Idebenone delays the onset of cardiac functional alteration without correction of Fe-S enzymes deficit in a mouse model for Friedreich ataxia

Hervé Séznec¹, Delphine Simon¹, Laurent Monassier², Paola Criqui-Filipe¹, Anne Gansmuller¹, Pierre Rustin³, Michel Koenig¹ and Hélène Puccio¹,*

¹Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC), CNRS/INSERM/Université Louis Pasteur, 67404 Illkirch cedex, CU de Strasbourg, France, ²Laboratoire de Neurobiologie et Pharmacologie Cardiovasculaire, INSERM E333, Faculté de Médecine, 67085 Strasbourg cedex, France and ³Unité de Recherche sur les Handicaps Génétiques de l’Enfant, INSERM U393, Hôpital Necker-Enfants Malades, 75015 Paris, France

Received January 8, 2004; Revised February 27, 2004; Accepted March 10, 2004

Friedreich ataxia (FRDA), a progressive neurodegenerative disorder associated with cardiomyopathy, is caused by severely reduced frataxin, a mitochondrial protein involved in Fe-S cluster assembly. We have recently generated mouse models that reproduce important progressive pathological and biochemical features of the human disease. Our frataxin-deficient mouse models initially demonstrate time-dependent intramitochondrial iron accumulation, which occurs after onset of the pathology and after inactivation of the Fe-S dependent enzymes. Here, we report a more detailed pathophysiological characterization of our mouse model with isolated cardiac disease by echocardiographic, biochemical and histological studies and its use for placebo-controlled therapeutic trial with Idebenone. The Fe-S enzyme deficiency occurs at 4 weeks of age, prior to cardiac dilatation and concomitant development of left ventricular hypertrophy, while the mitochondrial iron accumulation occurs at a terminal stage. From 7 weeks onward, Fe-S enzyme activities are strongly decreased and are associated with lower levels of oxidative stress markers, as a consequence of reduced respiratory chain activity. Furthermore, we demonstrate that the antioxidant Idebenone delays the cardiac disease onset, progression and death of frataxin deficient animals by 1 week, but does not correct the Fe-S enzyme deficiency. Our results support the view that frataxin is a necessary, albeit non-essential, component of the Fe-S cluster biogenesis, and indicate that Idebenone acts downstream of the primary Fe-S enzyme deficit. Furthermore, our results demonstrate that Idebenone is cardioprotective even in the context of a complete lack of frataxin, which further supports its utilization for the treatment of FRDA.

INTRODUCTION

Friedreich ataxia (FRDA), the most common form of autosomal recessive ataxia, is a neurodegenerative disease associated with increased incidence of diabetes and cardiomyopathy (1–4) which contributes to premature death as a consequence of arrhythmias and cardiac failure. The cardiac disease is characterized by a concentric hypertrophy of the ventricles (62%) or asymmetric septal hypertrophy (29%) along with diastolic function abnormalities (5). As the disease progresses, there is a natural transition from hypertrophy to dilatation, and organ failure. All these alterations are the consequence of severely reduced levels of the mitochondrial protein frataxin due to a large GAA repeat expansion within the first intron of the frataxin gene (6), causing inhibition of transcriptional elongation. Based on human, yeast and mouse data, the inferred frataxin function is closely involved in the early step of the iron-sulfur (Fe-S) cluster biosynthesis (7). Impairment in Fe-S cluster biosynthesis is generally associated with iron homeostasis and oxidative stress signalling alterations.

In FRDA patients, the pathogenic importance of oxidative stress was widely suspected, with elevated levels of oxidative stress markers, urine 8-hydroxy-2'-deoxyguanosine (8) and serum malondialdehyde (9) (indicative of DNA damage and lipid peroxidation, respectively). In addition, an impaired
response to oxidative challenge has been reported in cell lines derived from FRDA patients (10). This has therefore drawn attention to the development of pharmacological approaches that may alleviate the cellular damage caused by oxidative stress. Idebenone is a short-chain synthetic analogue of coenzyme Q10 that can function as an electron carrier in the mitochondrial respiratory chain and act as a potent free-radical scavenger both in vitro and in vivo (11,12).

