 1 diabetes mellitus may account, in part, for the mitochondrial morphological changes observed which could have downstream effects on mitochondrial functionality ultimately leading to cardiac contractile dysfunction. Electron micrographs from diabetic hearts revealed damaged mitochondrial cristae in mitochondria situated between the myofibrils when compared with the control hearts. Moreover, a significant decrease in mitochondrial internal complexity and granularity was observed in diabetic IFM. Our findings are in agreement with John *et al.* who investigated the impact of mitofilin down-regulation in HeLa cells. Their findings revealed a disorganized IMM as well as increased ROS production and apoptosis [16]. Moreover, their study revealed that mitofilin-deficient mitochondria fail to produce normal tubular cristae and cristae junctions. These author's observations share similarities to the structural abnormalities observed in diabetic IFM in our current study. In a similar study, Rabl *et al.* investigated the down-regulation of Fcj1, a putative orthologue of mammalian mitofilin, in yeast cells. The results of these studies revealed a lack of cristae junctions which was correlated with abnormal mitochondrial structure. Electron microscopy analyses from hearts overexpressing mitofilin revealed preservation of mitochondrial morphology and cristae structure in type 1 diabetic IFM. Moreover, restoration of mitochondrial internal complexity and granularity was observed in mitofilin diabetic IFM. Our findings are in agreement with a study in which overexpression of Fcj1 in yeast cells led to increased cristae junction formation and cristae branching, suggesting normal mitochondrial structure [3]. Taken together, data from the current study showing abnormal mitochondrial structure as a result of decreased mitofilin content in diabetic IFM, which was restored with mitofilin overexpression, are in agreement with others [3, 16, 17]. Finally, overexpression of mitofilin led to preservation of ejection fraction and fractional shortening, suggesting improved cardiac contractile function which may be the result of improvement in mitochondrial structure and ultimately, function.

The inner mitochondrial cristae membrane is the principal site for oxidative phosphorylation and ATP production [5]. Mitochondrial cristae structure increases the inner membrane surface area enabling a greater capacity for oxidative phosphorylation and ATP production. In the current study diabetic IFM with disrupted mitochondrial cristae structure and decreased internal complexity displayed significant decrements in ETC complexes I, III, IV as well as ATP synthase function which are in agreement with previous observations from our laboratory [34, 39]. Overexpression of mitofilin restored ETC complexes and ATP synthase activities in the mitofilin diabetic IFM which were associated with restoration of cristae morphology as well as mitochondrial internal complexity. Though not assessed in the current study, these findings may have been the result of an increased surface area leading to an enhanced capacity for oxidative phosphorylation and ATP generation. Nevertheless, it should be noted that mitofilin overexpression did not lead to changes in the absolute contents of ETC complexes. These findings suggest that the benefits imparted by mitofilin overexpression are not the result of increased ETC complexes, rather preservation of IMM integrity which could enhance stabilization of the ETC complexes. Additional studies

designed to determine changes in cristae surface area and their impact on ETC stabilization in the face of enhanced mitofilin presence would lend insight into the mechanisms contributing to the observed oxidative phosphorylation changes.

Several studies have shown enhanced ROS production from cardiac mitochondria of different type 1 diabetic animal models [34, 58]. The oxidative milieu resulting from enhanced ROS production promotes damage to mitochondrial membranes and proteins. We have previously observed increased oxidative damage as indexed through nitrotyrosine residues and lipid peroxidation in type 1 diabetic IFM [34]. In the current study, we observed similar increases in lipid peroxidation of diabetic IFM. Interestingly, overexpression of mitofilin attenuated lipid peroxidation in diabetic IFM which could be due to improved mitochondrial structure, and preservation of ETC function. Preservation of mitochondrial structure could potentially attenuate electron leakage resulting in decreased ROS production and downstream oxidative damage.

