Impaired cardiac mitochondrial oxidative phosphorylation and enhanced mitochondrial oxidative stress in feline hypertrophic cardiomyopathy

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1Department of Veterinary Clinical and Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark; 2Department of Biomedical Sciences, Center for Healthy Aging, University of Copenhagen, Copenhagen, Denmark; and 3Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark

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Christiansen LB, Dela F, Koch J, Hansen CN, Leifsson PS, Yokota T. Impaired cardiac mitochondrial oxidative phosphorylation and enhanced mitochondrial oxidative stress in feline hypertrophic cardiomyopathy. Am J Physiol Heart Circ Physiol 308: H1237–H1247, 2015. First published March 13, 2015; doi:10.1152/ajpheart.00727.2014.—Mitochondrial dysfunction and oxidative stress are important players in the development of various cardiovascular diseases, but their roles in hypertrophic cardiomyopathy (HCM) remain unknown. We examined whether mitochondrial oxidative phosphorylation (OXPHOS) capacity was impaired with enhanced mitochondrial oxidative stress in HCM. Cardiac and skeletal muscles were obtained from 9 domestic cats with spontaneously occurring HCM with preserved left ventricular systolic function and from 15 age-matched control cats. Mitochondrial OXPHOS capacities with nonfatty acid and fatty acid substrates in permeabilized fibers and isolated mitochondria were assessed using high-resolution respirometry. ROS release originating from isolated mitochondria was assessed by spectrophotometry. Thiobarbituric acid-reactive substances were also measured as a marker of oxidative damage. Mitochondrial ADP-stimulated state 3 respiration with complex I-linked nonfatty acid substrates and with fatty acid substrates, respectively, was significantly lower in the hearts of HCM cats compared with control cats. Mitochondrial ROS release during state 3 with complex I-linked substrates and thiobarbituric acid-reactive substances in the heart were significantly increased in cats with HCM. In contrast, there were no significant differences in mitochondrial OXPHOS capacity, mitochondrial ROS release, and oxidative damage in skeletal muscle between groups. Mitochondrial OXPHOS capacity with both nonfatty acid substrates and fatty acid substrates was impaired with increased mitochondrial ROS release in the feline HCM heart. These findings provide new insights into the pathophysiology of HCM and support the hypothesis that restoration of the redox state in the mitochondria is beneficial in the treatment of HCM.

Hypertrophic cardiomyopathy; mitochondria; oxidative stress

Hypertrophic cardiomyopathy (HCM) is a genetic cardiac disease characterized by unexplained left ventricular (LV) hypertrophy and abnormal diastolic function, with a prevalence of 0.2% in the adult general population (33, 53). Although the clinical manifestation is heterogeneous, HCM is a common cause of sudden cardiac death in young people and heart failure in adults (35). It has been demonstrated that mitochondrial dysfunction is involved in various cardiac diseases, including ischemia, dilated cardiomyopathy, and pacing-induced heart failure (27). Noninvasive studies have previously shown that myocardial energy metabolism is impaired in asymptomatic or mildly symptomatic HCM patients (20, 51), indicating that mitochondrial defects might emerge in the early phase of HCM and contribute to disease progression. Moreover, the mitochondria are known as a major source of ROS, which, in excessive amounts, can cause progressive damage to mitochondrial membrane phospholipids, DNA, and proteins (3). Furthermore, ROS act as signaling molecules, stimulating cardiac hypertrophy by activation of kinase signaling pathways (24, 46). Due to the limitation of invasive procedures such as endomyocardial biopsies from human hearts, the function of the mitochondria and the role of oxidative stress in human HCM have only sparsely been reported in the literature (38).

HCM is the most common cardiac disease in domestic cats (11, 42). Among them, gene mutations encoding cardiac myosin-binding protein C (MYBP-C3), which is known as a major mutation site in human HCM, have been found to be associated with HCM in two cat breeds (Maine Coon and Ragdoll cats) (36, 37). In other cat breeds, a genetic cause of HCM remains unknown. Because of their remarkable similarity of clinical and pathological features, cats have been strongly proposed as a useful investigation model for human HCM (12, 22). Although transgenic animal models of HCM have been previously studied (30, 31), there is a void of studies on myocardial mitochondrial function and redox state in spontaneously occurring animal models of HCM whose clinical course strongly resemble that of human HCM.

A recent clinical study (9) has demonstrated that skeletal muscle dysfunction may be related to familial HCM. Skeletal muscle energy metabolism is an important determinant of exercise capacity in patients with chronic heart failure (40). It has been reported that exercise capacity is limited in patients with HCM (13), which raises the possibility that mitochondrial function in skeletal muscle is also impaired in HCM.

Therefore, in the present study, we examined 1) whether capacities of mitochondrial oxidative phosphorylation (OXPHOS) with nonfatty acid substrates and fatty acid substrates were impaired and 2) whether mitochondrial oxidative stress was enhanced in the heart and skeletal muscle of domestic cats with spontaneously occurring HCM.

MATERIALS AND METHODS

Animals

The present study was approved by the Danish Animal Experimental Inspectorate (Authorization No. 2011/561-137) and conformed with EU Directive 2010/63/EU on the protection of animals used for...
scientific purposes. All cats were client-owned animals and were presented to the University Hospital for Companion Animals, University of Copenhagen, through a national screening and breeding program for feline HCM. Cats were enrolled in the study at the time their owners had requested euthanasia of cardiac or noncardiac causes. Written informed consent was obtained from all owners before the study.

