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Altered Expression of Cardiac Myosin Isozymes Associated with the Malignant Hyperthermia Genotype in Swine

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Background: **Anesthetic-induced malignant hyperthermia (MH) in humans and pigs is associated with dramatic alterations in cardiac function. However, it remains controversial as to whether MH-associated cardiac symptoms represent a primary difference of myocardium or a secondary alteration consequent to increases in the hyperthermic stress. Here the authors describe changes in myosin isoform expression in the hearts of MH-susceptible pigs with and without prior exposure to halothane.**

Methods: **One group of pigs was diagnosed as MH susceptible by halothane challenge and Hal-1843 nucleotide examination. To determine if there is an effect of halothane exposure, another group of pigs was diagnosed by simple MH genotyping without exposure to halothane. After diagnosis and genotyping, animals with and without exposure to halothane were killed to study cardiac myosin isozyme distributions, cardiac myofibrillar adenosine triphosphatase (ATPase) activity, and the steepness of the Ca^{2+}–ATPase activity relation in the hearts of normal and susceptible pigs. The altered myosin isozyme expression was analyzed by pyrophosphate gel electrophoresis.**

Results: **Malignant hyperthermia–susceptible animals with the prior halothane challenge showed an increased V1 myosin (−44%) expression, increased myofibrillar ATPase activity (−25%) and increased steepness of the Ca^{2+}–ATPase activity relation. Without exposure to halothane, no change of myofibrillar ATPase activity was found in the hearts of different genotyped pigs, but there was a small increase in expression of V1 myosin (−5%) in the mutant (TT).**

Conclusions: **The potential modulation of V1 myosin expression occurs in the hearts of MH-susceptible pigs. The added stress by halothane challenge would further cause a V3 → V1 shift, which may be attributed to the long-term effects of hyperthermic stress. (Key words: Hill plot analyses; mutagenetically separated polymerase chain reaction; pyrophosphate gel electrophoresis.)**

HYPERTHERMIA, muscle rigidity, and metabolic acidosis characterize malignant hyperthermia (MH), a serious and potentially lethal disease in humans and pigs.[1] Anesthetics such as halothane and muscle relaxants initiate the MH syndrome.[2] During the MH crisis there is a tachycardia, increased cardiac output, and increased myocardial oxygen consumption. This initial response is accompanied by an increase of blood catecholamine.[3] However, little is known about the long-term effects of hyperthermic stress on myofibrillar protein expression in MH-affected hearts.

The existence of three cardiac myosin isozymes (V1, V2, and V3) in mammalian ventricle, and modifications in their relative amounts under the developmental, hormonal, and functional states of the heart has been related to the heart's adaptation to new functional loads.[4] The adenosine triphosphatase (ATPase) activity of cardiac myosin is the highest for the V1 isozyme, intermediate for the V2 isozyme, and the lowest for the V3 isozyme. Shifts of V3 to V1 have been associated with an increased contraction speed, increased utilization of ATP (or increased oxygen demand) and reduced energetic efficiency.[5] Responses such as tachycardia and positive inotropy would occur in response to catecholamines regardless of the myosin isozyme composition. However, a shift of cardiac myosin isozyme from V3 to V1 in the MH-affected hearts would further compromise cardiac function in the face of hyperthermia and catecholamine stimulation.

The main purpose of the present study was to determine if changes in myosin isozyme expression occur in the hearts of MH-susceptible pigs. Myosin isozyme analysis was conducted on pigs with and without halothane challenge. It was therefore possible to distinguish between differences associated with the MH gene and differences that occur as a consequence of the halothane challenge. This study should clarify the roles of genotype and hyperthermia in the origin of the myocardial changes in MH.

Materials and Methods

Experimental Animals

To exclude the prior effect of halothane challenge performed to test MH susceptibility, two experimental groups of pigs with (Duroc and Landrace) and without (Berkshire) halothane challenge were used in this study. All animals (8 Landrace: 3 MH-positive/TT, 5 normal/CC; 6 Duroc: 2 MH-positive/TT, 4 normal/CC; and 96 Berkshire: 71 CC, 23 CT, and 2 TT)§ investigated were raised by the Taiwan Livestock Research Institute (Tainan, Taiwan). The reason for using different breeds of pigs in this study was to increase the population of MH-suscep-

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§ TT, CT, and CC indicate the genotype of MH for the homozygous normal, the heterozygous mutant, and the homozygous mutant, respectively.

tible animals, because there is a low incidence of porcine MH. However, in all comparative studies, pigs were chosen from the same parents or from the same breed to exclude crossbreed difference.