In contrast to our study, mitofilin was reported to be increased in pathological cardiac hypertrophy and was shown to promote cardiac hypertrophy in response to hypertrophic stimuli. In the current study we observed a decrease in heart weight with no change in heart weight/body weight ratio as a result of a parallel decrease in body weight following diabetes mellitus induction. One potential limitation of our study was the lack of assessment of heart weight/tibia length which may have been decreased provided tibia length remained constant which has been reported in previous low-dose STZ studies [59]. Moreover, mitofilin overexpression was shown to increase ROS generation and lower oxidative phosphorylation activity in animals subjected to a cardiac hypertrophy protocol [60]. The differences in the observed results between the two studies could be due to a multitude of reasons. Zhang *et al.* utilized whole tissues for assessing oxidative phosphorylation and frozen tissues for their ROS measure in comparison with isolated mitochondria used in our studies. Moreover, two different models of cardiomyopathy were being studied; diabetic cardiomyopathy and hypertrophy that depict a differential expression of mitofilin levels suggesting its distinct role in these pathologies. Furthermore, decrements in mitofilin content have also been observed in other human diseases such as Down's syndrome [24, 25], Parkinson's disease [26, 27], Epilepsy [28, 29] and Neurodegeneration [30, 31]. Hence, these varied changes of mitofilin observed in different human pathologies as well as different cardiac pathologies suggest unique responses and effects of mitofilin under different pathological stimuli.

In conclusion, we report for the first time, that overexpression of mitofilin in a type 1 diabetic mouse model preserves mitochondrial structure and function, resulting in cardiac contractile protection. Mitochondrial functional preservation is associated with the restoration of ETC complex activities, ATP synthase activity, state 3 respiration rates, and attenuation of lipid peroxidation which are the result of improved mitochondrial morphology, structure and internal complexity.

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References

- [1]. Mannella CA. The relevance of mitochondrial membrane topology to mitochondrial function. *Biochim Biophys Acta*. Feb; 2006 1762(2):140–7. [PubMed: 16054341]
- [2]. Zick M, Rabl R, Reichert AS. Cristae formation-linking ultrastructure and function of mitochondria. *Biochim Biophys Acta*. Jan; 2009 1793(1):5–19. [PubMed: 18620004]
- [3]. Rabl R, Soubannier V, Scholz R, Vogel F, Mendl N, Vasiljev-Neumeyer A, et al. Formation of cristae and crista junctions in mitochondria depends on antagonism between Fcjl and Su e/g. *J Cell Biol*. Jun 15; 2009 15(185):6–1047.
- [4]. Davies KM, Strauss M, Daum B, Kief JH, Osiewicz HD, Rycovska A, et al. Macromolecular organization of ATP synthase and complex I in whole mitochondria. *Proc Natl Acad Sci U S A*. Aug 23; 108(34):14121–6. [PubMed: 21836051]
- [5]. Gilkerson RW, Selker JM, Capaldi RA. The cristal membrane of mitochondria is the principal site of oxidative phosphorylation. *FEBS Lett*. Jul 10; 2003 546(2-3):355–8. [PubMed: 12832068]
- [6]. Vogel F, Bornhovd C, Neupert W, Reichert AS. Dynamic subcompartmentalization of the mitochondrial inner membrane. *J Cell Biol*. Oct 23; 2006 23(175):2–237.
- [7]. Wurm CA, Jakobs S. Differential protein distributions define two sub-compartments of the mitochondrial inner membrane in yeast. *FEBS Lett*. Oct 16; 2006 16(580):24–5628.
- [8]. DiMauro S, Bonilla E, Zeviani M, Nakagawa M, DeVivo DC. Mitochondrial myopathies. *Ann Neurol*. Jun; 1985 17(6):521–38. [PubMed: 3927817]
- [9]. Wallace DC. A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine. *Annu Rev Genet*. 2005; 39:359–407. [PubMed: 16285865]
- [10]. An J, Shi J, He Q, Lui K, Liu Y, Huang Y, et al. CHCM1/CHCHD6, novel mitochondrial protein linked to regulation of mitofilin and mitochondrial cristae morphology. *J Biol Chem*. Mar 2; 287(10):7411–26. [PubMed: 22228767]
- [11]. Darshi M, Mendiola VL, Mackey MR, Murphy AN, Koller A, Perkins GA, et al. ChChd3, an inner mitochondrial membrane protein, is essential for maintaining crista integrity and mitochondrial function. *J Biol Chem*. Jan 28; 286(4):2918–32. [PubMed: 21081504]
- [12]. Frezza C, Cipolat S, Martins de Brito O, Micaroni M, Beznoussenko GV, Rudka T, et al. OPA1 controls apoptotic cristae remodeling independently from mitochondrial fusion. *Cell*. Jul 14; 2006 14(126):1–177.
- [13]. Paumard P, Vaillier J, Coulary B, Schaeffer J, Soubannier V, Mueller DM, et al. The ATP synthase is involved in generating mitochondrial cristae morphology. *EMBO J*. Feb 1; 2002 1(21):3–221.
- [14]. Icho T, Ikeda T, Matsumoto Y, Hanaoka F, Kaji K, Tsuchida N. A novel human gene that is preferentially transcribed in heart muscle. *Gene*. Jul 8; 1994 8(144):2–301.
- [15]. Korner C, Barrera M, Dukanovic J, Eydt K, Harner M, Rabl R, et al. The C-terminal domain of Fcjl is required for formation of crista junctions and interacts with the TOB/SAM complex in mitochondria. *Mol Biol Cell*. Jun; 23(11):2143–55. [PubMed: 22496419]
- [16]. John GB, Shang Y, Li L, Renken C, Mannella CA, Selker JM, et al. The mitochondrial inner membrane protein mitofilin controls cristae morphology. *Mol Biol Cell*. Mar; 2005 16(3):1543–54. [PubMed: 15647377]