Nine adult purebred cats with spontaneously occurring HCM (six male and three female cats, mean age: 6.6 ± 0.8 yr) were enrolled in the study (Table 1). A control group of 15 age-matched cats (4 male and 11 female cats, mean age: 4.9 ± 0.7 yr) of Maine Coon (n = 10) and Norwegian Forest (n = 5) cat breeds with no evidence of cardiac, endocrine, or systemic disease were included in the study. Analysis of these control cats was also conducted to investigate tissue- and substrate-specific mitochondrial bioenergetics in healthy cats (8a).

Cats were defined as HCM positive if the thickness of the LV free wall (LVFW), interventricular septum (IVS), or both in end diastole exceeded 5.5 mm (14, 15) in the absence of differential diagnoses known to cause LV hypertrophy. Cats were defined as HCM negative when the LVFW and IVS were ≤5.0 mm in end diastole (16) and hypertrophy of the papillary muscles was absent. Cats with LVFW or IVS between 5.0 and 5.5 mm in end diastole were classified as equivocal and excluded from the study.

**Determination of Phenotype and Genotype**

General health status was assessed by a thorough clinical examination, measurement of blood pressure, routine hematology, serum biochemistry, and plasma thyroid hormone levels in all cats. Blood pressure was obtained by indirect oscillometry. In HCM cats, a systolic blood pressure of <160 mmHg at the time of initial diagnosis was considered normal (7). Genetic screening was performed on EDTA-stabilized blood from all Maine Coon cats (Laboklin, Bad Kissingen, Germany). Cardiac examination included auscultation, ECG, and echocardiography of all cats and thoracic radiographs where appropriate. Transthoracic echocardiographic measurements were performed using a Vivid 7 Dimension ultrasound unit (GE Healthcare, Horten, Norway) with a 10S multifrequency phased-array transducer (4–11.5 MHz), as previously described (15). LV hypertrophy was categorized as mild, moderate, or severe (5). LV outflow tract obstruction was subjectively evaluated by the presence of turbulent flow with systolic anterior motion of the mitral valve on two-dimensional imaging. Simpon’s method of disks, as previously described in dogs (55), in addition to fractional shortening (FS; in %).

**Tissue Sampling**

Euthanasia was performed by an intramuscular injection of detemordonine (0.04 mg/kg Dexdomitor, Orion Pharma, Turku, Finland) followed by an intravenous injection of pentobarbitone (150 mg/kg). The whole heart was then quickly excised and weighed. The LVFW was divided into four parts. The first part was stored in ice-cold relaxing solution [BIOPS; containing (in mmol/l) 2.77 CaK2EGTA, 7.23 EGTA, 20 tauarine, 6.56 MgCl2, 5.77 ATP, 15 phosphocreatine, 0.5 DTT, and 50 MOPS; pH 7.1] for measurements of mitochondrial OXPHOS and fatty acid oxidation capacities in permeabilized fibers at 37°C (Oxygraph 2k, Oroboros Instruments, Innsbruck, Austria). The second part was stored in an ice-cold isolation medium (containing 100 mmol/l sucrose, 100 mmol/l KCl, 50 mmol/l Tris-HCl, 1 mmol/l KH2PO4, 0.1 mmol/l EGTA, and 0.2% BSA; pH 7.4) for measurements of mitochondrial OXPHOS capacity and mitochondrial ROS release in isolated mitochondria. The third part was stored in 10% neutral buffered formalin for histopathological examination. The fourth remaining part was quickly frozen in liquid nitrogen and stored at −80°C for later analysis of enzymatic activities and oxidative damage. The left soleus skeletal muscle was also quickly excised, treated, and stored in the same way as the heart muscle.

**Pathology**

Formalin-fixed samples of cardiac muscle were processed using routine techniques, and 4- to 5-μm sections were cut and stained with hematoxylin and eosin and with Masson trichrome for the demonstration of collagen.

**Mitochondrial Enzymatic Activities**

Enzymatic activities of citrate synthase (CS) and aconitase, enzymes in the tricarboxylic acid (TCA) cycle, and β-hydroxyacyl-CoA dehydrogenase (HAD), an enzyme of fatty acid β-oxidation, were measured using spectrophotometry.

**Measurements of CS and HAD activities.** For measurements of CS and HAD activities, ~2 mg wet wt of cardiac muscle and 10 mg wet wt of soleus muscle were homogenized in 1.5 ml of 0.3 mol/l K2HPO4 with 0.05% BSA (pH 7.7) for 2 min on a Tissuelyser (Qiagen, Venlo, Limburg, The Netherlands). Fifteen microliters of 10% Triton X-100 was added, and samples were left on ice for 15 min before being stored at −80°C for later analysis.

For CS, the homogenate was diluted 50 times in a solution containing (in mmol/l) 0.4 acetyl-CoA, 0.6 oxaloacetate, 0.157 5,5'-dithiobis-(2-nitrobenzoic acid), and 39 Tris-HCl (pH 8.0). The change of 5,5'-dithiobis-(2-nitrobenzoic acid) to 5-thiobis-(2-nitrobenzoic acid) at 37°C was measured spectrophotometrically at 415 nm (44) on an automatic analyzer (Cobas 6000, C 501, Roche Diagnostics, Mannheim, Germany).