Halothane Contracture Test

The MH susceptibility in Duroc and Landrace pigs was determined by having animals inhale a mixture of 3% halothane, 2 l/min of oxygen, and 1 l/min of nitrous oxide according to Chang *et al.*[6] At the time of testing, animals were 8–12 weeks of age and weighed between 10 and 25 kg. During the 3-min inhalation of halothane, piglets with any one of four limbs presenting the muscle rigidity for 10 s were classified as MH-susceptible pigs. After the test, all animals were maintained with the usual laboratory conditions recommended by the Animal Care and Use Committee of Taiwan Livestock Research Institute (Tainan, Taiwan) until they were killed. A time range of 1–8 months elapsed between halothane exposure and myosin analysis.

Genotyping of Hal-1843 *Gene Mutation*

As described by Fujii *et al.*,[7] a single base substitution (*Hal-1843*) in both alleles relating to the gene encoding the ryanodine receptor in skeletal muscle has been linked to porcine MH. The technique known as mutagenically separated polymerase chain reaction was used to detect the genotype for the MH gene, according to the method of Lockley *et al.*[8] Blood samples collected from animals were used to extract the genomic DNA. The mutagenically separated polymerase chain reaction used three different allele-specific primers originally described by Fujii *et al.*[7] and was conducted in a total volume of 25 μl containing extracted DNA samples, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM dNTP, 0.1 μM primer for the normal allele, 0.8 μM primer for the homozygous mutant, 0.12 μM primer for the heterozygous mutant, and 1 U of *Taq* DNA polymerase. Amplification started with the following temperature setting: 94°C for 1 min, 69°C for 1 min, and 72°C for 1 min. Amplification was continued over 40 cycles of the following program: 94°C for 30 s, 69°C for 45 s, and 72°C for 45 s. It was then kept at 72°C for 5 min. The products were then loaded directly on an ethidium bromide stained gel for genotyping *Hal-1843* gene mutation.

Cardiac Myofibril Preparations

Animals (Landrace, body weight: 46.6–88 kg, age: 5–11 months; and Duroc, body weight: 23.6–32 kg, age: 3–4 months; Berkshire, body weight: 135–150 kg, age: 11–30 months) were anesthetized with thiopental sodium (15 mg/kg by intravenous injection; Pentothal, Abbott Australasia Pty. Ltd., Sydney, Australia) and killed by bleeding from the carotid arteries. The hearts were immediately removed from killed animals and put on ice and delivered to the laboratory.

Cardiac myofibrils were prepared according to Liou *et al.*,[9] following the original procedure of Solaro *et al.*[10] The preparation was conducted at 4°C. Left ventricles were cut into small pieces for homogenization in a Waring blender. The muscle was homogenized with 4 volumes of 0.3 M sucrose containing 10 mM MOPS (3-(N-morpholino)propanesulfonic acid; pH 7.0) for 1 min. The homogenate was centrifuged at 17,300*g* for 20 min. The pellet was resuspended with solution containing 60 mM KCl, 30 mM MOPS (pH 7.0), and 2 mM $MgCl_2$ (standard buffer solution). This suspension was then homogenized and centrifuged at 750*g* for 15 min. The sequence of resuspension, homogenization, and centrifugation was repeated four more times with the same solution. After this, the myofibrils were resuspended with the standard buffer solution containing 2 mM EGTA. For further purification of myofibrils, the EGTA-washed preparations were treated with 1% Triton X-100 (USB, Cleveland, OH) dissolved in the standard buffer solution. The myofibrils were then centrifuged at 750*g* for 15 min. To avoid foaming, the homogenizations were conducted using a Teflon–glass hand homogenizer (KONTES, Vineland, NJ). The Triton X-100 treatment was repeated once, followed four times by washing with the standard buffer solution. The isolated myofibrils were resuspended in the standard buffer solution containing 50% glycerol and stored in the freezer (−20°C) before use. Myofibrillar protein concentrations were determined by BCA Protein Assay Reagent (Pierce, Rockford, IL), using bovine serum albumin as a standard.

The actomyosin ATPase activity was measured by suspending myofibrils (−0.2 mg) in 10 mM MOPS (pH 7.0), 90 mM KCl, 5 mM $MgCl_2$, 2 mM EGTA, and various additions as indicated. The reaction was initiated by additions of varying concentrations of magnesium ATP. Reaction mixtures were shaken in a water bath at controlled temperature for 10 min. The ATP hydrolysis was stopped by additions of malachite green reagent, which contained 33% malachite green, 16.7% polyvinyl alcohol, and 16.7% ammonium molybdate.[11] The levels of inorganic phosphate released were determined by measuring the absorbance at 630 nm with a spectrophotometer (Hitachi U-2000; Hitachi, Tokyo, Japan). A calibration curve was constructed with KH_2PO_4 ranging from 20 to 200 nmol.