Based on in vitro experiments carried out on human heart homogenates showing the ability of Idebenone to protect against iron-induced lipid peroxidation and Fe-S inactivation (8,9), this drug was initially given to three FRDA patients and found to significantly improve their cardiac function (13). This initial study was further substantiated with a second open trial (40 patients over 2 years), which showed a 20–40% reduction of heart hypertrophy in half of the treated patients (14). During the last 3–5 years, many other therapeutic trials have assessed the clinical value of Idebenone (8,9,13–16), but the results remain controversial, mainly due to the clinical heterogeneity of the disease, and the lack of randomized placebo-controlled studies. In view of the many methodological difficulties in evaluating the effects of Idebenone in patients, the recent availability of mouse models give us a unique opportunity to evaluate the actual protective effect of Idebenone.

Since total disruption of the mouse frataxin gene generates embryonic lethal homozygotes (17) and knock-in intrinsic GAA expansions (18) and hypomorphic mutations in the frataxin gene may not result in a diseased phenotype during the comparatively short life-span of mice, we developed a mouse model with a tissue-targeted complete frataxin deficiency that resulted in a rapidly progressive disease (19). One of our models made use of the Cre/Lox mediated excision system under the control of the muscle creatine kinase promoter (MCK), allowing complete deletion of the frataxin gene in striated muscles by the Cre recombinase. This resulted in a pure cardiac phenotype, since skeletal muscles revealed no pathology, as in the human disease. This cardiac model (Frda/MCK mutant) recapitulates the major histological and biochemical features of the human disease (19), and therefore represents a valuable tool for treatment strategies. The aims of this study were: (i) to better understand the cardiac disease associated with FRDA by detailed functional, ultrastructural and biochemical investigations; and (ii) to carry out a placebo-controlled trial with Idebenone in order to test its cardioprotective effect in the context of total frataxin deficiency.

RESULTS

Frda/MCK mice develop a hypertrophic cardiomyopathy associated with a geometric remodelling

We previously reported the development of a hypertrophic cardiomyopathy which naturally evolved into a dilated cardiomyopathy in the Frda/MCK mutants based on histological data (19). To further assess the cardiac function and the evolution of the cardiac disease, detailed echocardiographic studies were performed in conscious mice between ages 3 and 8 weeks (Table 1). From 5 weeks and throughout the disease progression, Frda/MCK mutant hearts exhibited an increase of the left ventricle diastolic (LVEDD) and systolic (LVESD) diameters, indicating a dilatation of the heart (Table 1). This dilatation was associated with increased left ventricular posterior and septum wall thickness, which, together with the left ventricular enlargement, resulted in an increase of the left ventricular mass (LVM) (Table 1). Taken together, these data indicate that Frda/MCK mutant mice develop a hypertrophic cardiomyopathy, rapidly associated with an important geometric remodelling (Fig. 1A and B). In parallel, a reduction of the shortening (SF) and ejection (EF) fractions of mutants and control: *p < 0.05; **p < 0.01; ***p < 0.001; and between KO + ID and KO *p < 0.05; **p < 0.01 (ANOVA followed by Fisher and Scheffé tests).

Table 1. Echocardiographic parameters of cardiac-specific frataxin deleted Frda/MCK mutant mice and Idebenone-treated mutants