- [17]. Mun JY, Lee TH, Kim JH, Yoo BH, Bahk YY, Koo HS, et al. Caenorhabditis elegans mitofilin homologs control the morphology of mitochondrial cristae and influence reproduction and physiology. *J Cell Physiol. Sep*; 224(3):748–56. [PubMed: 20578245]
- [18]. Alkhaja AK, Jans DC, Nikolov M, Vukotic M, Lytovchenko O, Ludewig F, et al. MINOS1 is a conserved component of mitofilin complexes and required for mitochondrial function and cristae organization. *Mol Biol Cell. Jan*; 23(2):247–57. [PubMed: 22114354]
- [19]. Harner M, Korner C, Walther D, Mokranjac D, Kaesmacher J, Welsch U, et al. The mitochondrial contact site complex, a determinant of mitochondrial architecture. *EMBO J. Nov* 2; 30(21):4356–70. [PubMed: 22009199]
- [20]. Hoppins S, Collins SR, Cassidy-Stone A, Hummel E, Devay RM, Lackner LL, et al. A mitochondrial-focused genetic interaction map reveals a scaffold-like complex required for inner membrane organization in mitochondria. *J Cell Biol. Oct* 17; 195(2):323–40. [PubMed: 21987634]
- [21]. Pfanner N, van der Laan M, Amati P, Capaldi RA, Caudy AA, Chacinska A, et al. Uniform nomenclature for the mitochondrial contact site and cristae organizing system. *J Cell Biol. Mar* 31; 204(7):1083–6. [PubMed: 24687277]
- [22]. von der Malsburg K, Muller JM, Bohnert M, Oeljeklaus S, Kwiatkowska P, Becker T, et al. Dual role of mitofilin in mitochondrial membrane organization and protein biogenesis. *Dev Cell. Oct* 18; 21(4):694–707. [PubMed: 21944719]
- [23]. Zerbes RM, Bohnert M, Stroud DA, von der Malsburg K, Kram A, Oeljeklaus S, et al. Role of MINOS in mitochondrial membrane architecture: cristae morphology and outer membrane interactions differentially depend on mitofilin domains. *J Mol Biol. Sep* 14; 422(2):183–91. [PubMed: 22575891]
- [24]. Bernert G, Fountoulakis M, Lubec G. Manifold decreased protein levels of matrin 3, reduced motor protein HMP and hIrk in fetal Down's syndrome brain. *Proteomics. Dec*; 2002 2(12):1752–7. [PubMed: 12469345]
- [25]. Myung J, Gulesserian T, Fountoulakis M, Lubec G. Deranged hypothetical proteins Rik protein, Nit protein 2 and mitochondrial inner membrane protein, Mitofilin, in fetal Down syndrome brain. *Cell Mol Biol (Noisy-le-grand). Jul*; 2003 49(5):739–46. [PubMed: 14528910]
- [26]. Van Laar VS, Dukes AA, Cascio M, Hastings TG. Proteomic analysis of rat brain mitochondria following exposure to dopamine quinone: implications for Parkinson disease. *Neurobiol Dis. Mar*; 2008 29(3):477–89. [PubMed: 18226537]
- [27]. Van Laar VS, Mishizen AJ, Cascio M, Hastings TG. Proteomic identification of dopamine-conjugated proteins from isolated rat brain mitochondria and SH-SY5Y cells. *Neurobiol Dis. Jun*; 2009 34(3):487–500. [PubMed: 19332121]
- [28]. Furukawa A, Kawamoto Y, Chiba Y, Takei S, Hasegawa-Ishii S, Kawamura N, et al. Proteomic identification of hippocampal proteins vulnerable to oxidative stress in excitotoxin-induced acute neuronal injury. *Neurobiol Dis. Sep*; 43(3):706–14. [PubMed: 21669285]
- [29]. Omori A, Ichinose S, Kitajima S, Shimotohno KW, Murashima YL, Shimotohno K, et al. Gerbils of a seizure-sensitive strain have a mitochondrial inner membrane protein with different isoelectric points from those of a seizure-resistant strain. *Electrophoresis. Dec*; 2002 23(24):4167–74. [PubMed: 12481273]
- [30]. Wang Q, Liu Y, Zou X, An M, Guan X, He J, et al. The hippocampal proteomic analysis of senescence-accelerated mouse: implications of Uchl3 and mitofilin in cognitive disorder and mitochondrial dysfunction in SAMP8. *Neurochem Res. Sep*; 2008 33(9):1776–82. [PubMed: 18307031]
- [31]. Wishart TM, Paterson JM, Short DM, Meredith S, Robertson KA, Sutherland C, et al. Differential proteomics analysis of synaptic proteins identifies potential cellular targets and protein mediators of synaptic neuroprotection conferred by the slow Wallerian degeneration (Wlds) gene. *Mol Cell Proteomics. Aug*; 2007 6(8):1318–30. [PubMed: 17470424]
- [32]. Bugger H, Boudina S, Hu XX, Tuinei J, Zaha VG, Theobald HA, et al. Type 1 diabetic akita mouse hearts are insulin sensitive but manifest structurally abnormal mitochondria that remain coupled despite increased uncoupling protein 3. *Diabetes. Nov*; 2008 57(11):2924–32. [PubMed: 18678617]