Cardiac Myosin Preparations

A myosin extract was prepared from cardiac myofibril suspensions according to Hoh *et al.*[12] Myofibrils (−200 mg) were dissolved with 10 volumes of extraction buffer solutions containing 100 mM sodium pyrophosphate, pH 8.8, 5 mM EGTA, and 2 mM dithiothreitol at 2°C. The suspensions were centrifuged for 3 h at 48,000*g*. The supernatant containing extracted myosin was mixed with an approximately equal volume of glycerol and stored at −20°C. Myosin concentrations were

determined by Bio-Rad Protein Assay (Bio-Rad, Hercules, CA), based on the Bradford dye-binding procedure.[13] Purity of the isolated cardiac myosin was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis according to the methods of Fritz *et al.*[14]

The method used for polyacrylamide gel electrophoresis containing pyrophosphate buffer was described by Hoh *et al.*[12] Tube gel electrophoresis (Hoefer SE 600; Hoefer Scientific Instruments, San Francisco, CA) was run on 6-mm-ID tubes containing low percentage polyacrylamide gels (4% T and 3% C; T% = the total monomer concentration, and C% = the cross-linking monomer concentration) in 20 mM sodium pyrophosphate (pH 8.8) and 10% glycerol. Myosin samples (100–150 μl) in 50% glycerol were loaded directly on the top of the gels and run for 24 h with a constant current of 20 mA (Hoefer PS 500XT power supply, Hoefer Scientific Instruments). To avoid pH changes, the temperature of the recirculating cooling system supplied by a refrigerated circulator bath (B401-D; Firstek Scientific Corp., Taipei, Taiwan) was set at 0°C, and Shellzone antifreeze (Shell Oil Company, Houston, TX) was added. The running buffer was recirculated between the upper and lower tanks with a connection of tubing through a roller pump (EYELA RP-1000; Tokyo Rikakikai Co., Ltd., Tokyo, Japan). Gels were subsequently stained for protein with Coomassie Brilliant Blue R (BDH Laboratory Supplies, Poole, United Kingdom). The stained gels were photographed with a Kodak Digital Science DC 40 camera (Eastman Kodak Company, Rochester, NY) and analyzed by a computer program with Kodak Digital Science 1D Image Analysis Software.

To assay the myosin ATPase activity, 1–2 nmol cardiac myosin was included in a total volume of 10 μl reaction solution containing 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM KCl, 2.5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethane-sulphonyl fluoride, 100 mM ATP, and 0.5 mCi [α-^{32}P]ATP (3,000 Ci/mmol; Amersham Corp., Buckinghamshire, United Kingdom). Reactions were conducted at 37°C for 70 min and then terminated by the addition of EDTA to a final concentration of 20 mM. At the end of incubation, each reaction mixture (0.5 ml) was spotted on a thin-layer chromatographic plate (polyethyleneimine–cellulose; BDH Laboratory Supplies), and the plastic plate was developed with 0.5 M LiCl and 0.5 M formic acid for 70 min to separate the products of ATP hydrolysis.

The steady state measurements were also performed by incubating the extracted myosin (−0.041 mg) in 50 mM Tris-HCl, pH 7.5, 2.5 mM ATP, and 5 mM $CaCl_2$ for the Ca^{2+}–ATPase assay, and in 50 mM Tris-HCl, pH 7.5, 4 mM ATP, 4 mM EDTA, and 150 mM KCl for the K^+–ATPase determination. The inorganic phosphate was assayed by adding the malachite green reagent, as previously described.

Ca^{2+}-dependent Regulation of Cardiac Myofibrillar Adenosine Triphosphatase

To test if the different Ca^{2+}-dependent regulation of the actin–myosin interaction occurs in the hearts of normal and MH-susceptible pigs, the steepness of the Ca^{2+}–ATPase activity relation was measured in the two types of cardiac myofibrils. The measured ATPase activity at the actual pCa (−log [Ca^{2+}]) was subtracted from the activity at pCa 8.0. The subtracted activity (T_x) was normalized to the activity value (T_0) at saturating pCa. If the normalized ATPase ($U = T_x/T_0$) is used, then a straight line is obtained with the expression of log[U/(1 − U)] *versus* the logarithm of the Ca^{2+} concentration. This plot was fitted data to the Hill equation:

$$\log[U/(1 - U)] = n(\log[Ca_x]) + \log k$$

where $[Ca_x]$ is the actual Ca^{2+} concentration, n (Hill coefficient) is the slope, and k is the x-axis intercept of the fitted line. The Hill coefficient is a measure of cooperativity for the Ca^{2+}-activated ATPase activity. By using the constants derived from the Hill equation, the curves of the normalized ATPase activity (T_x/T_0) *versus* pCa were fit by computer with the equation:

$$(T_x/T_0) = [Ca_x]^n/((EC_{50})^n + [Ca_x]^n)$$

where EC_{50} is the Ca^{2+} concentration giving 50% activation of ATPase.