**Table 1. Phenotypic expression of cats with HCM and genotypes of MCO cats with HCM in the present study**

<table>
<thead>
<tr>
<th>Cat</th>
<th>Breed/Genotype</th>
<th>Sex</th>
<th>Age, yr</th>
<th>LV Hypertrophy</th>
<th>LV Outflow Tract Obstruction</th>
<th>Medication</th>
<th>Others</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCM1</td>
<td>MCO/GG</td>
<td>Female</td>
<td>10.3</td>
<td>Mild</td>
<td>-</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>HCM2</td>
<td>MCO(CC</td>
<td>Male</td>
<td>6.2</td>
<td>Severe</td>
<td>-</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>HCM3</td>
<td>MCO/MM</td>
<td>Male</td>
<td>2.2</td>
<td>Severe</td>
<td>+</td>
<td>None</td>
<td>Congestive heart failure</td>
</tr>
<tr>
<td>HCM4</td>
<td>BSH</td>
<td>Male</td>
<td>5.7</td>
<td>Moderate</td>
<td>+</td>
<td>β-Blocker, ACE inhibitor</td>
<td>Atrial fibrillation</td>
</tr>
<tr>
<td>HCM5</td>
<td>ESH</td>
<td>Male</td>
<td>5.9</td>
<td>Moderate</td>
<td>+</td>
<td>β-Blocker</td>
<td></td>
</tr>
<tr>
<td>HCM6</td>
<td>MCO/MM</td>
<td>Female</td>
<td>8.9</td>
<td>Mild</td>
<td>+</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>HCM7</td>
<td>BSH</td>
<td>Male</td>
<td>5.5</td>
<td>Moderate</td>
<td>+</td>
<td>ACE inhibitor</td>
<td></td>
</tr>
<tr>
<td>HCM8</td>
<td>MCO/MM</td>
<td>Female</td>
<td>5.8</td>
<td>Mild</td>
<td>+</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>HCM9</td>
<td>MCO/MM</td>
<td>Male</td>
<td>8.7</td>
<td>Mild</td>
<td>+</td>
<td>Beta-Blocker, ACE inhibitor</td>
<td>Atrial fibrillation</td>
</tr>
</tbody>
</table>

HCM, hypertrophic cardiomyopathy; LV, left ventricular; MCO, Maine Coon cats; GG, wild type negative for A31P myosin-binding protein-C3 mutation; CC, homozygous for the A31P myosin-binding protein-C3 mutation; BSH, British Shorthair cats; ESH, Exotic Shorthair cats; ACE, angiotensin-converting enzyme.
For HAD, the homogenate was diluted 70 times in a solution containing (in mmol/l) 0.33 acetacetyl-CoA, 0.18 NADH, 0.0417 EDTA, and 27.1 imidazole (pH 7.0). Changes in NADH at 37°C were measured spectrophotometrically at 340 nm (4) on an automatic analyzer (Cobas 6000, C 501, Roche Diagnostics).

Measurement of aconitase activity. For measurement of aconitase activity, ~5 mg wet wt of heart muscle was homogenized in 200 µl homogenization buffer (supplied with the Aconitase Assay Kit, Cayman Chemical, Ann Arbor, MI) for 2 min on a Tissuelyser (Qiagen, Hilden, Germany). Homogenates were sonicated at 40 kHz for 1 min and centrifuged at 800 g for 10 min, and supernatants were stored at −80°C for later analysis.

Samples were diluted 31.25 times in 1X assay buffer (supplied with the Aconitase Assay Kit, Cayman Chemical), and 50 µl of the dilutions were added to each well together with NADP⁺ and isocitric dehydrogenase (both supplied with the Aconitase Assay Kit, Cayman Chemical). Each sample was measured in duplicate with and without the addition of sodium citrate to initiate the enzymatic reaction. Finally, the formation of NADPH at 37°C was measured every minute for 30 min at 340 nm using a spectrophotometer (Multiscan FC, Thermo Fisher Scientific, Waltham, MA).

Preparation of Permeabilized Fibers

After careful manual dissection of the tissue, fiber bundles were permeabilized by gentle agitation for 30 min in ice-cold BIOPS solution with 50 µg/ml saponin, as previously described (47). After permeabilization, fibers were rinsed twice by agitation for 10 min in ice-cold respiration medium (MiR05; containing 110 mmol/l solution with 50 ng/ml saponin, as previously described (47). After the addition of permeabilized fibers (~1.5 mg heart muscle) and 200 µl of the following medium: 0.5 mmol/l MgCl₂, 20 mmol/l taurine, 10 mmol/l KH₂PO₄, and 20 mmol/l sucrose, 60 mmol/l K-lactobionate, 0.5 mmol/l EGTA, 0.1% BSA, in ice-cold respiration medium (MiR05; containing 110 mmol/l solution with 50 µl of the dilutions were added to each well together with NADP⁺ and isocitric dehydrogenase (both supplied with the Aconitase Assay Kit, Cayman Chemical). Each sample was measured in duplicate with and without the addition of sodium citrate to initiate the enzymatic reaction. Finally, the formation of NADPH at 37°C was measured every minute for 30 min at 340 nm using a spectrophotometer (Multiscan FC, Thermo Fisher Scientific, Waltham, MA).

Preparation of Isolated Mitochondria

Mitochondria were isolated from heart muscle in five cats with HCM and seven control cats as previously described (49). Briefly, tissue was minced and thoroughly rinsed with ice-cold mitochondrial homogenization buffer (supplied with the Aconitase Assay Kit, Cayman Chemical), and 50 µl of the dilutions were added to each well together with NADP⁺ and isocitric dehydrogenase (both supplied with the Aconitase Assay Kit, Cayman Chemical). Each sample was measured in duplicate with and without the addition of sodium citrate to initiate the enzymatic reaction. Finally, the formation of NADPH at 37°C was measured every minute for 30 min at 340 nm using a spectrophotometer (Multiscan FC, Thermo Fisher Scientific, Waltham, MA).