<table>
<thead>
<tr>
<th></th>
<th>LVEDD (mm)</th>
<th>LVESD (mm)</th>
<th>SF (%)</th>
<th>LVM (mg)</th>
<th>CO (ml/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctrl (n = 6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 4</td>
<td>2.9 ± 0.1</td>
<td>1.5 ± 0.2</td>
<td>50 ± 6</td>
<td>37 ± 5</td>
<td>44 ± 13</td>
</tr>
<tr>
<td>Week 5</td>
<td>2.7 ± 0.1</td>
<td>1.4 ± 0.1</td>
<td>50 ± 3</td>
<td>48 ± 6</td>
<td>66 ± 18</td>
</tr>
<tr>
<td>Week 6</td>
<td>3.2 ± 0.2</td>
<td>1.5 ± 0.2</td>
<td>50 ± 3</td>
<td>61 ± 6</td>
<td>56 ± 18</td>
</tr>
<tr>
<td>Week 7</td>
<td>2.6 ± 0.1</td>
<td>1.3 ± 0.2</td>
<td>51 ± 5</td>
<td>42 ± 3</td>
<td>67 ± 25</td>
</tr>
<tr>
<td>Week 8</td>
<td>2.9 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>59 ± 3</td>
<td>46 ± 4</td>
<td>63 ± 24</td>
</tr>
<tr>
<td>KO (n = 6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 4</td>
<td>3.1 ± 0.1</td>
<td>1.8 ± 0.2</td>
<td>43 ± 4</td>
<td>46 ± 7</td>
<td>52 ± 25</td>
</tr>
<tr>
<td>Week 5</td>
<td>3.3 ± 0.1*</td>
<td>2 ± 0.1†</td>
<td>38 ± 3†</td>
<td>85 ± 8†</td>
<td>59 ± 17</td>
</tr>
<tr>
<td>Week 6</td>
<td>3.8 ± 0.2‡</td>
<td>2.7 ± 0.2‡</td>
<td>31 ± 3‡</td>
<td>100 ± 8‡</td>
<td>45 ± 9‡</td>
</tr>
<tr>
<td>Week 7</td>
<td>4.4 ± 0.3‡</td>
<td>3.8 ± 0.4‡</td>
<td>17 ± 3‡</td>
<td>125 ± 18‡</td>
<td>38 ± 17‡</td>
</tr>
<tr>
<td>Week 8</td>
<td>4.9 ± 0.3‡</td>
<td>4.1 ± 0.4‡</td>
<td>16 ± 3‡</td>
<td>138 ± 17‡</td>
<td>22 ± 12‡</td>
</tr>
<tr>
<td>KO + ID (n = 6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 4</td>
<td>3 ± 0.1</td>
<td>1.5 ± 0.1</td>
<td>50 ± 3</td>
<td>46 ± 4</td>
<td>52 ± 27</td>
</tr>
<tr>
<td>Week 5</td>
<td>3.1 ± 0.2</td>
<td>1.6 ± 0.1</td>
<td>50 ± 2†</td>
<td>71 ± 8</td>
<td>65 ± 8</td>
</tr>
<tr>
<td>Week 6</td>
<td>3.2 ± 0.1†</td>
<td>1.8 ± 0.1†</td>
<td>43 ± 1†</td>
<td>76 ± 5†</td>
<td>52 ± 15</td>
</tr>
<tr>
<td>Week 7</td>
<td>3.8 ± 0.3</td>
<td>3.3 ± 0.3</td>
<td>23 ± 2</td>
<td>92 ± 15</td>
<td>41 ± 9</td>
</tr>
<tr>
<td>Week 8</td>
<td>4.2 ± 0.4</td>
<td>3.4 ± 0.6</td>
<td>24 ± 9</td>
<td>106 ± 16</td>
<td>37 ± 22</td>
</tr>
</tbody>
</table>

Ctrl: control littermate mice; KO: Frda/MCK mutant mice; KO+ID: Frda/MCK mutant mice treated with Idebenone (90 mg/kg/d from 3 to 9 weeks) (six animals/group); LVEDD and LVESD, left ventricular cross sectional internal diameters in end-diastole and in end-systole, respectively; SF, shortening fraction; LVM, left ventricular mass; CO, cardiac output. Statistical significance between KO and control: *p < 0.05; **p < 0.01; ***p < 0.001; and between KO + ID and KO *p < 0.05; **p < 0.01 (ANOVA followed by Fisher and Scheffé tests).

Fe-S enzyme deficiency precedes the physiological cardiac dysfunction in Frda/MCK mice

Biochemical analysis of the cardiac tissues revealed that the Fe-S enzyme deficiency in the Frda/MCK mutants is detectable...
early with 49 ± 16% reduction of complex II activities at 4 weeks ($P < 0.001$) (Fig. 2A). The enzyme activity gradually decreased over the course of the disease to reach 20% of residual activity at 8–9 weeks of age. Aconitase activity, representing the combined mitochondrial and cytosolic activities, presented a similar decline over the course of the disease (Fig. 2B), demonstrating a generalized and severe deficit of Fe-S enzyme activity. No significant cardiac dysfunction was observed by echocardiography at 4 weeks of age (Table 1), suggesting that 50% Fe-S enzyme activity is sufficient for normal physiologic function, although histological abnormalities are detected (see below). Direct measurements by atomic absorption spectroscopy of iron content in heart mitochondria-enriched fractions did not reveal significant mitochondrial iron accumulation at 7–8 weeks (despite rare mitochondrial iron deposits seen by electron microscopy). The iron concentration significantly increased between 8–9 weeks to reach almost 2-fold the normal iron content by death (Fig. 2D).