The pCa values were calculated by the computer program EQCAL (Biosoft, Cambridge, United Kingdom) with constants tabulated by Fabiato and Fabiato.[15] During our experimental conditions, the apparent stability constant for CaEGTA was taken to be 7.76×10^{10} M^{-1}.

Statistics

Quantitative values are presented as mean ± SD, except that data derived from Hill equation are expressed as mean ± SEM. Statistics were performed by a non-paired Student *t* test, with *P* values less than 0.05 considered significant.

Results

Examination of Porcine Malignant Hyperthermia

In addition to *Hal-1843* nucleotide examination, Duroc and Landrace pigs were diagnosed for MH susceptibility by halothane contracture test 1–8 months before the experiments. To differentiate between the effect of the MH gene by itself and the effect of halothane exposure, Berkshire pigs were genotyped without exposure to halothane. Experiments performed on these two different groups of MH-diagnosed animals were expected to determine the roles of the prior MH episode by halothane and the MH gene effect in the origin of myocardial changes in MH-affected hearts.

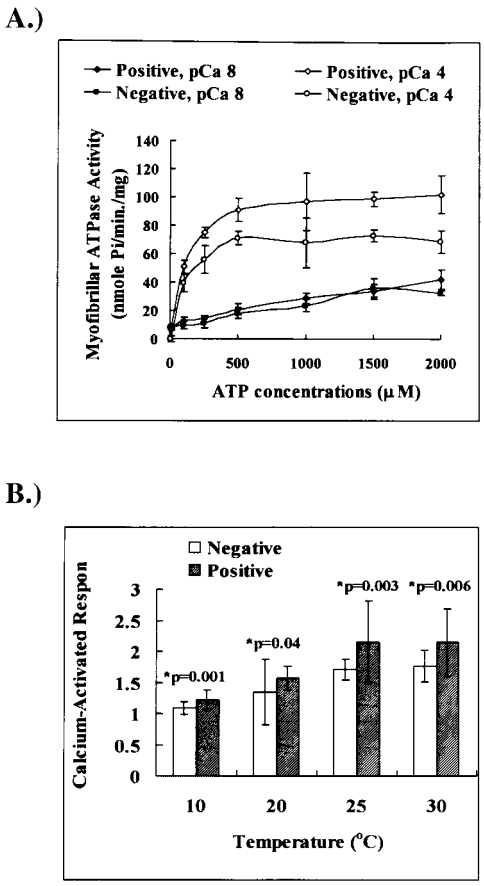

Fig. 1. Effects of varying the adenosine triphosphate (ATP) substrate concentrations (*A*) and temperatures (*B*) on cardiac myofibrillar ATPase activity (*A*) and changes of Ca^{2+}-activated response (*B*) in the hearts of normal and malignant hyperthermia (MH)-affected Landrace pigs. Different amounts of ATP (0.1–2 mM) were added to myofibril suspensions (−0.18 mg) for assaying the ATPase activity at pCa 8.0 and 4.0. Ca^{2+}-activated response was determined by the ratio of cardiac myofibrillar ATPase activity at pCa 4 to that at pCa 8. SD bars as indicated at each point. In all cases, data contain at least 10 measurements from three different animals.

Malignant Hyperthermia Effect on Cardiac Myofibrillar Adenosine Triphosphatase Activity in Halothane Prechallenged Pigs

It has been suggested that a defect in cardiac muscle is associated with the syndrome of MH.[16] However, the molecular basis of the defect is unknown. To determine if a defective site occurs in the myofibrillar proteins, we conducted measurements on the ATPase activity of cardiac myofibrils derived from MH-susceptible and nonsusceptible pigs. For experimental animals with halothane exposure, figure 1 shows that the Ca^{2+}-dependent ATPase activity of cardiac myofibrils is greater for MH-susceptible pigs than for normal animals over a wide range of ATP concentrations and temperatures. When the ATP concentrations were varied from 0.1 to 2 mM, the MH-affected cardiac myofibrils showed the greater Ca^{2+} activation of the enzymatic hydrolysis of ATP as compared with the normal animals (fig. 1A). The greater Ca^{2+} activation of the ATPase activity could be seen in the MH-affected porcine cardiac myofibrils in the temperatures varying from 10°C to 30°C as well (fig. 1B).