- [33]. Baseler WA, Dabkowski ER, Williamson CL, Croston TL, Thapa D, Powell MJ, et al. Proteomic alterations of distinct mitochondrial subpopulations in the type 1 diabetic heart: contribution of protein import dysfunction. *Am J Physiol Regul Integr Comp Physiol.* Feb; 300(2):R186–200. [PubMed: 21048079]
- [34]. Dabkowski ER, Williamson CL, Bukowski VC, Chapman RS, Leonard SS, Peer CJ, et al. Diabetic cardiomyopathy-associated dysfunction in spatially distinct mitochondrial subpopulations. *Am J Physiol Heart Circ Physiol.* Feb; 2009 296(2):H359–69. [PubMed: 19060128]
- [35]. He H, Giordano FJ, Hilal-Dandan R, Choi DJ, Rockman HA, McDonough PM, et al. Overexpression of the rat sarcoplasmic reticulum Ca²⁺ ATPase gene in the heart of transgenic mice accelerates calcium transients and cardiac relaxation. *J Clin Invest.* Jul 15; 1997 15(100):2–380.
- [36]. Hollander JM, Martin JL, Belke DD, Scott BT, Swanson E, Krishnamoorthy V, et al. Overexpression of wild-type heat shock protein 27 and a nonphosphorylatable heat shock protein 27 mutant protects against ischemia/reperfusion injury in a transgenic mouse model. *Circulation.* Dec 7; 2004 7(110):23–3544.
- [37]. Marber MS, Mestri R, Chi SH, Sayen MR, Yellon DM, Dillmann WH. Overexpression of the rat inducible 70-kD heat stress protein in a transgenic mouse increases the resistance of the heart to ischemic injury. *J Clin Invest.* Apr; 1995 95(4):1446–56. [PubMed: 7706448]
- [38]. Dabkowski ER, Williamson CL, Hollander JM. Mitochondria-specific transgenic overexpression of phospholipid hydroperoxide glutathione peroxidase (GPx4) attenuates ischemia/reperfusion-associated cardiac dysfunction. *Free Radic Biol Med.* Sep 15; 2008 15(45):6–855.
- [39]. Baseler WA, Dabkowski ER, Jagannathan R, Thapa D, Nichols CE, Shepherd DL, et al. Reversal of mitochondrial proteomic loss in Type 1 diabetic heart with overexpression of phospholipid hydroperoxide glutathione peroxidase. *Am J Physiol Regul Integr Comp Physiol.* Apr 1; 2013 304(7):R553–65. [PubMed: 23408027]
- [40]. Baseler WA, Thapa D, Jagannathan R, Dabkowski ER, Croston TL, Hollander JM. miR-141 as a regulator of the mitochondrial phosphate carrier (Slc25a3) in the type 1 diabetic heart. *Am J Physiol Cell Physiol.* Dec 15; 2012 303(12):C1244–51. [PubMed: 23034391]
- [41]. Croston TL, Shepherd DL, Thapa D, Nichols CE, Lewis SE, Dabkowski ER, et al. Evaluation of the cardiolipin biosynthetic pathway and its interactions in the diabetic heart. *Life Sci.* Sep 3; 2013 3(93):8–313.
- [42]. Williamson CL, Dabkowski ER, Baseler WA, Croston TL, Alway SE, Hollander JM. Enhanced apoptotic propensity in diabetic cardiac mitochondria: influence of subcellular spatial location. *Am J Physiol Heart Circ Physiol.* Feb.2009 298:2–H633. 42.
- [43]. Bauer M, Cheng S, Jain M, Ngoy S, Theodoropoulos C, Trujillo A, et al. Echocardiographic speckle-tracking based strain imaging for rapid cardiovascular phenotyping in mice. *Circ Res.* Apr 15; 108(8):908–16. [PubMed: 21372284]
- [44]. Palmer JW, Tandler B, Hoppel CL. Biochemical properties of subsarcolemmal and interfibrillar mitochondria isolated from rat cardiac muscle. *J Biol Chem.* Dec 10; 1977 10(252):23–8731.
- [45]. Croston TL, Thapa D, Holden AA, Tveter KJ, Lewis SE, Shepherd DL, et al. Functional deficiencies of subsarcolemmal mitochondria in the type 2 diabetic human heart. *Am J Physiol Heart Circ Physiol.* Jul 1; 2014 307(1):H54–65. [PubMed: 24778174]
- [46]. Dabkowski ER, Baseler WA, Williamson CL, Powell M, Razunguzwa TT, Frisbee JC, et al. Mitochondrial dysfunction in the type 2 diabetic heart is associated with alterations in spatially distinct mitochondrial proteomes. *Am J Physiol Heart Circ Physiol.* Aug; 2010 299(2):H529–40. [PubMed: 20543078]
- [47]. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* May 7.1976 72:248–54. [PubMed: 942051]
- [48]. Chance B, Williams GR. Respiratory enzymes in oxidative phosphorylation. I. Kinetics of oxygen utilization. *J Biol Chem.* Nov; 1955 217(1):383–93. [PubMed: 13271402]