Mitochondrial ROS Release

Because mitochondrial superoxide (O₂⁻) is a short-lived ROS and unsuitable for continuous measurement of ROS, H₂O₂ release from isolated mitochondria was measured at 37°C using spectrofluorometry (SAFAS Xenius, SAFAS Monaco, Monaco) after the conversion of mitochondrial O₂⁻ into H₂O₂ by the addition of SOD, as previously described (17). H₂O₂ reacts with Amplex red (Molecular Probes, Carlsbad, CA) in a 1:1 stoichiometry catalyzed by horseradish peroxidase, which yields the fluorescent compound resorufin (excitation: 560 nm and emission: 590 nm). Resorufin was continuously monitored throughout the experiment.

Isolated mitochondria were added to 500 µl MiR05 medium with 90 U/ml SOD, 0.05 mmol/l Amplex red, and 12 U/ml horseradish peroxidase in the cuvette. We used the following two protocols.

Protocol 1. In protocol 1, substrates, ADP, and inhibitors were added in the following order: 1) 10 mmol/l pyruvate (complex I-linked substrate) + 2 mmol/l malate, 2) 5 mmol/l ADP, 3) 10 µmol/l cytochrome c, 4) 0.5 µmol/l rotenone (complex I inhibitor), 5) 10 mmol/l succinate, and 6) sequential titration of FCCP (uncoupler) with 0.25 µmol/l in each step.

Mitochondrial OXPHOS Capacity with Fatty Acid Substrates in Permeabilized Fibers

Both a long-chain fatty acid (palmitoyl-l-carnitine) and a medium-chain fatty acid (octanoyl-l-carnitine) were used as substrates. Because excess of fatty acid, especially long-chain fatty acid, has potentially an uncoupling effect on mitochondrial OXPHOS (56), before the study, optimal concentrations of fatty acid were determined in each muscle by the following titration protocols: 0.1 mmol/l (heart muscle) and 0.2 mmol/l (skeletal muscle) palmitoyl-l-carnitine and 0.625 mmol/l (heart muscle) and 1.25 mmol/l (skeletal muscle) octanoyl-l-carnitine.

Substrates, ADP, and inhibitors were added in the following order: 1) fatty acid (palmitoyl-l-carnitine or octanoyl-l-carnitine) + 2 mmol/l malate, 2) 5 mmol/l ADP, 3) 10 µmol/l cytochrome c, 4) 0.5 µmol/l rotenone (complex I inhibitor), 5) 10 mmol/l succinate, and 6) sequential titration of FCCP (uncoupler) with 0.25 µmol/l in each step.

Mitochondrial OXPHOS Capacity with Fatty Acid Substrates in Permeabilized Fibers

After the addition of permeabilized fibers (~1.5 mg heart muscle and 2.5 mg soleus muscle) to the chamber in the respirometer, substrates, ADP, and inhibitors were added in the following order: 1) 10 mmol/l glutamate + 2 mmol/l malate (complex I-linked substrates), 2) 5 mmol/l ADP, 3) 10 µmol/l cytochrome c, 4) 0.5 mmol/l succinate (complex II-linked substrate), 5) 2 µg/ml oligomycin (complex V inhibitor), 6) 2.5 mmol/l antimycin A (complex III inhibitor), and 7) 0.5 mmol/l N,N,N',N'-tetramethyl-p-phenylenediamine + 2 mmol/l ascorbate (complex IV-linked substrates). All measurements were carried out in duplicate. The integrity of the outer mitochondrial membrane was tested by the addition of cytochrome c, and data were eliminated when the increase in O₂ consumption rate was >10% as a sign of a damaged outer mitochondrial membrane. Respiratory rates (i.e., O₂ consumption rates) are expressed as O₂ flux normalized to muscle weight (in pmol s⁻¹ mg wet wt muscle tissue⁻¹). The respi-
Table 2. Baseline characteristics of the cats

<table>
<thead>
<tr>
<th></th>
<th>Control Cats</th>
<th>HCM Cats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of cats</td>
<td>15</td>
<td>9</td>
</tr>
<tr>
<td>Age, years</td>
<td>4.9 ± 0.7</td>
<td>6.6 ± 0.8</td>
</tr>
<tr>
<td>Sex, male castrated/female castrated/female intact</td>
<td>4/2/9</td>
<td>6/3/0</td>
</tr>
<tr>
<td>Body weight, kg</td>
<td>5.3 ± 0.3</td>
<td>5.7 ± 0.3</td>
</tr>
<tr>
<td>Heart weight, g</td>
<td>17.1 ± 1.0</td>
<td>25.6 ± 2.4*</td>
</tr>
<tr>
<td>Heart weight/body weight, g/kg</td>
<td>3.29 ± 0.11</td>
<td>4.50 ± 0.33*</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE. *P < 0.05 vs. control cats.

Statistical Analysis

All data are expressed as means ± SE. Student’s t-test was performed to compare means between cats with HCM and control cats. All statistical analyses were performed using GraphPad Prism (version 6.0, GraphPad Software, La Jolla, CA). P values of < 0.05 were considered statistically significant.

RESULTS

Clinical Characteristics and Echocardiographic Data

HCM cats and control cats were comparable with respect to age and body weight (Table 2). The heart weight-to-body weight ratio was significantly higher in cats with HCM compared with control cats (Table 2). End-diastolic thickness of the LVFW and IVS was significantly higher in cats with HCM compared with control cats (Table 3), indicating LV hypertrophy. The left atrium-to-aorta ratio was also significantly increased in HCM cats (Table 3). There were no significant differences in FS between HCM and control cats, and EF was significantly higher in HCM cats (Table 3). Moreover, all cats had normal FS and EF, indicating preserved LV systolic function.