**Oxidative stress modifications in Frda/MCK mice**

In order to test whether Fe-S deficiency and/or iron accumulation would be the cause of cardiac hypertrophy through production of free radicals, we measured the levels of oxidative stress markers (lipoperoxides, oxidized proteins) in total heart extracts of Frda/MCK mutant animals. At 2 weeks of age and until 5 weeks, the level of oxidative stress markers (oxidized proteins) was slightly but not significantly increased in mutant animals compared to control littermates (Fig. 2C). In contrast, from 7 weeks of age until death (around 10–12 weeks), a clear and significant decrease of the oxidized protein level was observed (Fig. 2C). This decrease was also observed for lipid peroxide levels in Frda/MCK mutants at 9 weeks of age (data not shown). The decrease of the respiratory chain activity that results from the severe deficiency of the Fe-S enzymes past 5 weeks of age (Fig. 2A and B) is most likely the cause of reduced reactive oxygen species production and hence the observed lower levels of oxidative stress markers in the heart of mutant mice.

**Electron microscopy reveals mitochondrial degeneration**

The cardiac tissue of Frda/MCK mutant mice (ages 2–12 weeks) was examined by optical and electron microscopy (EM) (a minimum of two animals per genotype per age). The first visible alterations seen at 4 weeks consisted of excessive cellular lipid accumulation (verified by oil red staining, data not shown) with the presence of rare degenerated fibers with loss of myofibrils, which are consequently invaded by sarcoplasm (Fig. 3A). Between 5 and 6 weeks, we observed a gradual decrease in lipid droplets and a concomitant appearance of swollen mitochondria, few with lucent matrix or disorganized cristae, and disorganization of cardiac muscle fibers (data not shown). At 6–7 weeks of age, a prominent accumulation of mitochondria was observed (Fig. 3B). In addition to the swollen mitochondria, others presented parallel stacks of cristae membranes or contained dense material reminiscent of iron deposits (Fig. 3B, double arrow). At 12 weeks, Frda/MCK cardiac tissues presented some affected fibers with the presence of large rounded clear ‘vacuoles’ that were interspersed between normal appearing fibers (Fig. 3C, inset), with the ‘vacuoles’ preferentially located between fibers in areas where mitochondria and lipids were

![Figure 1. Echocardiographic and electrocardiographic analysis for evaluation of the cardiac phenotype. (A) 2D short axis of left-ventricular diameter end-diastolic (LVEDD) images of control (left) and Frda/MCK mutant (right) mice at 7 weeks of age. Diameter of the left ventricle is shown. (B) M-mode tracings of the left ventricle in control (left) and Frda/MCK mutant (right) mice at 7 weeks of age (same mice as in A). (C) Electrocardiograms at 9 weeks of age in control mouse (left) and Frda/MCK mutant mouse with a complete atrioventricular block (right).](image-url)
expected (Fig. 3C, EM). The frequent observation of residual membranous material within the large vacuoles (Fig. 3C, single arrow), strongly suggests that these represent degenerated mitochondria. Transversally, an excessive accumulation of abnormal mitochondria closely packed and displacing the fibers to the periphery was observed (Fig. 3C, bottom). The
mitochondria showed disoriented and fused cristae and many presented large electron dense material suggestive of iron deposits (Fig. 3C, double arrow). The ferric nature of these deposits was further demonstrated by Perl’s staining (19).

