Structural and biochemical evidence of mitochondrial depletion in pigs with hypertrophic cardiomyopathy

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Abstract

Pig hearts with naturally occurring hypertrophic cardiomyopathy (HCM) were isolated to investigate the effects of mitochondrial deficiency at biochemical and molecular levels. Enzyme activities of mitochondrial-encoded cytochrome c oxidase and NADH dehydrogenase in the HCM hearts ($n = 12$) were lower than that in the controls ($n = 12$) by $41 \pm 29\%$ ($P < 0.01$) and $43 \pm 21\%$ ($P < 0.001$), respectively. Additionally, Southern blot analysis was conducted to quantify the relative amount of mitochondrial DNA (mtDNA) from the HCM and controls. The relative amount of mtDNA in the HCM hearts was significantly $57 \pm 19\%$ ($P < 0.001$) lower than that in the controls. Both mitochondrial enzyme deficiency and mtDNA depletion were significantly correlated with the degree of cardiac hypertrophy judged based on the ratio of heart/body weight. In conclusion, our results reveal that a secondary effect of tissue-specific mtDNA depletion and mitochondrial dysfunction is in response to the HCM.
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1. Introduction

Mitochondria are the major source of cellular ATP providing the energy for cardiac function by mitochondrial oxidative phosphorylation (OXPHOS). The cellular organelle contributes to myocyte injury via loss of physiologic function. Therefore, the physiologic significance of damage to mitochondrial DNA (mtDNA), proteins, or lipids should be established at the level of the mitochondrion, myocyte, and whole heart (Lesnefsky et al., 2001). Morphologic abnormalities of mitochondria and mitochondrial dysfunction occur in association with heart failure (Sharov et al., 2000), as well as in cardiomyocytes of ischemic, dilated, and hypertrophied hearts (Jarreta et al., 2000; Marin-Garcia et al., 1997, 1998).

Hypertrophic cardiomyopathy (HCM) is a cardiac disease in which the most characteristic morphologic features are hypertrophied, nondilated ventricles, and myofibrillar disarray with increased amounts of matrix (Maron, 1997). This disease affects approximately 1 in every 500 of the general population (Spirito et al., 1997). Notably, over 50% of all reported cases of HCM are inherited, presenting disease-causing mutations in several genes encoding sarcomeric protein (Burch and Blair, 1999; McKenna et al., 1998) or in mtDNA (Marin-Garcia and Goldenthal, 1997). Although in some cases a correlation between genetic defects and decreased specific mitochondrial enzymatic activities has been noted, few investigations have focused on the characterization of respiratory chain function and the findings have often been contradictory (Marin-Garcia et al., 1997, 1998; Ozawa et al., 1990). The lack of work on the area could be due to the limitations of a lack of available heart specimens in humans. Furthermore, the question of whether alterations in genetically mtDNA molecule and mitochondrially enzymatic defects play a primary or secondary role in HCM remains essentially unanswered.

Recently, the authors developed a pig model for naturally occurring HCM (Huang et al., 1996; Liu et al., 1994). The morphologic and pathologic features of pig HCM hearts comprise hypertrophic ventricles with

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cellular disorientation, fibrosis, and occluded intramural coronary arteries (Chiu et al., 1999; Dai et al., 1997; Liu et al., 1994). These characteristics all resemble those found in humans (Maron, 1997). The novel pig model also found mitochondrial damage in HCM hearts, which we hypothesized that oxidative stress caused the damage (Lin et al., 1997). Clinically, mitochondrial dysfunction associated with human cardiac disease has been evaluated at the biochemical and molecular levels (Anan et al., 1995; Marin-Garcia and Goldenthal, 1997). Mitochondrial regulation and deficiencies associated with HCM disease therefore can be profitably explored.

This study reports on pigs with HCM and abnormal activity levels of mitochondrial respiratory enzymes in the cardiomyocytes. The data also revealed a significant reduction in myocardial mtDNA compared with the controls. Therefore, mtDNA depletion may play a role in the pathogenesis of HCM.

