Regional absence of mitochondria causing energy depletion in the myocardium of muscle LIM protein knockout mice

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Abstract

Objective: Defects in myocardial mitochondrial structure and function have been associated with heart failure in humans and animal models. Mice lacking the muscle LIM protein (MLP) develop morphological and clinical signs resembling human dilated cardiomyopathy and heart failure. We tested the hypothesis that defects in the cytoskeleton lead to dilated cardiomyopathy through mitochondrial dysfunction in the MLP mouse model.

Methods and results: Oxidative phosphorylation activity was determined in left ventricles of MLP knockout (KO) mice and control littermates by measuring complex activities of the electron transport chain (I–IV) and ATP synthase (complex V). All complexes and citrate synthase (CS) showed decreased activities in the KO mice, although activity per amount of CS, a measure for mitochondrial density, was normal. Light and electron microscopy revealed a disorganization of mitochondria and a dramatic decrease in mitochondrial density, even revealing regions completely lacking mitochondria in the KO hearts. Real-time PCR analysis showed decreased transcript levels of mtDNA and nuclear encoded mitochondrial genes and of peroxisome proliferator activated receptor gamma co-activator 1\textsuperscript{a} (PGC-1\textsuperscript{a}), a key regulator of mitochondrial biogenesis. MtDNA copy number (ratio mtDNA/nuclear DNA) was slightly increased in the MLP KO mice.

Conclusion: Our results show that the absence of MLP causes a local loss of mitochondria. We hypothesize that this is caused by a disturbed interaction between cytoskeleton and mitochondria, which interferes with energy sensing and energy transfer. Recovery of energy depletion by stimulating mitochondrial biogenesis might be a useful therapeutic strategy for improving the energy imbalance in heart failure.

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Keywords: Cardiomyopathy; Heart failure; Cytoskeleton; Mitochondria; Oxidative phosphorylation

1. Introduction

Defects in mitochondrial structure and function have been associated with cardiovascular diseases such as hypertrophic cardiomyopathy (HCM) \cite{1}, dilated cardiomy-
opathy (DCM) [2] and idiopathic cardiomyopathy (IDC) [3] and primary, causative mutations have been reported in the mitochondrial DNA (mtDNA) and in nuclear genes encoding mitochondrial proteins (nDNA) [4]. Mitochondrial dysfunction in cardiomyopathy can also be secondary to other, often unknown, genetic or environmental causes [5,6]. Nonetheless, the exact role for mitochondrial dysfunction in different stages of cardiac disease remains to be elucidated. In the heart, energy production mainly relies on mitochondrial oxidative phosphorylation (OXPHOS), consisting of the electron transport chain (complexes I–IV) and the ATP synthase (complex V). These complexes consist of subunits encoded by mtDNA and nDNA [4]. The enzyme activities of the OXPHOS complexes can be either increased, normal or decreased in HCM, DCM and heart failure (HF) [5,7,8]. One of the major reasons for these discrepancies is that human cardiomyopathy and HF are complex disorders with a heterogeneous etiology, in which a variety of factors, like genetic background, environment and drug treatment, can be involved [9]. Thus, to get further insight into the role of the OXPHOS system in the transition to HF, it would be advantageous to use an animal model that is more genetically and environmentally homogeneous. Several genetically altered mouse models of cardiac hypertrophy and failure exist, including defects in contractile, cytoskeletal proteins and Ca2+-regulatory proteins, cell surface receptors, cell-signaling molecules, transcription factors, growth factors and mitochondria [10].

Knocking out cytoskeletal genes in a number of mice models illustrates the importance of the intermediate filament (IF) network in mitochondrial behaviour and function. Abnormalities in number, distribution and morphology of mitochondria have been observed in hearts of desmin KO mice [11] or skeletal muscle of dystonin KO [12] and plectin KO mice [13]. In addition, downregulation of nuclear encoded mitochondrial transcripts has been reported in patients with Duchenne muscular dystrophy and α-sarcoglycan deficiency [14] and other kinds of myofibrillar myopathy in human skeletal muscle, indicating an important contribution of structural abnormalities to the metabolic crisis in these myopathies [15].

The cytoskeletal protein muscle LIM protein (MLP) is a conserved positive regulator of cardiac muscle development and MLP-deficient mice develop DCM with myocyte hypertrophy and HF [16]. MLP has been found to be an important component of the cardiac mechanical stretch sensing machinery and recently, human MLP mutations were found to be associated with DCM [17,18] and HCM [19]. To test the hypothesis that this defect in the cytoskeleton leads to DCM through mitochondrial abnormalities, we studied mitochondria and mitochondrial functioning in the myocardium of MLP KO mice and identified an uncompensated local loss of mitochondria leading to energy depletion.