Idebenone delays the cardiac progressive dilatation and hypertrophy

Since the Frda/MCK mice represent an excellent model for a rapidly progressing FRDA cardiomyopathy, we used it in a placebo-controlled trial with Idebenone, the controversial but currently the only potential source of therapy for the disease. Idebenone was orally administered at a minimal ingested dose of 90 mg/kg/day that is 18-fold the dose commonly given to FRDA patients (5 mg/kg/day). With the exception of their idebenone content, hand made food pellets were similarly prepared for treated and untreated mice, and administration was blinded to the experimentator. No difference in body weight was seen among groups at any time during the study (data not shown). As compared to placebo-treated Frda/MCK mutants, Idebenone-treated mutant mice exhibited a reduction in the left ventricular hypertrophic process (−24% left ventricular mass; *P < 0.01 at week 6; n=6 per group). The increase of left ventricular diameters was slowed down, a clear reduction of the dilatation being observed at 6 weeks of age (−16%; *P < 0.01). Moreover, at this age, although the dilatation was already present in Idebenone-treated animals, the left ventricular shortening and ejection fractions were preserved (Table 1 and Fig. 4A). The delay in the onset of the cardiac alterations was about 1 week, Idebenone-treated animals at 8 weeks of age being similar to placebo-treated mutants around week 7. Idebenone increased the life span of the Frda/MCK mutants by 10% (*P<0.012; n=15), with an average survival rate of 79±9 days versus 71±9 days for placebo-treated mutant animals (Fig. 4B).

To determine whether the effect of Idebenone involved a partial rescue of the decrease in the Fe-S enzymes activities, we monitored complex II activity in heart homogenate from treated and placebo mutant animals at 4 and 5 weeks of age. No difference in the activity of this Fe-S protein was seen between the two groups (Fig. 4C). No effect of Idebenone was also detected on the status of lipid peroxidation by TBARS measurements in total heart extract [Idebenone-treated mutant 0.26±0.08 O.D. (n=7) versus non-treated mutants 0.29±0.07 O.D. (n=6)] nor on mitochondrial extract [0.20±0.06 O.D. (n=7) versus 0.21±0.06 O.D. (n=6)] at 9 weeks of age.

Discussion

In the present study, we demonstrate that murine FRDA cardiomyopathy is characterized by an early onset of dilatation with development of left ventricular hypertrophy followed by reduced systolic function. Furthermore, we determine precisely...
the onset and evolution of the Fe-S deficiency in the cardiac disease progression. Fe-S deficiency occurs very early in the disease pathology (at 4 weeks), with 50% residual activity, while the iron accumulation occurs at the end stage of the disease. Moreover, in the heart of the 7-week-old Frda/MCK mutant mice (and after), indexes of lipid and protein oxidation levels were significantly lower than in the control littermate mice. We demonstrate, however, that Idebenone, a short chain CoQ derivative with well-established antioxidant properties, is clearly protective against the cardiomyopathy by delaying by 1 week the disease progression, at about 5–6 weeks of age, when indexes of oxidative stress are still high, but without correcting the Fe-S deficiency.

Myocardial involvement is a cardinal feature of FRDA, but is variable in its clinical and electric presentation. The main and most frequent clinical manifestations are rhythm disturbances and myocardial insufficiency terminating in a congestive heart failure. Electrocardiographic abnormalities are observed in 75–100% of patients, with non-specific repolarization alterations, cardiac arrhythmias and atrioventricular block (20). Abnormalities at echocardiogram are less frequent (50–70%) and are mainly represented by a left ventricular concentric hypertrophy while asymmetrical septal hypertrophy represents a minority of cases (20). As the disease progresses, there is a natural transition from hypertrophy to dilatation, and organ failure. At the biochemical level, a specific deficiency in Fe-S cluster-containing subunits of mitochondrial respiratory complexes I-III and aconitase has been reported in FRDA endomyocardial biopsies (21). Intracellular iron deposits in cardiomyocytes are a specific finding in FRDA cardiomyopathy (22). Therefore, our FRDA cardiac mouse model develops the major physiological, biochemical and histological features of the human disease. Function-wise, our mouse model mimics the human form of hypertrophic cardiomyopathy associated with a geometric remodelling. These modifications result in a rapid alteration of the systolic function leading to a severe decrease in resting cardiac output, typical of decompensated cardiomyopathy. The complete absence of frataxin in the mouse model certainly accounts for the increased severity of the mouse compared to the human disease, such as simultaneous hypertrophy and dilatation.