Four of five Maine Coon cats with HCM were homozygous for the A31P mutation in MYBP-C3 (Table 1), and one cat was wild type negative. In the control group, Maine Coon cats were negative (n = 9) or heterozygous (n = 1) for the A31P mutation.

Histopathology

On histopathological examination, secondary changes, including perimysial and endomyosial fibrosis, enlarged nuclei and cardiomyocytes, and intracellular fat vacuolization, were present in the hearts of HCM cats. Myocyte disarray was an inconsistent finding, present only in severely hypertrophied HCM hearts (Fig. 1).

Mitochondrial Enzymatic Activities

Enzymatic activities of CS and HAD in cardiac muscle, but not in skeletal muscle, were significantly decreased in HCM cats compared with control cats (Fig. 2, A and B). However, there was no significant difference in the HAD-to-CS ratio between HCM cats and control cats in cardiac muscle (0.63 ± 0.07 vs. 0.75 ± 0.04) and in skeletal muscle (0.95 ± 0.05 vs. 1.00 ± 0.04). Moreover, the enzymatic activity of aconitate did not differ in both cardiac and skeletal muscle between HCM and control cats (Fig. 2C).

Mitochondrial OXPHOS Capacity With Nonfatty Acid Substrates in Permeabilized Fibers

In cardiac muscle, non-ADP-stimulated resting respiration (LeakN with glutamate and malate normalized to muscle mass was lower in HCM cats than in control cats (12.0 ± 1.7 vs. 21.0 ± 1.6 pmol·s⁻¹·mg wet wt⁻¹, P < 0.05). However, there was no significant difference in LeakN with these substrates between HCM and control cats after normalization to CS activity (0.098 ± 0.02 vs. 0.12 ± 0.009 pmol·s⁻¹·CS activity⁻¹). ADP-stimulated state 3 respiration with glutamate and malate normalized to muscle mass in the heart was also significantly decreased in HCM cats, which was consistent with lower RCR in HCM cats compared with control cats (Fig. 3A), indicating impaired complex I-linked mitochondrial OXPHOS capacity. State 3 respiration with glutamate and malate remained significantly reduced in HCM hearts, even after normalization to CS activity (Fig. 3A). State 3 respiration with complex I + II-linked substrates, including glutamate, malate, and succinate, tended to be lower in hearts from HCM cats (Fig. 3B). There was no significant difference in complex IV capacity in the hearts (Fig. 3C). Moreover, respiration with oligomycin (Leakomy) and residual O2 consumption with antimycin A in the heart was comparable between HCM and control cats (Leakomy: 61.6 ± 5.1 vs. 67.1 ± 3.3 pmol·s⁻¹·mg wet wt⁻¹ and residual O2 consumption: 14.9 ± 1.5 vs. 17.6 ± 0.9 pmol·s⁻¹·mg wet wt⁻¹). Because in permeabilized cardiac muscle fiber from cats, Leakomy, which was thought to be close to LeakN, was much higher than LeakN, we calculated RCR as state 3/LeakN instead of state 3/Leakomy. In contrast, there was no significant difference between HCM and control cats in mitochondrial OXPHOS capacity with nonfatty acid substrates in skeletal muscle (Fig. 3, A–C).

Mitochondrial OXPHOS Capacity With Fatty Acid Substrates in Permeabilized Fibers

In cardiac muscle, LeakN with malate and long-chain or medium-chain fatty acids was significantly decreased in HCM cats compared with control cats (palmitoyl-L-carnitine: 8.9 ± 1.6 vs. 14.7 ± 0.8 pmol·s⁻¹·mg wet wt⁻¹, P < 0.05, and octanoyl-L-carnitine: 9.9 ± 1.6 vs. 15.4 ± 1.6 pmol·s⁻¹·mg wet wt⁻¹, P < 0.05). State 3 respiration with malate and fatty acids, in which complex I and electron transfer flavoprotein are the main acceptors for reducing equivalents, was also significantly decreased in hearts from HCM cats (Fig. 3, D and E), and it remained significantly decreased when normalized to CS activity (Fig. 3, D and E). In line with the results of state 3 respiration, RCR with malate and fatty acids in the heart was significantly decreased in HCM cats compared with control cats (Table 3).

Table 3. Summary of echocardiographic data

<table>
<thead>
<tr>
<th></th>
<th>Control Cats</th>
<th>HCM Cats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of cats</td>
<td>15</td>
<td>9</td>
</tr>
<tr>
<td>Left atrium-to-aorta ratio</td>
<td>1.18 ± 0.03</td>
<td>1.94 ± 0.26*</td>
</tr>
<tr>
<td>Intraventricular septal end-diastolic thickness, mm</td>
<td>4.0 ± 0.1</td>
<td>6.5 ± 0.3*</td>
</tr>
<tr>
<td>LV free wall end-diastolic thickness, mm</td>
<td>3.9 ± 0.1</td>
<td>7.5 ± 0.6*</td>
</tr>
<tr>
<td>LV end-diastolic diameter, mm</td>
<td>14.8 ± 0.5</td>
<td>14.1 ± 0.6</td>
</tr>
<tr>
<td>Fractional shortening, %</td>
<td>42.0 ± 2.6</td>
<td>47.7 ± 2.7</td>
</tr>
<tr>
<td>Ejection fraction, %</td>
<td>69.3 ± 2.0</td>
<td>78.1 ± 7.9*</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE. *P < 0.05 vs. control cats.
lower in HCM cats than in control cats (palmitoyl-L-carnitine: 1.3 ± 0.1 vs. 2.1 ± 0.1, P < 0.05, and octanoyl-L-carnitine: 1.9 ± 0.2 vs. 3.0 ± 0.2, P < 0.05). Moreover, complex II-linked state 3 respiration with succinate and octanoyl-L-carnitine, but not with succinate and palmitoyl-L-carnitine, was significantly decreased in hearts from HCM cats (Fig. 3, F and G). In skeletal muscle, there was no significant difference in mitochondrial OXPHOS capacity with fatty acid substrates (Fig. 4, D–G). We finally added FCCP, an uncoupler, by titration to evaluate the maximal capacity of the electron transfer system (ETS). However, there was no additional increase in O2 consumption rate in the heart and skeletal muscles, respectively (data not shown), indicating that mitochondrial OXPHOS capacity with fatty acids, malate, and succinate was almost maximally activated in these muscles from cats (i.e., there was no excess capacity).