2. Materials and methods

2.1. Animal source, care, feeding, and management

Landrace pigs raised in the Nuclear Breeding Center of Northern Taiwan were bred specifically for the study of HCM (Huang et al., 1996). The pigs were fed 17.5% crude protein and 3050 kcal of metabolizable energy per kg. Feed and water were administered ad libitum. All pigs were reared in a natural lighting environment. The animals were treated according to guidelines established by the National Science Council of the Republic of China (National Science Council, 1993). The HCM pigs were prescreened by Echocardiography assessment with a commercial VingMed (CFM-800) 3.25 MHz transducer by 2D and M-mode methods, as previously described (Dai et al., 1997; Lin et al., 2000), then diagnosed by gross and histopathologic findings after sacrifice. Other age-matched control pigs were randomly selected from the same herd. This study examined 24 pigs aged 6–18 months, including 12 pigs with mild to severe HCM and 12 pigs without detectable pathologic HCM as normal controls.

2.2. Gross and histologic examination

Hearts were removed and weighed after sacrifice, which was performed with anesthesia, electric shock, and stunning. Gross examinations of the hearts were conducted as described by Liu et al. (1994), including measurement of the thickness of the middle left ventricle (LV) and interventricular septum (IVS) free walls. Tissues from the IVS were dissected 2 cm below the atrioventricular groove for further histologic examination and measurements of mitochondrial enzyme activity and mtDNA content.

Fresh IVS tissues were cut into 1 mm³ blocks and immediately fixed in 0.1 phosphate buffered 2.5% glutaraldehyde for transmission electron microscopic studies performed as described by Dai et al. (1997). Fixed cubes were washed in 0.1 M phosphate buffer, 5% sucrose, postfixed in 1% osmium tetroxide, dehydrated in graded concentrations of ethanol, and embedded in Spurr's resin. Thin sections, stained with uranyl acetate and lead citrate, then were viewed using a Hitachi H-600 electron microscope. Additional IVS tissue was fixed for 7 days in 4% (v/v) formalin containing 0.1 M sodium phosphate (pH 7.5) for histologic examination by Hematoxylin–eosin and Masson's trichrome blue staining.

2.3. Measurements of enzyme activity

Skeletal muscles between the 10th and 11th ribs and livers were also collected for comparison with myocardial tissues. All samples were immediately frozen in liquid nitrogen and stored at −80 °C for subsequent preparation of tissue homogenates.

Isolated tissues were thoroughly washed with 50 mM potassium phosphate buffer (pH 8.0) and sliced into pieces weighing about 0.5 g each. Tissue homogenization and total protein concentration were measured as described previously (Lin et al., 1997).

Activities of mitochondrial-encoded mitochondrial cytochrome c oxidase (COX) (respiratory complex IV) and NADH-dehydrogenase (ND) (complex I) in the IVS extracts were measured by spectrophotometric assays, as reported by Dimauro et al. (1980). Moreover, citrate synthase (CS) activity was also measured following the method described previously (Dimauro et al., 1980). CS, a nuclear-encoded mitochondrial enzyme, has frequently been employed to gauge mitochondrial number in cells.

2.4. Primers and DNA probes preparation

Total cellular DNA extracted from the heart, muscle, and liver tissues was used as a template in polymerase chain reaction (PCR) employing oligonucleotide primers derived from specific sequences of porcine mitochondrial D-loop (mtDNA) or nuclear ribosomal gene (rDNA) to generate the ^{32}P-labeled probes. The primers for amplifying the sequence of mitochondrial D-loop region for probe preparation were mt-F (GCACAAAC ATACAAATATGTGACCC) and mt-R (TTATT TAA GGGGAAAGAGTGGGCGA). The primers for rDNA as an internal standard in the quantitative measurement of mtDNA were rD-F (ACAAGAATTCGTTTCCCAC GCTCTCATTT) and rD-R (GGCCCAAGCTTTCC AGCGACAGGCG). The primer sequences of mt-F/mt-R and rD-F/rD-R were designed based on sequence information available in the GenBank database of AF039171 (porcine mtDNA; Lin et al., 1999) and AF039170 (porcine rDNA), respectively.