2. Methods

2.1. Animal model

The MLP-deficient mice have been extensively described before [16]. Studies were performed in eight male MLP KO mice and five male control mice at 12 weeks of age. MLP KO mice at this age consistently show left ventricular hypertrophy as indicated by an increased left ventricle weight (LVW)/body weight (BW) ratio and an increased mRNA expression of the hypertrophy marker atrial natriuretic factor (ANF) [16]. In addition, 12-week-old KO mice show dilation with reduced cardiac function [16], which was confirmed by echocardiographic measurements. Wild-type mice of 129Sv/B6 strain were used as controls. After the mice were sacrificed, hearts were harvested and frozen in liquid nitrogen. Experiments were performed in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996) and were approved by the Committee for Animal Research of the Maastricht University, The Netherlands.

2.2. OXPHOS activities

Frozen left ventricles from MLP KO mice and controls were ground in 0.25 M sucrose, 10 mM N-[2-hydroxyethyl]piperazine-N’-[2 ethylsulfonic acid] (HEPES) and 1 mM ethylene diamine triaceatic acid (EDTA), pH 7.4. Citrate synthase (CS) activity (μmol/min/g muscle) was measured according to Srere [20] to correct for mitochondrial density and protein (mg/g muscle) was determined using the Lowry method to correct for total protein content [21]. All enzyme activities were measured at 37 °C in heart homogenates by spectrophotometric methods as described before [22–25].

2.3. Electron microscopy

From two MLP KO and control mice, freshly isolated hearts were perfused with 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. For light microscopic analysis, left ventricle tissue was stained with toluidine blue. For electron microscopy, the tissue was postfixed in 1% osmium tetroxide, dehydrated in 70–100% ethanol, incubated in propylene oxide, then embedded in Embed 812 resin (Electronic Microscopic Science). Sections were observed and imaged with a Philips CM 100 electron microscope. Quantitative analysis of mitochondrial size and regions lacking mitochondria were carried out at a magnification of 23,800 and 4550, respectively.

2.4. MtDNA copy number

MtDNA content was determined by comparing the ratio of mtDNA (ND1, NADH-ubiquinone oxidoreductase sub-
unit 1) to nDNA (18S ribosomal RNA) measured in triplo by real-time quantitative PCR (TaqMan®) using the SYBR® Green PCR Core Reagents (Applied Biosystems). DNA was amplified in a 20-μl volume reaction mixture containing SYBR Green PCR buffer, 4 mM MgCl₂, 1.25 mM dNTP, 0.25 U AmpliTaq Gold® DNA Polymerase and 1.25 pmol of the forward or reverse primer. PCR conditions were as follows: first, 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s/60 °C for 1 min. Immediately after PCR, samples were heated to 95 °C for 15 s, 60 °C for 1 min, and then heated to 95 °C in 20 min, followed by cooling to 4 °C, to create a dissociation curve to check for non-specific amplification. Primers used for amplification are shown in Table 1.

2.5. Gene expression

Total left ventricular RNA was isolated using the TRIzol reagent (Invitrogen) and purified with the RNeasy clean-up kit (Qiagen). Reverse transcription into cDNA was performed in a 40-μl volume with 1.25 μg total RNA as a template. After denaturing the RNA at 65 °C for 10 min, a reaction mixture containing first-strand buffer, 1.25 mM dNTPs, 200 U Superscript II (Invitrogen), RNAsin and a mixture of 0.75 μg oligo(DT) primer and 0.75 μg random primers (Invitrogen) was added and incubated for 1 h at 42 °C followed by 5 min at 95 °C. Primers were created using Primer Express® software (Applied Biosystems). Sequences are presented in Table 1. Analysis of gene expression was performed in duplo by real-time quantitative PCR (TaqMan®), using the SYBR® Green PCR Core Reagents (Applied Biosystems). Real-time PCR conditions for the amplification of cDNA were the same as described for the mtDNA copy number study. Transcription values were normalized to the housekeeping gene cyclophilin.