Mitochondrial OXPHOS Capacity in Isolated Mitochondria

In cardiac muscle, mitochondrial state 3 respiration with malate and pyruvate was significantly decreased in cats with HCM compared with control cats (Fig. 4A), indicating that complex I-linked OXPHOS capacity was impaired in isolated mitochondria from HCM hearts. State 2 (Fig. 4B) and state 4 (data not shown) were comparable between HCM cats and control cats. RCR with malate and pyruvate tended to be lower in HCM hearts (Fig. 4C). In contrast, there was no significant difference in mitochondrial OXPHOS capacity with malate and pyruvate in isolated mitochondria from skeletal muscle between the groups (Fig. 5).

Mitochondrial ROS Release

Mitochondrial ROS release during state 3 with malate and pyruvate (complex I-linked substrates) was significantly higher
in hearts from HCM cats than from control cats (Fig. 5A). Moreover, HCM hearts had a higher mitochondrial ROS release per mitochondrial O₂ consumption during state 3 with these substrates (Fig. 5B). During state 2 and state 4 with these substrates, there was no difference in cardiac mitochondrial ROS release between groups (data not shown). Mitochondrial ROS release with succinate (complex II-linked substrate) and with rotenone was comparable between the groups (Fig. 5C). In skeletal muscle, mitochondrial ROS release with complex I-linked and complex II-linked substrates, respectively, was similar between groups (Fig. 5, A and C).

Oxidative Damage

In cardiac muscle, TBARS were significantly higher in HCM cats than in control cats (Fig. 5D), indicating greater oxidative damage in HCM hearts. In contrast, in skeletal muscle, TBARS were similar between groups (Fig. 5D).

DISCUSSION

A major novel finding of the present study is that spontaneously occurring HCM cats with preserved LV systolic function had an impaired mitochondrial OXPHOS capacity, specifically in the myocardium. Moreover, ROS release, originating from isolated mitochondria during ADP-stimulated state 3 with complex I-linked substrates, was greater in HCM hearts than in normal hearts. In accordance with increased mitochondrial ROS release, myocardial oxidative damage, as assessed by lipid peroxides, was higher in HCM cats. In contrast, there were no significant differences in mitochondrial function and mitochondrial oxidative stress in skeletal muscle. These findings support the hypothesis that myocardial mitochondrial defects and mitochondrial oxidative stress are molecular mechanisms contributing to the development and progression of HCM.

Advantage of Using an Animal Model of Spontaneously Occurring HCM

In the present study, we investigated the pathophysiology of HCM, focusing on mitochondrial respiratory capacity and oxidative stress in domestic cats with spontaneously occurring HCM and without comorbidities. In clinical studies, there is a limitation on performing such invasive diagnostic procedures as endomyocardial biopsy, making it difficult to conduct a thorough investigation of myocardial mitochondrial function and redox balance in HCM.

In animal studies, transgenic cardiac hypertrophy models are often used to elucidate the pathogenesis of HCM. Until now, disease-causing mutations in at least nine genes encoding sarcomeric proteins, including MYBP-C3, have been found in up to two-thirds of patients with HCM (54). However, there are still 25–35% of HCM patients with undetected gene mutation or unknown causes (43). Therefore, there may be an advantage in using cats with spontaneously occurring HCM, whose natural history and clinical features closely resemble those of human HCM, to investigate the pathophysiology of HCM.

Abnormal Mitochondrial Fatty Acid Metabolism in the HCM Heart

In the healthy heart, the primary energy sources are fatty acids, and 60–70% of ATP comes from mitochondrial fatty acid oxidation (39). Previous studies (41, 48) have shown that myocardial uptake of free fatty acids, as measured by 123I-labeled 15-(p-iodophenyl)-3-((R,S)-methylpentadecanoic acid, is reduced in patients with HCM (41, 48). Here, we demon-