In the PCR, each aliquot of 100 μl PCR mixture contained 200 μM of each dNTP, 3 μCi of [α-^{32}P]dCTP, 0.5 units of *Taq* DNA polymerase (HT Biotechnology), 10 mM Tris–HCl (pH 9.0), 1.5 mM MgCl$_2$, 50 mM KCl, 0.01% Triton X-100, 0.001% gelatin, and 0.2 μM of primer pair, mt-F/mt-R or rD-F/rD-R. The reaction mixtures were preheated to 94 °C for 3 min; then PCR was performed for 36 cycles of denaturing at 94 °C for 30 s, annealing at 55 °C for 30 s, and extending at 72 °C for 30 s. The PCR products were purified to remove free isotopes, then electrophoresed and counted to verify the ^{32}P-labeled probes of mitochondrial D-loop (392 bp) and rDNA (476 bp) fragments.

2.5. Quantification of mtDNA

MtDNA was quantified by comparing the ratio of mtDNA to rDNA by Southern blot analysis. The quantification approach was essentially like that employed in previous studies but with slight modifications (Moraes et al., 1991; Tritschler et al., 1992). Each cell normally contains hundreds of copies of rDNA genes, providing a suitable control for quantification of mtDNA (Young et al., 1976). Total cellular DNA was extracted from 50 mg of heart, skeletal muscle, and liver according to the method reported by Zeviani et al. (1988), then digested with restriction enzyme *Pst*I. Five μg of digested DNA were separated electrophoretically in a 0.8% agarose gel and then transferred onto a nitrocellulose membrane for Southern hybridization with the mtDNA D-loop and rDNA probes. The radioactive bands on the membrane were detected, integrated, and analyzed using the PhosphorImage system and Image Quant software, as described previously (He et al., 1998).

The ratio of mtDNA/rDNA for each DNA sample was calculated by dividing the mtDNA signal by the rDNA signal. Moreover, the relative amount of mtDNA was expressed as the ratio of the signal of the mitochondrial probe (mtDNA) to that of the nuclear probe (rDNA).

2.6. Statistics

All quantitative data were expressed as means ± standard deviations (SD). Differences between groups were evaluated by a Students' *t* test. *P* values less than 0.01 were considered statistically significant.

3. Results

3.1. Characterization of HCM

Table 1 lists the physical characteristics of the 12 HCM pigs and the 12 unaffected pigs (normal controls).

Table 1
Age, body weight, heart weight, heart to body weight ratio, and thickness of ventricular free walls in the control and HCM groups

	Control	HCM
Number of animals	12	12
Age (month)	11.9 (3.9)	12.3 (3.6)
Body weight (kg)	123 (22)	150 (43)
Heart weight (g)	512 (103)	848 (272)**
Heart/body weight (g/kg)	3.97 (0.39)	5.81 (1.44)**
Ventricular thickness (mm)		
Left ventricle	19.9 (2.1)	26.3 (5.6)*
Interventricular septum	19.8 (2.1)	25.7 (5.2)*

Values are means ± SD. Significant differences of *$P < 0.01$ and **$P < 0.001$ were determined with respect to the normal control.
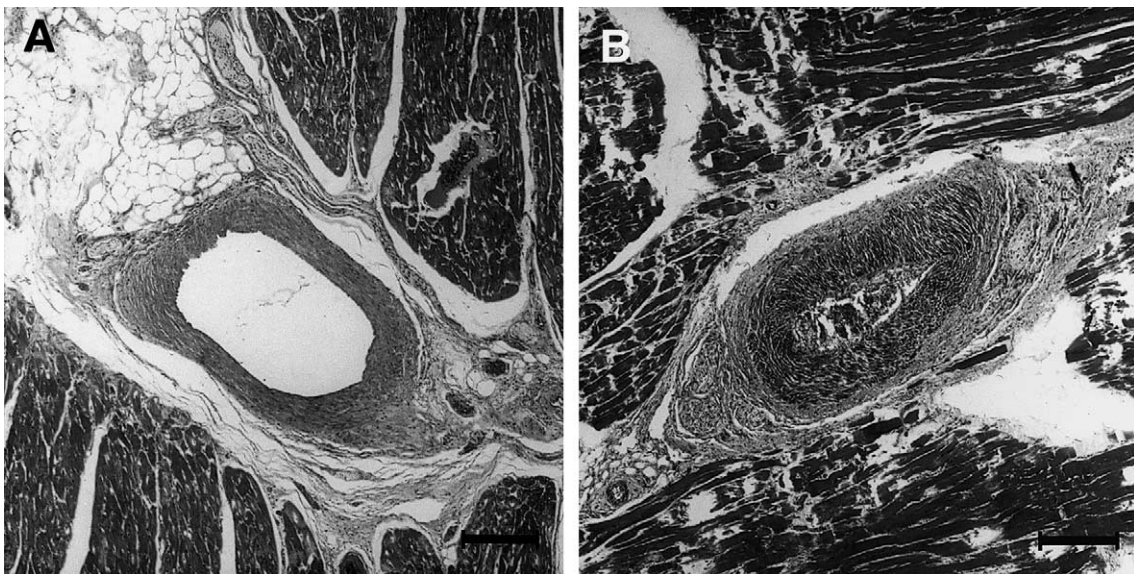