- [49]. Chance B, Williams GR. Respiratory enzymes in oxidative phosphorylation. VI. The effects of adenosine diphosphate on azide-treated mitochondria. *J Biol Chem.* Jul; 1956 221(1):477–89. [PubMed: 13345836]
- [50]. Warshaw JB. Cellular energy metabolism during fetal development. I. Oxidative phosphorylation in the fetal heart. *J Cell Biol.* May; 1969 41(2):651–7. [PubMed: 5783881]
- [51]. Trounce IA, Kim YL, Jun AS, Wallace DC. Assessment of mitochondrial oxidative phosphorylation in patient muscle biopsies, lymphoblasts, and transmittochondrial cell lines. *Methods Enzymol.* 1996; 264:484–509. [PubMed: 8965721]
- [52]. Feniouk BA, Suzuki T, Yoshida M. Regulatory interplay between proton motive force, ADP, phosphate, and subunit epsilon in bacterial ATP synthase. *J Biol Chem.* Jan 5; 2007 282(1):1–764.
- [53]. Pullman ME, Penefsky HS, Datta A, Racker E. Partial resolution of the enzymes catalyzing oxidative phosphorylation. I. Purification and properties of soluble dinitrophenol-stimulated adenosine triphosphatase. *J Biol Chem.* Nov. 1960 235:3322–9. [PubMed: 13738472]
- [54]. Rosca MG, Okere IA, Sharma N, Stanley WC, Recchia FA, Hoppel CL. Altered expression of the adenine nucleotide translocase isoforms and decreased ATP synthase activity in skeletal muscle mitochondria in heart failure. *J Mol Cell Cardiol.* Jun; 2009 46(6):927–35. [PubMed: 19233197]
- [55]. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* Aug 15; 1970 227(5259):680–701.
- [56]. Ferreira RM, Vitorino R, Padrao AI, Moreira-Goncalves D, Alves RM, Duarte JA, et al. Spatially distinct mitochondrial populations exhibit different mitofilin levels. *Cell Biochem Funct.* Jul; 2005 30(5):395–9. [PubMed: 22438066]
- [57]. Shen X, Zheng S, Thongboonkerd V, Xu M, Pierce WM Jr, Klein JB, et al. Cardiac mitochondrial damage and biogenesis in a chronic model of type 1 diabetes. *Am J Physiol Endocrinol Metab.* Nov; 2004 287(5):E896–905. [PubMed: 15280150]
- [58]. Song Y, Du Y, Prabhu SD, Epstein PN. Diabetic Cardiomyopathy in OVE26 Mice Shows Mitochondrial ROS Production and Divergence Between In Vivo and In Vitro Contractility. *Rev Diabet Stud.* Fall; 2007 4(3):159–68. [PubMed: 18084673]
- [59]. Motyl K, McCabe LR. Streptozotocin, type I diabetes severity and bone. *Biol Proced Online.* 2009; 11:296–315. [PubMed: 19495918]
- [60]. Zhang Y, Xu J, Luo YX, An XZ, Zhang R, Liu G, et al. Overexpression of Mitofilin in the Mouse Heart Promotes Cardiac Hypertrophy in Response to Hypertrophic Stimuli. *Antioxid Redox Signal.* May 2.