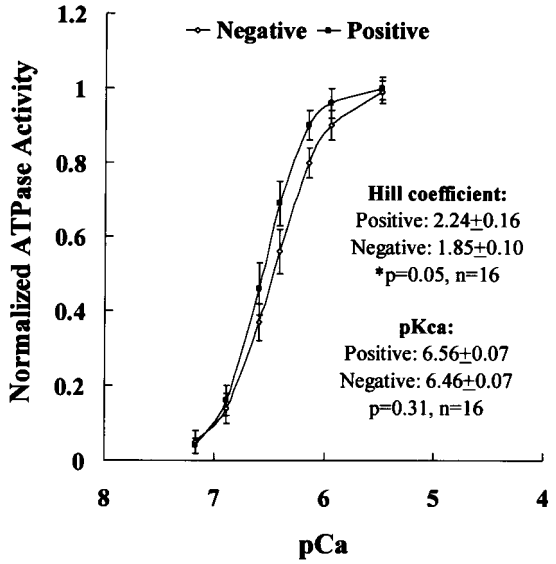

Fig. 2. Cumulative plots of cardiac myofibrillar adenosine triphosphatase (ATPase) activity *versus* pCa in cardiac muscles of normal and malignant hyperthermia–susceptible pigs. Relative myofibrillar ATPase activity at each pCa was obtained by subtracting the ATPase activity measured at each pCa from that at pCa 8.0, and then normalizing the value to the difference at pCa 4.0. The curve was fitted to the data using the Hill equation as described in Methods. Hill coefficient and the pCa value for giving 50% activation of ATPase activity (pKca) is indicated. All values were presented as mean ± SEM. At least three different animals were killed for the measurements. Numbers of measurement (n) as indicated. A Student *t* test was used to calculate *P* value. Asterisk indicates significant difference between the two myocardial preparations.

Malignant Hyperthermia Effect on the Ca^{2+} Regulation of Cardiac Activation in Halothane Prechallenged Pigs

To further characterize the effect of MH on the Ca^{2+} dependence of cardiac myofibrillar ATPase activity, a plot of the normalized ATPase activity *versus* pCa was studied in normal and MH-affected cardiac myofibrils (fig. 2). The steepness of the Ca^{2+} ATPase activity relation (Hill coefficient) is apparently greater for the myocardial preparations in MH-susceptible pigs than in normal animals. However, the Ca^{2+} concentrations giving half-maximal activation (pKca) are not significantly different in these two groups. Thus, a greater cooperativity with no changes in the Ca^{2+} sensitivity was observed in cardiac myofibrils of MH-susceptible pigs as compared with normal animals. As reported by Harrison and Bers,[17] varying temperatures altered the Ca^{2+} sensitivity of skinned fibers from rat, guinea pig, and frog ventricular muscle. In agreement with their findings, our results (data not shown) showed that Ca^{2+} sensitivity was reduced by decreasing the temperature from 30°C to 20°C in both types of cardiac myofibrils.

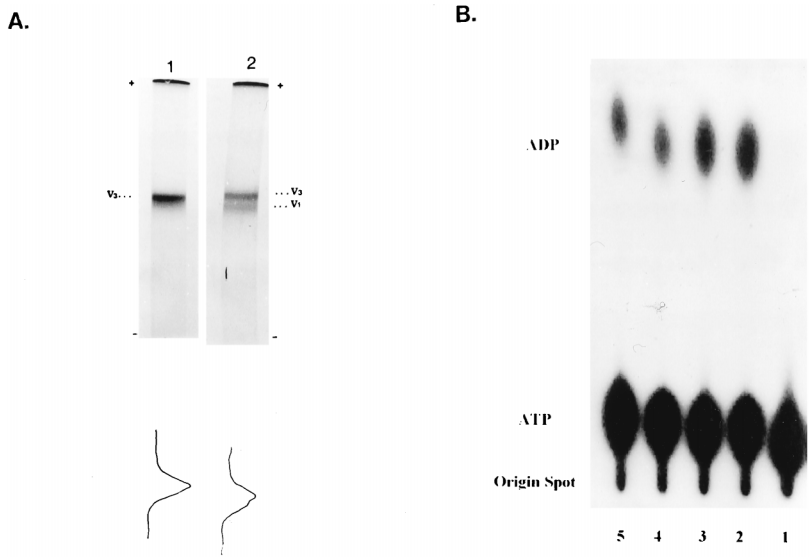

Fig. 3. (*A*) The electrophoretic profile and gel scanning of cardiac myosin isozymes from the hearts of normal and malignant hyperthermia–susceptible Landrace pigs with prior halothane challenge. Lane 1: myosin isolated from normal hearts; lane 2: myosin from malignant hyperthermia–affected hearts. V1 and V3 myosin is indicated. Myosin (50 μl per tube) was loaded on each gel. (*B*) The myosin adenosine triphosphatase assay for cardiac myosin isolated from the two types of porcine hearts. Animals were diagnosed for malignant hyperthermia susceptibility by halothane contracture test 1–8 months before the experiments. The assay was run on thin-layer chromatography as described in Methods. Lane 1 is the mixture without myosin; lanes 2 and 3 are the mixtures with 1 and 2 nmol of malignant hyperthermia–affected cardiac myosin, respectively; lanes 4 and 5 are the mixtures with 1 and 2 nmol of normal cardiac myosin, respectively. The original spot, adenosine triphosphate (ATP), and adenosine diphosphate (ADP) position are as indicated.