Fig. 1. Typical example of histologic features of normal (A) and occluded intramural coronary artery (B) stained with Masson's trichrome in an HCM heart (magnification 40×). Scale bars indicate 0.1 mm.

The control and HCM groups displayed no significant differences in either age or body weight, but the heart weight and heart/body weight ratio of the HCM pigs significantly exceeded those of the control group ($P < 0.001$). Additionally, the thickness of the ventricular free walls in the HCM pigs significantly surpassed those of the control pigs ($P < 0.01$). The physical characteristics of the HCM hearts thus were consistent with our previous reports (Chiu et al., 1999; Liu et al., 1994). Further histologic evaluation consistently revealed cardiomyocyte disorientation, cellular fibrosis, and occluded intramural coronary arteries in the HCM hearts (Fig. 1), as in our previous studies (Chiu et al., 1999; Dai et al., 1997; Liu et al., 1994).

3.2. Ultrastructural examination

Electron microscopic investigation depicted morphologically normal cardiomyocytes in the HCM hearts. However, the myofibrils in the cells appeared disorientated and disrupted. Myofibril free areas occupied by clusters of mitochondria were observed and most of the mitochondria displayed swollen and/or disrupted cristae. Additionally, the HCM hearts exhibited electron-dense and lipid droplets (Fig. 2).

3.3. Biochemical examinations

Biochemical analysis revealed that the specific activity levels of mitochondrial respiratory complexes I and IV, i.e., ND and COX, in the HCM hearts were significantly lower than that in the controls ($n = 12$) by $41 \pm 29\%$ ($P < 0.01$) and $43 \pm 21\%$ ($P < 0.001$), respectively. CS activity in the HCM tissues exceeded the control level by 13%; however, the difference was not statistically significant ($P = 0.15$) (Table 2).

3.4. MtDNA content

mtDNA/rDNA ratios were quantified to represent the relative amount of mtDNA (Fig. 3). The mtDNA/rDNA ratio in the control hearts was 3.00 ± 0.38 (range from 2.21 to 3.54), while the ratio in the HCM hearts was 1.30 ± 0.57 (range from 0.38 to 2.21) (Table 3). The relative amount of mtDNA in the HCM samples ranged from 13% to 73% of the average for the 12 control

Table 2
Mitochondrial enzymes in the control and HCM hearts

	Control	HCM
Number of animals	12	12
COX	180 (55)	106 (52)*
ND	51 (13)	29 (11)**
CS	361 (56)	408 (91)