Highlights

- Mitofilin is decreased in interfibrillar mitochondria as a result of diabetes.
- Overexpression of mitofilin preserves mitochondrial morphology following diabetes.
- Overexpression of mitofilin attenuates cardiac dysfunction resulting from diabetes.

Figure 1. Schematic of mitofilin transgenic construct and protein expression

(A) The generation of mitofilin transgenic mice was accomplished by insertion of human mitofilin cDNA into the XbaI and XhoI cloning sites of pCAGGS as SgfI and XhoI fragment and released by SspI and BamHI digestion. Western blot analysis of mitofilin protein expression in isolated (B) SSM and IFM mitochondria. Lanes 1-2 are controls, lanes 3-4 are mitofilin transgenic, and lanes 5-6 are mitofilin transgenic diabetic samples for both SSM and IFM mitochondria. (C) Bar graph representation for SSM and IFM mitochondria protein levels. (D) Western blot analysis of mitofilin protein expression in total heart mitochondria from control (lanes 1-2) and mitofilin transgenic (lanes 3-4) mouse. COX IV, GAPDH and ponceau staining were used as loading controls for the blots. Values are expressed as means $\pm$ SEM. *P < 0.05 vs. control; n=4 per each group. SSM: subsarcolemmal mitochondria, IFM: interfibrillar mitochondria.