Malignant Hyperthermia Effect on Cardiac Myosin Isozyme Expression in Halothane Prechallenged Pigs

One possible explanation for the increased myofibrillar ATPase activity and the steepness of the Ca^{2+}–ATPase activity relation in the hearts of MH-susceptible pigs is that there is a shift of cardiac myosin isozyme in MH-affected hearts. A method for resolving different cardiac myosin isoforms on pyrophosphate gel electrophoresis, as described by Hoh *et al.*,[12] was used to test this possibility. A typical electrophoretic profile and gel scanning for cardiac myosin extracted from normal and MH-affected hearts is shown in figure 3A. Ventricular myosin from MH-affected porcine hearts exhibited two myosin bands. One myosin band (V1) has a faster mobility than the other band (V3). In contrast, myosin extracted from the control cardiac myofibrils only had one major V3 band, in agreement with a previous report by Lompre *et al.*[18] The presence of the V1 myosin is associated with the increase in the myosin ATPase activity, as assayed by analysis of hydrolytic products on the thin-layer chromatography (fig. 3B). A greater spot area of adenosine diphosphate in MH-affected cardiac myosin (lanes 2 and 3) than in normal myosin (lanes 4 and 5) indicated that the increased ATP hydrolysis by the myosin appears in the hearts of MH-susceptible pigs.

Table 1 shows the quantitative analysis of relative amounts of myosin isozymes and adenosine diphosphate production by cardiac myosin isolated from the ventricles of MH-susceptible (n = 3) and normal (n = 3) Landrace pigs. The amount of V1 myosin relative to total myosin (V1 + V3) in the hearts of MH-affected pigs was 44.3 ± 9.4%. This increased V1 myosin (−44%) expression in MH-affected hearts is accompanied by an increase of myosin ATPase activity (−25%) in MH-affected hearts, as quantitated by the adenosine diphosphate release. In addition, the myosin ATPase activity was also measured by a more commonly used method in which the steady-state inorganic phosphate release by the myosin was determined by adding malachite green reagent.[10] Means of triplicate measurements obtained with the Ca^{2+}-activated myosin ATPase activity (nmoles of inorganic phosphate per milligram myosin per minute) were −352 and −280 for the MH-affected and normal porcine cardiac myosin, respectively. Estimation by this assay of the inorganic phosphate release is in good agreement with that by the thin-layer chromatography assay of the adenosine diphosphate release. Thus, a shift in cardiac myosin isozyme expression from the V3 to V1 is correlated with increases in the myosin ATPase activity in the hearts of MH-susceptible pigs that were diagnosed by a halothane contracture test.

Table 1. Percentages of Myosin Isozymes and ADP Release Activity of Cardiac Myosin Isolated from Ventricles of Halothane-positive and -negative Landrace Pigs

	Positive	Negative
Myosin content (n = 5)		
Relative amounts of V1 (%)	44.3 ± 9.4	
Relative amounts of V3 (%)	55.7 ± 9.4	100
ADP release activity (n = 4)		
(/nM myosin/70 min)	0.35 ± 0.03	0.28 ± 0.03*

Percentages of cardiac myosin isozymes (V1 and V3) were obtained by quantitative analyses of pyrophosphate gels shown in figure 3A. Adenosine diphosphate (ADP) release activity of the two types of cardiac myosins was quantitated by calculating the ratio of the spot area of ADP to the sum of the spot areas of ADP and adenosine triphosphate (ATP) on the thin-layer chromatography radiogram shown in figure 3B. Values are mean ± SD of measurements (n) from three different Landrace pigs. *P* values were calculated by Student *t* test.

* Significant difference between the two myosin preparations; $P = 0.004$.