COX indicates mitochondrial cytochrome *c* oxidase (complex IV); ND, mitochondrial NADH dehydrogenase (complex I); CS, citrate synthase. Enzyme activity of COX, ND, and CS are expressed as nmol/min/mg protein. Data represent means $\pm$ SD from three independent measurements. Significant difference of *$P < 0.01$ and **$P < 0.001$ determined with respect to the normal control.
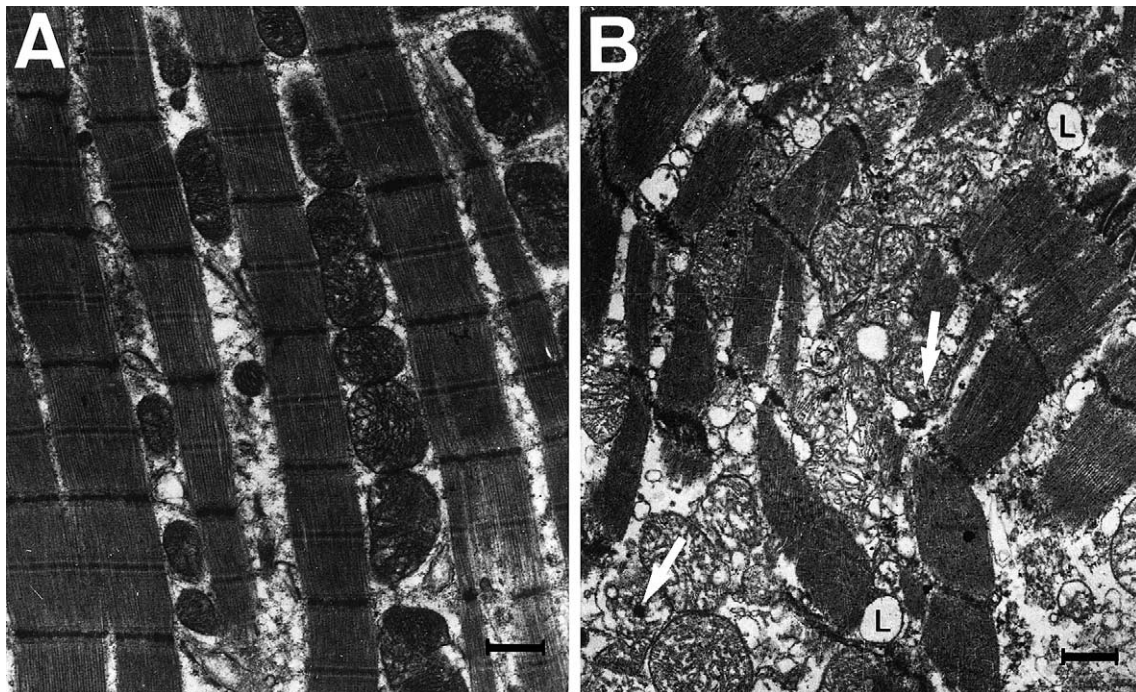

Fig. 2. Transmission electron microscopic sections from hearts without (A) and with (B) HCM. Disorderly myofibrils and damaged mitochondria were observed in the HCM specimen. L denotes lipid droplets and the arrows indicate electron-dense droplets (magnification 8000×). Scale bars indicate 1 μm.
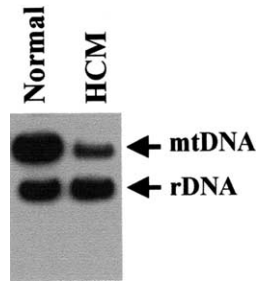
Fig. 3. Autoradiogram of Southern hybridization. Total cellular DNA of porcine specimens was isolated, digested with *Pst*I, electrophoresed, and blotted onto a nitrocellulose membrane. The membrane then was simultaneously hybridized with two ^{32}P-labeled probes, a nuclear ribosomal gene (rDNA) and a mitochondrial D-loop (mtDNA). Next, autoradiographic signals after hybridization were measured on blots with PhosphorImager (Molecular Dynamics). The relative amount of mtDNA in the specimens was expressed as the ratio of the signal of the mtDNA probe (indicated by an arrow and labeled with mtDNA) to that of the rDNA probe (indicated by an arrow and labeled with rDNA), i.e., mtDNA/rDNA ratio. Lane 1: labeled Normal, displayed the DNA isolated from the hearts without pathologic HCM, i.e., normal tissue; lane 2: labeled HCM, displayed the DNA isolated from the HCM heart.

Table 3
The mtDNA/rDNA ratios in heart, skeletal muscle, and liver of the control and HCM groups

	Control	HCM
Number of animals	12	12
Heart	3.00 (0.38)	1.30 (0.57)**
Skeletal muscle	3.08 (0.46)	2.88 (0.35)
Liver	0.75 (0.28)	0.79 (0.37)

Data represent means ± SD from two independent experiments and the duplicate total cellular DNA isolated from each subject were loaded on the same membrane for DNA hybridization in each experiment. Significant difference of **$P < 0.001$ were determined with respect to the normal control.