Our results support a necessary role of frataxin for efficient Fe-S cluster synthesis, although non-essential, since, despite absence of detectable frataxin at birth, there is still 50% Fe-S enzyme activity at 4 weeks of age. Therefore, in agreement with recently published results in the yeast model (23–25), in the absence of frataxin, Fe-S cluster biosynthesis would occur at a very reduced rate. Furthermore, our results indicate that mitochondrial iron accumulation occurs 4–5 weeks after the onset of heart pathology and Fe-S deficit. This further demonstrates that, while mitochondrial iron status might be involved early (26), the iron accumulation per se cannot be causative of these abnormalities (19), being rather a marker of disease terminal stage. Moreover, our data suggest that the very specific loss of Fe-S protein activity reported in this model (19) does not generate dramatic increase of oxidative stress during cardiac hypertrophy in the context of complete lack of frataxin. The stable, or slight, non-significant increase of oxidative stress markers may, in fact, reflect a more significant increase that is hidden by the concomitant decrease of the respiratory chain activity (as testified by the marked decrease of complex II activity), which is the major source of reactive oxygen species production within the cell. The progressive decline of the respiratory chain activity also explains the decrease of oxidative stress indexes as the disease progresses. This observation is certainly different from the observation previously performed in human samples, where residual level of frataxin is linked to a moderately reduced activity of the respiratory chain still compatible with an overproduction of reactive oxygen species. In addition, the significant reduction of oxidative stress indexes at the end stage of the disease argues against a possible role of the concomitant iron accumulation and deposit in the generation of reactive oxygen species through the Fenton reaction. In the context of low respiratory chain activity, this iron is presumably oxidized and hence non-toxic.

We found that Idebenone effectively delayed the progressive cardiac hypertrophy and dilatation, and preserved ventricular contractility by 1 week thus increasing the life span of the animal by 10%. However, in contrast with the recent observations made on one FRDA patient heart biopsy (27), Idebenone did not restore the Fe-S enzyme activity. Also in contrast with measurements of urine 8-hydroxy-2'-deoxyguanosine in patients (8), we found no evidence of protection against oxidative stress.

It appears that Idebenone is protective in our model in a precise time window, after the onset of the disease but just before the dramatic collapse of Fe-S enzyme activities. Past this point, the sharp decrease of the respiratory chain activity that should ensue, leading to the observed reduction of reactive oxygen species production prevents Idebenone from acting as an antioxidant.

The difference between FRDA patients and our mouse model is most likely directly related to the residual frataxin expression in the FRDA patients. The Frda/MCK mouse model develops a more severe disease with no frataxin expression, and therefore with a more abrupt decrease of Fe-S synthesis compared to the patients expressing residual frataxin. In the present study, we saw a protective effect of Idebenone in a time window (5–6 weeks), where the severity of the cardiomyopathy presumably matches that in the human disease. In this respect, the results of our mouse trials are in perfect agreement with the patients trials, where the cardioprotective effects of Idebenone lasted for more than 6 months. The results from our placebo-controlled double-blind trial therefore strengthen the results obtained on the patients trials, as most of them were open trials without placebo controls and support the use of Idebenone for the human disease.

MATERIALS AND METHODS

All methods employed in this work are in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publications No. 85-23, revised 1996).

Echocardiography

Animals were weighted and analysed for cardiac anatomy and function on a Sonos 5500 (Hewlett Packard) with a 15 MHz linear transducer (15L6). All the examinations were performed...
in the conscious state as described (28–30), by two operators blinded to the genotype and treatment protocol, every week, from the age of 3 to 9 weeks (six points of evaluation per animal). The heart was first imaged in the 2-dimensional (2D) mode in the parasternal long-axis view to obtain the aortic root dimensions. The aortic flow velocity and the heart rate (HR) were measured with pulsed-wave Doppler on the same section. The sample volume cursor was placed in the aortic root and the transducer angled slightly, which allowed to parallel the aortic flow with the interrogation beam so that maximum aortic flow velocity was obtained easily. The cardiac output (CO) was calculated from the following equation

\[
\text{CO} = 0.785 \times D^2 \times \text{VTI} \times HR
\]

where \(D\) is the internal diameter of the aortic root and VTI is the velocity–time integral of the Doppler aortic spectrum. Left ventricular cross-sectional internal diameters in end-diastole (LVEDD) and in end-systole (LVESD) were obtained by an M-mode analysis of a 2D short axis view at the papillary muscle level. The shortening fraction was calculated as