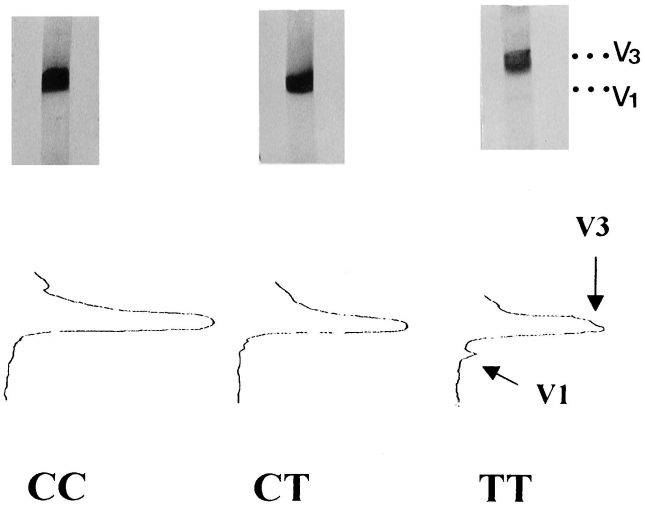

Fig. 4. The electrophoretic profile (*top*) and gel scanning (*bottom*) of cardiac myosin isozymes in the hearts of different malignant hyperthermia–genotyped (CC, CT, and TT) pigs without exposure to halothane. Malignant hypothermia genotyping of animals is described in Methods. Myosin was overloaded (−250 μl per tube) to detect the V1 myosin on the gel.

Cardiac Myosin Isozyme Distribution in the Hearts of Different Malignant Hyperthermia–Genotyped Pigs

As reported by Roewer *et al.*,[19] halothane exposure initiates cardiac symptoms in MH-susceptible pigs. It is possible that the changes in cardiac myosin isozyme distribution and ATPase activity observed in this study may not be related to a primary difference in the hearts of MH-susceptible pigs, but is a result of the episode of MH induced by halothane. To differentiate between these two possibilities, animals were genotyped without halothane challenge. Figure 4 shows that a significant expression of V1 myosin was detected in the heart of the homozygous mutant (TT), but not in the carrier (CT) or the normal (CC). The relative amount of V1 myosin to total myosin in the TT mutant was quantitated as −5% (table 2). Consistent with the low V1 content, the myofibrillar ATPase activity was not significantly different among the three different genotyped porcine hearts (table 2).

Table 2. Myofibrillar ATPase Activity and Myosin Isozyme Distribution in the Hearts of Different MH-genotyped Berkshire Pigs

Genotype	Myofibrillar ATPase Activity (nM Pi · min^{-1} · mg^{-1})	Myosin Isozyme (%) V1	Myosin Isozyme (%) V3
CC	48.66 ± 7.84 (N = 71)		100 (N = 10)
CT	51.18 ± 7.0 (N = 23)		100 (N = 8)
TT	49.16 (N = 2)	5.05	94.94 (N = 2)

Animals were genotyped by Hal-1843 nucleotide examination. Cardiac myofibrillar adenosine triphosphatase (ATPase) activity at pCa 4.0 (−log[Ca^{2+}]) was assayed as described in the Methods. Cardiac myosin isozymes were analyzed by pyrophosphate-containing gel electrophoresis according to Hoh's method. Values are mean ± SD. Numbers (N) of animals studied are indicated. Each animal contains at least three measurements.

MH = malignant hyperthermia; Pi = phosphate.

Discussion

Myocardial defects have been recognized in human and porcine MH.[3] However, it is not known whether the defects reflect a primary difference in the myocardial cells or are a consequence of the hyperthermic stress. Fenoglio and Irey[20] reported that patients suffering from MH have similar pathologic changes in the ultrastructure of both myocardium and skeletal muscle. Huckell *et al.*[21] reported that ventricular arrhythmias or chest pain occur in MH-susceptible patients in the absence of pyrexic crises. These findings suggest that the occurrence of primary defects in cardiac muscles might contribute to cardiac dysrhythmias during the MH crisis. On the other hand, an *in vitro* study by Roewer *et al.*[22] showed that in the absence of triggering agents, the electrophysiologic properties of ventricular muscle cells of MH-susceptible pigs did not differ from those of normal animals. These investigators also showed that halothane exposure caused abnormal alterations in membrane action potentials of cardiac muscles from MH-susceptible pigs. Thus, they suggested a latent disorder in the hearts of MH-susceptible pigs that becomes apparent in the presence of halothane. In the present study, we found another abnormality in cardiac myofibrils that occurs in association with porcine MH. In the absence of halothane challenge, there is no significant difference in myofibrillar ATPase activity in the hearts of different MH-genotyped pigs (CC, CT, and TT), whereas there is a small but significant increase in V1 myosin expression (−5%) of the mutant (TT). In contrast, MH-susceptible pigs with prior halothane exposure showed a greater increase in the V1 myosin expression (−44%) associated with an increase in myosin ATPase activity (−25%). A simple interpretation for the difference seen between the two experimental groups of MH-susceptible pigs is that there is a genetic shift of cardiac myosin isozyme in MH-susceptible pigs, and this could be further induced in these animals by halothane. Because different strains of pigs (Berkshire as opposed to Duroc and Landrace) were investigated, the result obtained does not rule out the possibility for a strain-related difference between the halothane-exposed and genotyped pigs. Nevertheless, the present study provides evidence of an increased V1 myosin expression in MH-susceptible pigs with and without prior exposure to halothane.