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Fig. 3. Mitochondrial oxidative phosphorylation (OXPHOS) capacity in permeabilized heart and SKM fibers from control cats (n = 15) and HCM cats (n = 9). Mitochondrial OXPHOS capacity was normalized to muscle mass (left) and CS activity (right), respectively. A–G; state 3 respiration and respiratory control ratio (RCR) with complex I-linked substrates [glutamate + malate (Mal); A], state 3 respiration with complex I + II-linked substrates [glutamate + Mal + succinate (Suc); B], complex IV capacity induced by the artificial electron donors ascorbate and N,N,N',N'-tetramethyl-p-phenyldiamine (C), state 3 respiration with electron transfer flavoprotein (ETF) and complex I-linked substrates [palmitoyl-l-carnitine (Pal) + Mal; D], state 3 respiration with ETF and complex I-linked substrates [octanoyl-l-carnitine (Oct) + Mal; E], state 3 respiration with complex II-linked substrates [Pal + Mal + Suc + rotenone (Rot); F], and state 3 respiration with complex II-linked substrates (Oct + Mal + Suc + Rot; G). All data are expressed as means ± SE. *P < 0.05 vs. control cats.
strated that myocardial mitochondrial OXPHOS capacity with both long-chain and medium-chain fatty acids and HAD activity were severely depressed in cats with HCM, which suggests that fatty acid metabolism in the HCM heart is impaired via not only reduced uptake of fatty acids but also via limited capacity of fatty acid \( \beta \)-oxidation and mitochondrial OXPHOS. In addition, complex II-linked state 3 respiration with succinate and each fatty acid had different results, which depends on the kinds of fatty acid. This difference may be attributable to different uptake of fatty acid into the mitochondria, because palmitoyl-L-carnitine, not octanoyl-L-carnitine, is dependent on carnitine palmitoyl transferases I and II, which are located on mitochondrial membranes.

In HCM, gene mutations in sarcomeric protein can increase \( \text{Ca}^{2+} \) sensitivity, ATPase activity, and the energetic cost of myocyte contraction, which may lead to excessive myocardial energy demands (2). Fatty acids are inherently energy-rich substrates, generating more ATP per gram substrate than glucose, however, at the expense of high amounts of \( \text{O}_2 \) (45). Taken together, limited myocardial fatty acid metabolism may be a compensatory change as a metabolic adaptation to meet a higher energy demand under \( \text{O}_2 \)-limited conditions in the HCM heart. Indeed, a recent randomized clinical trial has showed that perhexiline, an agent considered to shift substrate use from fatty acids to glucose via partial inhibition of fatty acid oxidation, improved cardiac high-energy phosphate metabolism and exercise capacity in patients with symptomatic HCM (1).

**Impaired Mitochondrial OXPHOS Capacity in the HCM Heart**

In the present study, myocardial mitochondrial OXPHOS capacity with nonfatty acid substrates, complex I-linked substrates, was also impaired in cats with HCM, which is consistent with a previous study (31) in a transgenic mouse model of HCM. Moreover, activity of CS (an enzyme of the TCA in the mitochondrial matrix) in the heart was decreased in HCM cats. A previous study (21) has demonstrated that CS activity was decreased in patients with chronic heart failure due to HCM; however, to our knowledge, this is the report of early manifestation of lowered CS activity in HCM with preserved LV...
systolic function. It is well known that CS activity strongly correlates with mitochondrial content (25). Accordingly, decreased mitochondrial content may affect mitochondrial dysfunction in HCM hearts as well as lower OXPHOS capacity per mitochondrion. As >90% of ATP generation relies on mitochondrial OXPHOS in the heart, the HCM heart may be in an energy-compromised state, even if substrate utilization is shifted from fatty acids to glucose. The notion of energy depletion in the HCM heart is also supported by a previous study (20) of 31P magnetic resonance spectroscopy, which demonstrated abnormal cardiac muscle energy metabolism (i.e., lower ratio of phosphocreatine to ATP) in patients with asymptomatic HCM.

Enhanced Mitochondrial Oxidative Stress in the HCM Heart

It has been reported that systemic oxidative stress, evaluated by serum levels of 8-iso-PGF_2α, is enhanced in patients with HCM (10). Furthermore, oxidative damage in the right ventricular side of the septum has been shown to be increased in patients with HCM (38), which is consistent with our findings of higher myocardial TBARS in cats with HCM. In animal studies (30, 32), the antioxidant N-acetylcysteine could reverse myocardial hypertrophy or interstitial fibrosis in mouse or rabbit models of human HCM. Therefore, oxidative stress may be involved in the pathogenesis or disease progression of HCM. However, the precise mechanism of enhanced oxidative stress, including the source of ROS, in HCM has not yet been fully identified.

In the present study, we found that mitochondrial ROS release during ADP-stimulated state 3 with complex I-linked substrates in the heart was increased in association with impaired mitochondrial OXPHOS capacity with these substrates in cats with HCM. Mitochondria are the major source of ROS as a byproduct of OXPHOS, induced by a reduction of O_2 to O_2^- at electron transport chain complexes via electron leakage (23). In particular, damaged mitochondria can release excessive ROS, and complex I has been shown to be a major source of ROS in failing hearts (18). Moreover, we demonstrated higher mitochondrial ROS release/O_2 consumption during state 3 with complex I-linked substrates in HCM heart, which raises the possibility that an inefficient and higher energy demand in the HCM heart may influence the greater ROS release from the mitochondria due to constantly activated mitochondrial OXPHOS in HCM, although we evaluated merely ex vivo mitochondrial ROS release. Moreover, by their proximity to mitochondrial ROS, mitochondrial proteins, phospholipids, and DNA are targets of oxidative damage, which may result in creating a vicious cycle in the mitochondria (3). In the present study, the enzymatic activity of aconitase in the TCA cycle, which is known as one of the markers of mitochondrial oxidative damage due to containing iron-sulfur proteins (50), was not significantly decreased in HCM hearts. The unchanged aconitase activity suggests that we might observe early stage of mitochondrial oxidative damage before aconitase activity is impaired as all cats with HCM had preserved LV systolic function, and except one HCM cat, were asymptomatic. Another possible explanation of unchanged aconitase activity in HCM hearts is that frataxin, an iron-binding protein, may interact with aconitase and prevent decreases in aconitase activity (8). Although we could not identify whether increased release of mitochondrial ROS was a cause or consequence of mitochondrial defects in the HCM heart, the potential link between mitochondrial ROS and impaired mitochondrial OXPHOS capacity may, at least in part, contribute to the disease progression in HCM.