samples, and the average mtDNA depletion in the HCM pigs was $43 \pm 19\%$ compared with the controls ($P < 0.001$). Otherwise, the amount of internal rDNA in the heart tissues with or without HCM did not differ significantly. The mtDNA/rDNA ratios in the liver and skeletal muscle with or without HCM were also measured, and the measurement results revealed statistically insignificant differences between the HCM and control specimens (Table 3). The data revealed low mtDNA in the HCM hearts specifically. Furthermore, the cardiac mtDNA/rDNA ratios were significantly negatively correlated ($P < 0.001$) with the degree of cardiac hypertrophy as judged by heart/body weight ratio (Fig. 4A). The relationships between activities of mitochondrial enzyme and heart/body weight ratios in the HCM and control subjects were also significantly negatively correlated ($P < 0.001$) (Figs. 4B and C). However, the enzyme activity of nuclear encoding CS was not correlated with the heart/body weight ratio in either group (Fig. 4D).
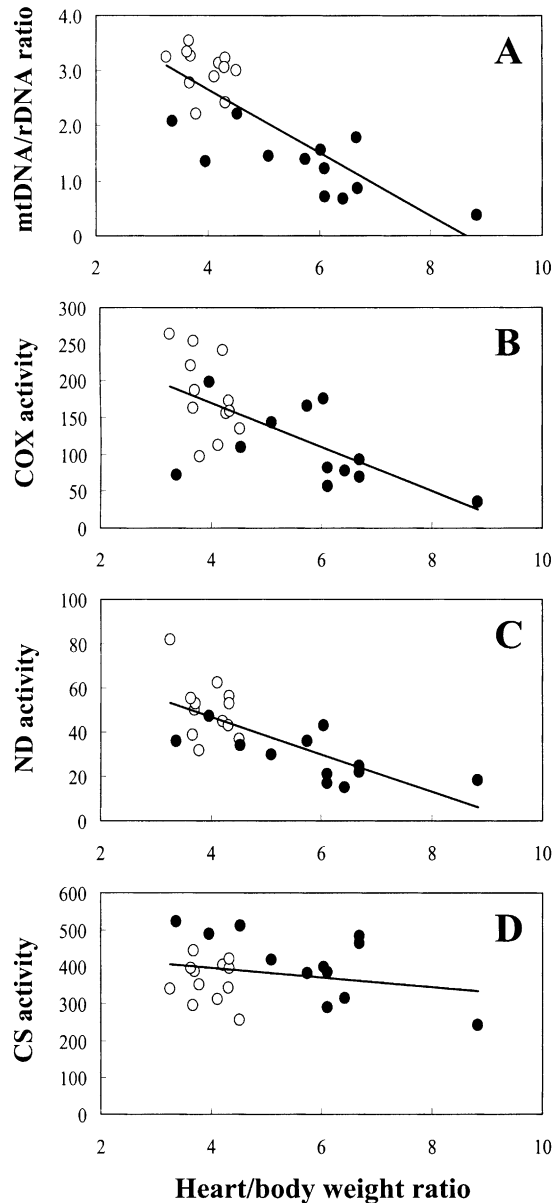

Fig. 4. Relationship between mtDNA/rDNA ratio, mitochondrial COX, and mitochondrial ND of the heart and heart/body weight ratio in normal ($n = 12$, ○) and HCM ($n = 12$, ●) animals. Linear regression was carried out for all data points: COX indicates mitochondrial cytochrome *c* oxidase (complex IV); ND, mitochondrial NADH dehydrogenase (complex I); CS, citrate synthase. Enzyme activity of COX, ND, and CS was expressed as nmol/min/mg protein. Notably, linear regression revealed the following: A (mtDNA/rDNA ratio vs. heart/body weight ratio), $r^2 = 0.60$, $P < 0.001$; B (COX vs. heart/body weight ratio), $r^2 = 0.40$, $P < 0.001$; C (ND vs. heart/body weight ratio), $r^2 = 0.46$, $P < 0.001$; D (CS vs. heart/body weight ratio), $r^2 = 0.08$, $P = 0.18$.