Lompre *et al.*[23] demonstrated that ablation of the thyroid gland in adult rats caused a cardiac myosin V1 → V3 shift that was reversed by administration of thyroxine. Their data showed that the half-life of the α-myosin heavy chain (MHC) mRNA and the protein is −3 days and −7 days, respectively. It takes approximately 3–9 weeks to complete the changes in myosin isozyme V1 → V3 shift in the hearts of thyroid-ectomized rats. A similar

experiment needs to be performed on the time course of the halothane-induced change in myosin isoform expression. In the present study, with the time ranges of 1–8 months elapsing between halothane exposure and myosin analysis, all susceptible pigs with the prior halothane exposure showed an increased V1 myosin expression. Along with the finding on an additional group of MH-susceptible pigs based on genotyping without prior exposure to halothane, our study suggests that an intrinsic effect of MH would contribute to an increased expression of V1 myosin in susceptible animals. However, the mechanism for the effect of halothane exposure on a shift of cardiac myosin isozyme in MH-susceptible pigs is not currently understood.

As previously indicated, MH is characterized by increased levels of circulating catecholamine.[24] More recently, Scholz *et al.*[25] showed that during halothane-induced porcine MH, there are substantial changes in cardiovascular performance and myocardial metabolism in association with increased catecholamine concentrations. Studies of isoproterenol effects on force generation of trabeculae isolated from the right ventricles of normal (Yorkshire) and MH-affected (Pietrain) porcine hearts indicated that there is a greater β-adrenergic activation in MH-affected hearts.[26] Levels of the second messenger of inositol triphosphate were also found to be increased in the hearts of MH-susceptible pigs.[25] All of these effects of catecholamines–halothane would cause a tachycardia and positive inotropic effect, which is presumably what occurs during MH.

There are short- and long-term effects of increased β-adrenergic activation of cardiac muscle. The short-term effect involves an enhanced intracellular Ca^{2+} transient with a resultant increase in myocardial contractility, as well as effects on pacemaker cells to alter heart rate. The long-term effect involves changes in gene expression with a V3 to V1 shift. Studies with heterotopically transplanted rat hearts by Geenen *et al.*[27] showed that repeated catecholamine surges caused an increased synthesis of MHC. The present study involving exposure of MH-susceptible pigs to halothane suggests that the long-term effect of catecholamine–halothane increased the V1 myosin expression in the hearts of susceptible animals. Future studies are needed to correlate catecholamine levels with the degree of cardiac myosin isozyme shift in cardiac muscle cells of MH-susceptible pigs.

Changes in the expression of cardiac MHC isoforms would cause alterations of contractile function of the hearts. A recent study by Nakao *et al.*[28] showed that decreases in mRNA levels of α-MHC gene in ventricular cardiocytes are correlated with systolic dysfunction in human heart failure. Their study suggested that the potential modulation of V1/V3 ratio could be adapted to compromise the altered cardiac function in pathologic conditions. The observed change of cardiac myosin isozyme distribution in MH-susceptible pigs would lead to an increased oxygen demand and reduced energetic efficiency of the heart. Such changes, occurring in addition to the hyperthermic stress and increased catecholamine levels, would further compromise cardiac function.

In the recent report by Metzger *et al.*,[29] the hypothyroid rat was used as an animal model for studying the effect of a shift of myosin heavy chain $\alpha \rightarrow \beta$ in the heart on the thin filament activation of cardiac muscle. Their results showed that an $\alpha \rightarrow \beta$ isoform shift was associated with a reduction in skinned fiber Ca^{2+} sensitivity with no change in cooperativity. However, experimental differences such as different pathologic conditions and different species of animals studied may contribute to the disparate results. As found in this study, the cooperativity of the Ca^{2+}-activated myofibrillar ATPase was increased in MH, although the Ca^{2+} sensitivity was unchanged (fig. 2). This increased cooperativity is very likely caused by a shift of cardiac myosin activity and a change of thin-filament protein–protein interactions. Using electron microscopy and optical diffraction, Weisberg and Winegrad[30] showed that the structure of the thick filaments from the ventricles with the V1 myosin is different from that with the V3 myosin. A rigid configuration of crossbridges exists in the thick filaments with the V1 myosin, whereas crossbridges in filaments with the V3 myosin are more flexible. This difference in flexibility might influence the kinetics of crossbridge attachment and detachment. Thus, the cardiac myofibrils of MH-susceptible pigs with substantial amounts of the V1 myosin would produce a greater cooperativity of the contractile response to the Ca^{2+} binding.

In summary, there is a genetic shift in myosin isoform expression in the hearts of MH-susceptible pigs. Alterations of cardiac myosin isozyme distribution and contractile ATPase activity could be further enhanced by halothane exposure.

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