Other Possible Sources of Enhanced Oxidative Stress in the HCM Heart

In addition to ROS originating from the mitochondria, other sources of ROS, such as NAD(P)H oxidase and xanthine oxidase, may be involved in the enhanced oxidative stress in HCM hearts, as previous studies (34, 57) have demonstrated their role in the development of LV hypertrophy in pressure overload- or drug-induced animal models, which should be further explored in an animal model of HCM or patients with HCM. Moreover, in general, oxidative stress is determined by an imbalance between ROS and antioxidant-defense capacity, and it has been reported that the activity of catalase, an antioxidant, is decreased in hearts from a porcine model of spontaneously occurring HCM (28), which suggests that lower antioxidant capacity may also contribute to the enhanced oxidative stress in HCM hearts.

No Excess Capacity of the ETS With Complex II-Linked Substrates in Feline Cardiac and Skeletal Muscle

FCCP titration was performed to evaluate ETS (noncoupled) capacity after the addition of complex II-linked substrates in cardiac and skeletal muscles from cats. In clinical studies (6, 26), ETS capacity exceeds OXPHOS capacity with complex I- or complex I + II-linked substrates in cardiac and skeletal muscles. However, in the present study, ETS capacity did not exceed OXPHOS capacity with complex II-linked substrates in cardiac and skeletal muscles not only from control cats (8a) but also from HCM cats, suggesting that there was no excess capacity of the ETS in these muscles from cats. One of the possible explanations of this difference between human and feline muscles is due to a difference in species, but another one is that we evaluated ETS capacity with complex II-linked substrates, not complex I+II-linked substrates, in feline muscles, which can lead to an underestimation of maximal ETS capacity. Therefore, we cannot completely exclude the possibility of excess capacity of the ETS when it is evaluated with complex I + II-linked substrates in feline muscles.

Limitations

There are several limitations that should be acknowledged. First, the sex and breed of cats were not matched between groups. However, all male cats were castrated, and there was no specific difference in body shape, daily physical activity, and cardiac and skeletal muscle function between breeds of cats. Second, four of nine cats with HCM received angiotensin-converting enzyme inhibitors and/or β-blockers, which may influence mitochondrial function and oxidative stress levels; however, there was a statistically significant difference in these parameters, despite medication. Third, we did not measure mitochondrial membrane potential (ΔΨ_m). Although there is ΔΨ_m-independent ROS release as well as ΔΨ_m-dependent ROS release (52), we could not completely exclude the possibility that higher ΔΨ_m due to the decreased OXPHOS may contribute to the higher mitochondrial ROS release during state.
3. With complex I–linked substrates in feline HCM hearts. Finally, we evaluated skeletal muscle function using the soleus muscle, most of which consists of slow-twitch type-I muscle fibers. Although there were no significant differences in mitochondrial function and oxidative stress in the soleus muscle between groups, we could not completely exclude the possibility of any difference in skeletal muscle function in other muscle types, including type II-rich fibers.

Conclusions

We observed impaired mitochondrial OXPHOS capacity with both nonfatty acid and fatty acid substrates and increased release of mitochondrial ROS in hearts from cats with spontaneously occurring HCM with preserved LV systolic function. Mitochondrial defects and mitochondrial oxidative stress may contribute to the pathogenesis and disease progression in HCM. Our findings support the possibility of a potential target of mitochondrial ROS, such as mitochondria-targeted antioxidant drugs, in the prevention or treatment of HCM.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES

The image contains a page from a scientific paper. The page number and citation details are not visible. The text appears to be a discussion on mitochondrial defects and oxidative stress in hypertrophic cardiomyopathy (HCM), with references to various studies on the topic. The paper cites numerous authors and sources, indicating a comprehensive review of the subject. The text is formatted in a standard academic style, typical of a scientific journal, with references formatted in APA style.

The content includes discussions on the role of antioxidant enzymes in naturally occurring hypertrophic cardiomyopathy, resolution of established cardiac hypertrophy and fibrosis, and prevention of systolic dysfunction in a transgenic rabbit model of human cardiomyopathy through thiol-sensitive mechanisms. It also mentions the antioxidant effects of N-acetylcysteine in a mouse model of hypertrophic cardiomyopathy mutation, and the prevention of oxidative stress in the nucleus caused by Nox4 via mediation of HDAC4 and cardiac hypertrophy.

The text references a wide range of sources, including peer-reviewed articles, books, and reviews, covering topics such as mitochondrial dysfunction, inflammation, oxidative stress, and cellular metabolism in the context of HCM. The references include journals like "Journal of the American College of Cardiology" (JACC), "Circulation", "Biochemical and Biophysical Research Communications" (BBRC), and "American Journal of Physiology - Heart and Circulatory Physiology" (AJP-Heart). The authors cited range from Lin CS, Liu CY, Sun YL, Chang LC, Chiu YT, Huang SY, Lin JH, Lesnefsky EJ, Moghaddas S, Tandler B, Kerner J, Hoppel CL, Lombardi R, Rodriguez G, Chen SN, Ripplinger CM, Li W, Chen J, Lucas DT, Aryal P, Szweda LI, Koch WJ, Leinwand LA, and Maron BJ.

The text is a comprehensive discussion on the mechanisms and implications of mitochondrial defects and oxidative stress in HCM, providing a detailed overview of the current understanding and research in the field.