2.6. Statistical analysis of real-time PCR data

The data collected was modelled using the Gaussian distribution. The inference criterion used for comparing the models is their ability to fit the observed data, i.e. models are compared directly through their minimized minus log-likelihood. When the numbers of parameters in models differ, they are penalized by adding the number of estimated parameters, a form of the Akaike information criterion [26]. All OXPHOS activity models allowed differences between controls and MLP KO mice. The effect of CS activity, protein content and a different variance for each group was included when necessary. In the gene expression study, cyclophilin was included in all models as a housekeeping gene. A difference between control and knockouts (group effect) was also included. For the mtDNA copy number study, the model included the ratio mtDNA/nDNA, control/knockouts (group effect), and differences in mtDNA and nDNA within controls and knockouts.

3. Results

3.1. Mitochondrial content and enzyme activities

Left ventricles of MLP KO mice were enlarged compared to control littermates (data not shown). The total protein content in mg/gram muscle was decreased to 75% of the controls (statistically not significant), whereas CS activity was significantly decreased to 38% of the controls, suggesting a strong decrease in mitochondrial number. Mitochondrial enzyme activities for complexes I, II, IV and V were significantly decreased to 43%, 32%, 37% and 30%, respectively, of the controls (Fig. 1). Complex III was decreased to 69% of the controls, although not statistically significant. After correction for CS in the MLP KO mice, complex activities were comparable with controls, indicating that the mitochondria have normal OXPHOS activities.

Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Length (bp)</th>
<th>Sequence forward primer</th>
<th>Sequence reverse primer</th>
</tr>
</thead>
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<tr>
<td>ND1</td>
<td>184</td>
<td>5'-CAG GAT GAG CCT CAA ACT CC-3'</td>
<td>5'-GTT CAG GCT GCC AGA AGT AA-3'</td>
</tr>
<tr>
<td>ND2</td>
<td>204</td>
<td>5'-AGG GAT CCC ACT GCA CAT AG-3'</td>
<td>5'-CCT ATG TGG GCA ATT GAT GA-3'</td>
</tr>
<tr>
<td>Cytb</td>
<td>200</td>
<td>5'-AGC TCC TTC CAT GAC GAC AA-3'</td>
<td>5'-GAG GTG AAC GAT TGC TAG GG-3'</td>
</tr>
<tr>
<td>COII</td>
<td>172</td>
<td>5'-AGC AAA TCA ACA ACC CCG TA-3'</td>
<td>5'-GTC AGG CAC CGG TCA TG-3'</td>
</tr>
<tr>
<td>ATP6</td>
<td>79</td>
<td>5'-AAT TAC AGG CTT CCG ACA CAA AC-3'</td>
<td>5'-TGG AAT TAG TGA AAT TGG ATG AAC-3'</td>
</tr>
<tr>
<td>SDHC</td>
<td>69</td>
<td>5'-ACA AAT GGT CTC TTC CTA GCA CA-3'</td>
<td>5'-CCC CTC CAC TCA AGG TTA CTT-3'</td>
</tr>
<tr>
<td>Cox5a</td>
<td>72</td>
<td>5'-CTT TAA ATG AGT TGG GAA TCT CCA C-3'</td>
<td>5'-GCC CAT CGA AGG GAG TTT ACA-3'</td>
</tr>
<tr>
<td>Tafm</td>
<td>204</td>
<td>5'-GTC ATG AGG CAC CGT ATG GC-3'</td>
<td>5'-GCC ATG CTG GAA AAA AAC CAC TT-3'</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>198</td>
<td>5'-CAA TGA ATG CAG CCG TCT TA-3'</td>
<td>5'-GTG TGA GGA GGG TCA TCG TT-3'</td>
</tr>
<tr>
<td>ANT1</td>
<td>71</td>
<td>5'-ACT TCG CCT TCA AAG ACA AGT ACA-3'</td>
<td>5'-GCC CCA GAA CTG CTT AGT G-3'</td>
</tr>
<tr>
<td>Cyclophilin</td>
<td>71</td>
<td>5'-CAG ATG CTC GAC CAA ACA CAA-3'</td>
<td>5'-GCC ATC CAC CCA TTC AGT CT-3'</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>61</td>
<td>5'-TGC AGG CCC TGT AAT TGG AA-3'</td>
<td>5'-GCC CCC AAT GGA TCC TCG TT-3'</td>
</tr>
</tbody>
</table>

ND1 and ND2, NADH-ubiquinone oxidoreductase 1 and 2; Cytb, cytochrome b; COII, cytochrome c oxidase subunit II; ATP6, ATP synthase 6. Mitochondrial primers are based on accession AB042432 (mus musculus mtDNA), SDHC, succinate dehydrogenase C (NM_025321); Cox5a, cytochrome c oxidase subunit 5a (NM_007747); Tafm, mitochondrial transcription factor A (NM_009360); PGC-1α, peroxisome proliferator activated receptor gamma co-activator 1 (NM_008904); ANT1, adenine nucleotide transporter 1 (XM_134169); cyclophilin (NM_008907); 18S rRNA (X00868).
and that the overall decrease in OXPHOS activity is not due to mitochondrial dysfunction and increased production of oxygen radicals.