Figure 2. Mitofilin protein expression analysis in control and diabetic mitochondria subpopulations from mouse and human samples

(A) Control SSM (lanes 1 and 2) and diabetic SSM (lanes 3 and 4) (B) control IFM (lanes 1 and 2) and diabetic IFM (lanes 3 and 4) were analyzed for mitofilin protein expression. Bar graph representation of mitofilin protein levels over COX IV in SSM (C) and IFM (D) subpopulations. Analysis of mitofilin protein content in non-diabetic and type 1 diabetic human patient samples in SSM (E) and IFM (F) subpopulations. Bar graph representation of mitofilin protein levels over COX IV in SSM (G) and IFM (H) isolated from human patient samples. Values are presented as means $\pm$ SE; *P < 0.05 for control vs. diabetic. Control for protein loading was confirmed with COX IV (mitochondria loading control); n=5 for each group. SSM: subsarcolemmal mitochondria, IFM: interfibrillar mitochondria.

Figure 4. Mitochondrial membrane potential

Isolated mitochondria from control, diabetic, mitofilin control and mitofilin diabetic hearts were incubated with JC-1, and 100,000 gated events were analyzed per sample in (A) SSM and (B) IFM subpopulations. Values are expressed as means $\pm$ SEM. * $P < 0.05$ for control vs. diabetic and # $P < 0.05$ for diabetic vs. mitofilin diabetic; $n=8$ per each group. SSM: subsarcolemmal mitochondria, IFM: interfibrillar mitochondria.

Figure 5. Lipid peroxidation by-products

Oxidative damage to lipids was assessed in control, diabetic, mitofilin control and mitofilin diabetic (A) SSM and (B) IFM subpopulations by measuring lipid peroxidation by-products malondialdehyde (MDA) and 4-hydroxyalkenals (4-HAE) using a colorimetric assay. Results were compared against a standard curve of known 4-HAE and MDA concentrations. Values are expressed as means $\pm$ SEM. * $P < 0.05$ for control vs. diabetic and # $P < 0.05$ for diabetic vs. mitofilin diabetic; $n=4$ per each group. SSM: subsarcolemmal mitochondria, IFM: interfibrillar mitochondria.

Figure 6. Mitochondrial structure

Electron micrographs ($\times 16.0K$) from left ventricle of (A) Control, (B) Diabetic, (C) Mitofilin control, and (D) Mitofilin diabetic mice hearts showing mitochondrial morphology and cristae structures in the IFM subpopulations of type 1 diabetic heart. IFM: interfibrillar mitochondria : myofibrils.

Figure 7. Mitochondrial Internal Complexity

Mitochondrial internal complexity was assessed in control, diabetic, mitofilin control and mitofilin diabetic hearts. Forward scatter and side scatter were used to analyze isolated mitochondria as seen by representative histograms of (A) SSM and (B) IFM. The ratio of side scatter to forward scatter was used to calculate internal complexity of the mitochondria in (C) SSM and (D) IFM mitochondria. Values are expressed as means $\pm$ SEM. * $P < 0.05$ for control vs. diabetic and # $P < 0.05$ for diabetic vs. mitofilin diabetic; $n=8$ per each group. SSM: subsarcolemmal mitochondria, IFM: interfibrillar mitochondria.

Figure 8. Western Blot analyses of mitochondrial dynamics

Protein expression of mitochondrial fission, fusion, Chchd3 and Opa1 proteins were analyzed in control, mitofilin, mitofilin diabetic, and diabetic cardiac (A) SSM and IFM mitochondria. Control for protein loading was confirmed with COX IV (mitochondria loading control); n=4 for each group. SSM: subsarcolemmal mitochondria, IFM: interfibrillar mitochondria.

Table 1

Cardiac contractile function.