\[
\text{SF} = \frac{\text{LVEDD} - \text{LVESD}}{\text{LVEDD}} \times 100
\]

and the ejection fraction using the equation

\[
\text{EF} = \frac{\text{LVEDA} - \text{LVESA}}{\text{LVEDA}} \times 100
\]

where LVEDA and LVESA are respectively LV end-diastolic and LV end-systolic areas. From this view, the diastolic septum (S) and posterior wall (PW) thicknesses were measured. The left ventricular mass (LVM) was calculated with the following formula (29,30)

\[
\text{LVM} = 1.055 \times [(S + PW + LVEDD)^3 - (LVEDD)^3]
\]

All the measurements were performed on at least three beats, according to the guidelines of the American Society of Echography.

**Electrocardiograms**

Eight-week-old mice, anaesthetized with tribromoethanol (2.5% solution, 13 \(\mu\)g body weight), were recorded with the four arms of the electrocardiogram (ECG) leads attached at the origin of each paw by unipolar and bipolar lead derivations. The signal was recorded by an ECK (EKG-Burdick, Siemens) connected to a data acquisition system (MP100 and Acknowledge Software, Biopac Systems Inc.).

**Histopathology and electron microscopy**

Paraformaldehyde fixed tissues were embedded in paraffin, sectioned on a microtome, and stained with haematoxylin and eosin or Perl's staining (19). Lipid analysis by oil red staining was performed on cryostat sections of unfixed tissues (31).

For electron microscopy, cardiac tissue was prepared and examined with a Philips 208 electron microscope, operating at 80 kV (19).

**Biochemical analysis**

The activities of the respiratory chain enzyme complexes succinate dehydrogenase (complex II) and cytochrome c oxidase (complex IV) were determined on liquid nitrogen frozen tissues (32). The activities of the isocitrate dehydrogenase (IDH) and mitochondrial and cytosolic aconitases (Aco) were determined as described (32). Aliquots of 100 \(\mu\)l of mitochondria-enriched subcellular heart fractions (from 100 to 300 mg heart wet weight) were assayed for iron levels by atomic absorption spectroscopy (19). Iron content was expressed in ng Fe/mg protein.

**Oxidative stress assays**

The thiobarbituric acid reactive substances (TBARS) were measured by incubating 100 \(\mu\)l of heart homogenate (total or mitochondria-enriched fraction) with two volumes of 15% (w/v) trichloroacetic acid, 0.375% (w/v) thiobarbituric acid, 0.25 N HCl solution for 15 min at 100°C, cooled at 25°C and centrifuged at 1000g for 10 min. TBARS were measured on the supernatant by spectrophotometry at 535 nm. The lipoperoxide content and the oxidation of proteins of heart homogenates were measured using the Lipid Hydroperoxide Assay Kit (Cayman Chemical, Ann Arbor) and the Oxyblot Oxidation Protein Detection Kit (Intergen, Temecula), respectively, according to the manufacturer's protocols.

**Animal care and idebenone treatment**

Animal care and mutant generation were as previously described (19). A total of 15 mutant animals per treatment group were analysed, issued from four different breeding pairs. Six animals in each group were investigated for echocardiographic examinations; and Dr R. Kahn for the MCK transgenic mice. This work was supported by funds from the Muscular Dystrophy Association of America (MDA), the Muscular Dystrophy Association of America (MDA), the CNRS (grant #96084), and the European Community (contract QLG1-CT-1999-00584) and by the INSERM and the Hopitaux Universitaires de Strasbourg.

**ACKNOWLEDGEMENTS**

We wish to thank L. Reutenauer, N. Lagarde and J. Hergueux for technical support; A. Guimond and L. El Fertak for echocardiographic examinations; and Dr R. Kahn for the MCK transgenic mice. This work was supported by funds from the Association Francaise contre les Myopathies (AFM), the Muscular Dystrophy Association of America (MDA), the CNRS (grant #96084), and the European Community (contract QLG1-CT-1999-00584) and by the INSERM and the Hopitaux Universitaires de Strasbourg.

**REFERENCES**