3.2. Microscopic analysis of mitochondria

Light microscopic analysis of myocardial structure in controls and MLP KO mice showed a decrease in mitochondrial number and revealed several regions totally lacking mitochondria in the knockouts (Fig. 2B). Additionally, mitochondria appear to be less well organized than in the control hearts. Ultrastructural analysis by electron microscopy revealed a disturbed mitochondrial alignment along myofibrils (Fig. 3B and D), although there were also regions present in which the alignment was preserved. Furthermore, the overall size of the mitochondria was

![Fig. 1. Activities of complexes I–V and citrate synthase (CS) in muscle homogenates from MLP KO and wild-type (WT). All complexes and citrate synthase show decreased activities in the KO mice (n=8) compared to the WT mice (n=5), although complex III was not significant. Protein content (mg/g muscle) is also decreased, but not significant. Data for all activities are expressed as mean absolute activities in μmol/min/gram muscle (±S.D.).](image1)

![Fig. 2. Mitochondrial organization in MLP KO and controls. Transversely sectioned left ventricle tissue from controls (A) and knockouts (B) were stained with toluidine blue and observed with a light microscope. The dark spots within cardiomyocytes represent the mitochondria (arrowheads). The mitochondria in the knockouts (B) are less well organized and the cardiomyocytes even show regions lacking mitochondria (arrows). Bar = 200 μm.](image2)
significantly decreased in the knockouts compared to controls: 0.48 ± 0.04 versus 0.67 ± 0.07 μm².

The regional mitochondrial loss was quantified using electron microscopy by comparing 50 healthy cardiomyocytes in the control mice versus 50 cardiomyocytes in the KO mice. Local areas in control cardiomyocytes without mitochondria covered at most ~5% of the total cell area. One third of the cardiomyocytes in the MLP KO mice showed local areas without mitochondria covering at least ~40% of the total area. The remaining cells showed regions lacking mitochondria of ~10–40% of the total cell area. Only 5 out of the 50 cardiomyocytes investigated in the MLP KO mice showed similar mitochondrial occupation as in controls, although the disturbed organization was still visible in these cells. The observed OXPHOS deficiency is most likely caused by this local loss of mitochondria.

3.3. MtDNA copy number

To test if the decrease in mitochondrial number in the MLP KO mice is caused by defects in mtDNA replication, we determined the relative mtDNA copy number by comparing the amount of the mitochondrial encoded ND1 gene to the nuclear encoded 18S gene. KO hearts showed an increase in the ratio mtDNA/nDNA (ND1/18S rRNA) by a factor 1.4 compared to WT. Measurements were performed by real-time quantitative PCR. Values are presented as fold change and corresponding confidence intervals.

Fig. 3. Ultrastructural changes in MLP KO and controls. Electron micrographs represent longitudinally sectioned left ventricles from controls (A and C) and knockouts (B and D). Knockouts reveal a disturbed mitochondrial alignment along myofibrils and the overall size of the mitochondria is smaller. Bar = 2 μm (A and B) and 1 μm (C and D).

Fig. 4. Mitochondrial copy number in MLP KO compared to wild-type (WT). MLP KO mice show an increase in the ratio mtDNA/nDNA (ND1/18S rRNA) by a factor 1.4 compared to WT. Measurements were performed by real-time quantitative PCR. Values are presented as fold change and corresponding confidence intervals.
increase in the ratio mtDNA/nDNA by a factor 1.4 compared to control hearts (Fig. 4). Despite the decrease in the number of mitochondria, the mtDNA content in the knockouts is increased.