Contractile Parameter	Control	Diabetic	Mitofilin Control	Mitofilin Diabetic
BW, g	28.0 ± 0.8	25.5 ± 0.5 *	27.1 ± 0.7	24.6 ± 0.4 *
HW, mg	102.7 ± 4.4	94.3 ± 2.5 *	103.0 ± 2.1	91.3 ± 1.9 *
HW/BW, mg/g	3.7 ± 0.1	3.7 ± 0.1	3.8 ± 0.1	3.7 ± 0.1
Ejection fraction, %	70.6 ± 1.4	65.2 ± 0.8 *	70.6 ± 1.8	74.7 ± 1.5 #
Fractional shortening, %	39.0 ± 1.1	34.8 ± 0.6 *	39.4 ± 1.4	42.4 ± 1.4 #
Cardiac Output, ml/min	18.1 ± 1.2	14.2 ± 1.4 *	17.5 ± 1.1	16.0 ± 2.7
Diameter; s, mm	2.0 ± 0.1	2.1 ± 0.1	2.0 ± 0.2	1.8 ± 0.1
Diameter; d, mm	3.2 ± 0.1	3.3 ± 0.1	3.4 ± 0.1	3.1 ± 0.1
Volume; s, µl	13.8 ± 0.6	14.4 ± 1.5	12.8 ± 2.6	10.1 ± 1
Volume; d µl	41.4 ± 4	44.7 ± 3.5	47.4 ± 4.3	37.9 ± 2.4
Stroke volume, µl	32.3 ± 1.9	28.1 ± 2.3	34.6 ± 2.1	28.3 ± 1.7
Heart rate, bpm	517.0 ± 15.7	487.0 ± 13.6	506.0 ± 14.5	486.0 ± 21.6

Cardiac contractile measurements were assessed using the Vevo 2100 imaging system. Values are means ± SEM.

BW: body weight, HW: heart weight, s: systole, d: diastole, bpm: beats per minute.

* $P < 0.05$ diabetics vs. their respective control,

$P < 0.05$ diabetic vs. mitofilin diabetic; n=20 for BW, HW and n=6 for other parameters.

Table 2

Mitochondrial functional assessment.

Group	Complex I	Complex III	Complex IV	Complex V	State 3	State 4
SSM Control	32.2 ± 4	98.7 ± 17	12.5 ± 2.4	12.1 ± 0.9	16.1 ± 1.1	2.6 ± 0.5
SSM Diabetic	29.2 ± 2.7	88 ± 7.6	10.7 ± 1.5	12.9 ± 0.9	18.1 ± 0.7	1.9 ± 0.3
SSM Mitofilin	30.4 ± 3.7	86 ± 11.7	10.4 ± 1.9	16.8 ± 1.4 [*]	17.9 ± 2.1	3.6 ± 0.6
SSM Mitofilin Diabetic	29.9 ± 1.9	88.4 ± 13.7	12 ± 2.6	14.7 ± 1.2	22.2 ± 2.6	2 ± 0.3
IFM Control	46.8 ± 1.9	197 ± 8.8	39 ± 7	24.5 ± 1.3	21.8 ± 0.9	4.2 ± 0.6 [†]
IFM Diabetic	31.3 ± 2.8 [*]	151 ± 6.6 [*]	19.5 ± 2 [*]	20.6 ± 0.6 [*]	14.7 ± 0.6 [*]	2 ± 0.2
IFM Mitofilin	44.1 ± 6.1	173 ± 13.6	31.9 ± 2.7	23.9 ± 0.7	19.2 ± 1.4	2.4 ± 0.3
IFM Mitofilin Diabetic	45.9 ± 1.7 [#]	206 ± 11 [#]	37.7 ± 4 [#]	26.6 ± 1.1 [#]	22.3 ± 3.5 [#]	2.2 ± 0.1

Mitochondrial electron transport chain (ETC) complex activities and respiration rates were examined in control, diabetic, mitofilin control and mitofilin diabetic mitochondrial subpopulations. ETC complexes I, III, IV and V activities were assessed spectrophotometrically by measuring the oxidation of NADH (complex I), reduction of cytochrome c (complex III), oxidation of cytochrome c (complex IV) and an assay coupled with pyruvate kinase (complex V). Enzymatic activities for complexes I, III and IV are expressed as activity per minute per milligram of protein and complex V is expressed as nmol of NADH per minute per milligram of protein. State 3 and state 4 respiration rates were determined in the presence of the substrates glutamate-malate, and state 3 respiration was examined upon addition of ADP. Values are expressed as means ± SEM.

SSM: subsarcolemmal mitochondria, IFM: interfibrillar mitochondria.

^{*} $P < 0.05$ vs. control,

[#] $P < 0.05$ vs. diabetic

[†] $P < 0.05$ against all groups; n=8 per each group.