4. Discussion

Mitochondrial abnormalities at the biochemical and molecular level are increasingly recognized as playing a role in heart disease (Anan et al., 1995; Marin-Garcia and Goldenthal, 1997). Respiratory enzyme deficiencies

and defects in mtDNA, including point mutations (Merante et al., 1994; Santorelli et al., 1996), deletions (Ozawa et al., 1990), and depletion (Marin-Garcia et al., 1997, 1998), have been frequently associated with human HCM. However, the correlation between specific mtDNA defects, respiratory deficiencies, and cardiac pathogenesis remains poorly defined. Related studies have been limited to humans owing to a lack of heart samples, and most specimens have been taken post-mortem. Therefore, the HCM pig established at our institute is a potential animal model for studying the pathologic role of mitochondrial functions in HCM (Huang et al., 1996; Liu et al., 1994).

This study found that reduced activity of COX and ND, the mitochondrial-encoded mitochondrial enzymes involved in OXPHOS, indicated mitochondrial dysfunction in HCM hearts. Thus, the investigation confirmed that most mitochondria in HCM hearts are damaged using Ultrastructural examination (Fig. 2). Determination of mitochondrial respiratory complex activities also demonstrated mitochondrial deficiency in HCM hearts at the biochemical level. Deficiencies in complex III and IV activities have previously been associated with mutant mitochondrial tRNA alleles in fatal infantile cardiomyopathy (Merante et al., 1994; Santorelli et al., 1996; Zeviani et al., 1988). Defective mitochondrial transcription or translation has most likely reduced levels of both respiratory complex IV activity and mitochondrial-encoded COX subunit 2 content (Marin-Garcia et al., 1997). Consequently, the existence of mitochondrial defects at the molecular level, such as in mtDNA depletion associated with respiratory defects, is an important area for future study. Although a few studies have demonstrated mutations of mitochondrial tRNA genes involved in human maternally inherited HCM (Merante et al., 1994; Santorelli et al., 1996), no evidence yet exists of inheritance in the HCM pig model.

The number of mtDNA molecule per mitochondrion has been estimated to vary between 2 and 10 in different tissues (Robin and Wong, 1988; Shmookler Reis and Goldstein, 1983). The reduction of mtDNA in the HCM subjects therefore suggests that each mitochondrion contains less than 2–10 genomes/organelles normally present, and some organelles actually must be totally depleted of mtDNA because of HCM cardiomyocytes having an abnormally increased number of mitochondria. In spite of mtDNA depletion in hearts with HCM remains obscure, we proposed that a secondary effect of mtDNA depletion and mitochondrial dysfunction is in response to the cardiac pathology. At genetic issue, mutation in the region for replicating the mitochondrial genome could explain the present findings, but the pig herd affected with HCM displayed no evidence of maternal inheritance. Possibly, mtDNA depletion may be a secondary result of an unknown primary defect unrelated to alterations in copy number or caused by mutations in nuclear genes that control mtDNA levels in cardiomyocytes, such as mitochondrial transcription A (mtTFA) (Poulton et al., 1994) or DNA polymerase γ (Naviaux et al., 1999). The HCM and control pigs used in the present study came from the same herd and have similar genetic backgrounds. Therefore, it is greatly suspicious that genetic deficiency is associated with the HCM pigs.

The present findings strongly suggest that the observed mtDNA depletion is a tissue-specific response to HCM hearts, since other tissues, such as liver and skeletal muscle, from HCM individuals displayed undetectable levels of mtDNA depletion compared with the control individuals. The tissue-specific mtDNA defect in the HCM hearts may correspond to ischemic damage caused by the frequent occluded intramural coronary arteries in HCM, which may relate to a reduced vasodilator capacity in producing ischemia (Dilsizian et al., 1993; Maron et al., 1986; Yoshida et al., 1998). The mitochondria in the HCM hearts may proliferate to compensate for the mismatch of energy metabolism in cardiomyocytes in the ischemic condition. Furthermore, mitochondria and mtDNA damage caused by the generation and action of oxidative free radicals is known to occur during ischemia (Marin-Garcia et al., 1996; Piantadosi and Zhang, 1996).

In summary, this investigation has revealed abnormal mitochondria, reduced enzyme activity of respiratory complexes I and IV, and reduced mtDNA level in HCM pig hearts. The present pig model provides an important opportunity to elucidate the mitochondrial defects at the biochemical and molecular levels in the pathogenesis of HCM.

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