3.4. Gene expression differences

The decreased mitochondrial content could also be caused by a decrease in mitochondrial protein synthesis. The mitochondrial encoded transcripts for complexes I, III, IV and V (NADH-ubiquinone oxidoreductase subunit 2 (ND2), cytochrome b (Cytb), cytochrome c oxidase subunit II (COII) and ATP synthase 6 (ATP6)) were significantly downregulated to 64%, 70%, 57% and 73% of control levels (Fig. 5). Transcript levels of the nuclear encoded mitochondrial genes for complexes II and IV (succinate dehydrogenase C (SDHC) and cytochrome c oxidase subunit 5a (Cox5a)) showed a significant decrease to 61% and 69% of the controls. Key regulators of mtDNA transcription and regulation, such as mitochondrial transcription factor A (Tfam), peroxisome proliferator activated receptor gamma co-activator 1 (PGC-1α) and adenine nucleotide transporter 1 (ANT1) transcripts were also significantly downregulated to 77%, 67% and 55%, respectively, of the controls.

4. Discussion

In this study, we investigated the role of mitochondria and mitochondrial functioning in the MLP KO mouse model. Our results demonstrate an energy deficiency in the myocardium of the MLP KO mice, as indicated by a decrease in overall mitochondrial OXPHOS activity. This is most likely caused by a regional absence of mitochondria in the myocardium of the MLP KO mice. Also, the overall size of mitochondria is smaller, although OXPHOS activity per mitochondrion is normal, as indicated by normalization of OXPHOS activities after correction for mitochondrial density. The absence of mitochondria is associated with a downregulation of mitochondrial and nuclear encoded transcripts, indicating a decreased mitochondrial biogenesis. This is corroborated by a downregulation of key regulators of this process, like Tfam, PGC-1α and ANT1. The decrease of Tfam transcripts contrasts with the observed increase in mtDNA content, which may be reminiscent of an initial, physiological increase in mtDNA due to an increased energy demand.

The absence of MLP leads to disorganization of the Z-lines and myofibrils [16]. The Z-disc is involved in sarcomeric organization, force generation and transmission and acts as an interface between contractile proteins, other cytoskeletal elements and signaling molecules [27,28]. Muscle mitochondria are present as laterally aligned strands between myofibrils and known to be anchored to the intermediate filament (IF) network of the Z-line. The cytoskeleton defects may influence mitochondrial number and function through alterations in the interactions between outer mitochondrial membrane proteins and specific cytoskeletal(-associated) proteins [29]. The integrity of these contact sites [30] and the associated functional complexes with sarcomeres and sarcoplasmic reticulum (i.e. ICEUs, intracellular energetic units) are important for a variety of mitochondrial functions, such as energy coupling and oxidative metabolism [31–34]. An increased energy demand usually results in activation of PGC1-α, the key regulator of mitochondrial biogenesis and gene expression in heart and other tissues [35,36]. It can be expected that loss of proper alignment of mitochondria, as shown in the MLP KO mice, probably in combination with mechanical stretch, results in mitochondrial loss and in interference with the compensa-
hypothesis. Phospholamban (PLB) ablation [38] or inhibition of β-adrenergic receptor kinase (βARK1) [39] have been shown to prevent the cardiomyopathic phenotype in the MLP mice. One explanation for the rescue of the MLP phenotype is that the enhanced cardiac relaxation leads to the elimination of the mechanical stretch stimulus. This results in a restoration of the defect in the stretch sensor function, which in normal hearts activates compensatory cardiomyocyte survival and hypertrophy downstream signals [17]. However, an alternative explanation could be that the cardiac energy status will be recovered by improving the interaction between the cytoskeleton and the mitochondria. This could be tested by evaluation of mitochondria, mitochondrial function and biogenesis in the double MLP/PLB KO mice and MLP KO/βARKct mice.

The role of energy imbalance and mitochondrial abnormalities has been implicated in several studies, where mutations in mtDNA or mitochondrial genes have been described in heart failure [6]. Also, AMP-activated protein kinase (AMPK) mutations were found to result in skeletal and cardiac disease [37,40]. AMPK regulates expression of both CaMKIV and PGC-1α [41]. Human MLP mutations [17–19] and the telethonin (T-cap) and α-actinin mutations, both disrupting the stable complex with MLP [17,18], lead to DCM, presumably by the same mechanism. It would be interesting to test if the transition to heart failure in these patients is due to a defective stretch sensor accompanied by increased wall stress, a defect in energy supply or a combination of these factors.

In summary, our findings indicate a direct link between cytoskeletal defects and local energy deficiency in affected hearts of the MLP KO mice. Treatment aimed at recovery of energy depletion might be useful in escaping the vicious cycle of energy mismatch. Furthermore, stimulation of the transcriptional cascades involved in mitochondrial biogenesis may also be an approach for increasing myocardial energetics.

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