J Pharmacol Sci **111**, 405 – 415 (2009)

Journal of Pharmacological Sciences

Full Paper

Alterations in Dystrophin-Related Glycoproteins in Development of Right Ventricular Failure in Rats

Takuya Daicho[1], Yoriko Daisho[1], Suguru Kojima[1], Saori Takano[1], Yugo Tejima[1], Tetsuro Marunouchi[1], Norio Takagi[1], Satoshi Takeo[1], and Kouichi Tanonaka[1,*]

[1]*Department of Molecular and Cellular Pharmacology, Tokyo University of Pharmacy and Life Sciences, Hachioji 192-0392, Japan*

Received July 8, 2009; Accepted October 13, 2009

Abstract. Genetic depletion of the dystrophin-related glycoprotein (DRGP) complex causes cardiomyopathy in animals and humans. The present study was undertaken to explore the possible involvement of alterations in DRGP in the development of the right ventricular failure in monocrotaline-administered rats (MCT rats). At the 6th and 8th weeks after subcutaneous administration of 60 mg/kg monocrotaline, echocardiographic examination showed that cardiac output indices were decreased and that the right ventricular Tei indices were increased, suggesting that right ventricular failure occurs, at the latest, by 6 weeks after monocrotaline-administration. The levels of α- and β-sarcoglycan and β-dystroglycan in the right ventricle of the MCT rats at the 6th and 8th weeks were markedly decreased, and these decreases were inversely related to the increase in the right ventricular Tei index of the MCT-administered animals. The content and activity of the Ca^{2+}-activated neutral protease m-calpain in the right ventricle of the MCT rats were increased at the 4th to 8th weeks and those of matrix metalloproteinase-2, at the 6th and 8th weeks. These results suggest that m-calpain– and/or matrix metalloproteinase-2–mediated alterations in the contents of α-sarcoglycan, β-sarcoglycan, and β-dystroglycan may be involved in the development of right ventricular failure in MCT rats.

Keywords: dystrophin-related glycoprotein, right ventricular failure, calpain, matrix metalloproteinase, monocrotaline

Introduction

Pulmonary artery hypertension is a progressive disease caused by a variety of pulmonary and/or cardiac disorders. Pulmonary artery hypertension is commonly characterized by increases in the pulmonary vascular resistance or in the right ventricular systolic pressure. A rise in the right ventricular systolic pressure induces right ventricular hypertrophy and right ventricular failure, eventually leading to premature death. It is well recognized that a single subcutaneous administration of the pyrrolizidine alkaloid monocrotaline (MCT) to various species of experimental animals causes cardiovascular and pulmonary disorders similar to those seen in patients with pulmonary artery hypertension (1), including severe right ventricular hypertrophy (2) and right ventricular failure (3). Despite extensive studies, the fundamental mechanisms responsible for the development and progression of right ventricular failure have not been fully elucidated.

Dystrophin-related glycoprotein (DRGP) complexes, which contain dystrophin, sarcoglycans, and dystroglycans, have been suggested to play an important role in the structural stabilization of the sarcolemmal integrity (4, 5). Inherent mutation or depletion of sarcoglycans and/or dystrophin genes causes muscular dystrophy and dilated cardiomyopathy in humans and hamsters (6, 7). Recent studies have shown that δ-sarcoglycan gene transfection mediated by adeno-associated virus improves cardiac function, sarcolemmal stability, and survival of TO-2 hamsters (8), which are an animal model mimicking human dilated cardiomyopathy. Furthermore, it has been reported that degradation of DRGP occurs in enterovirus-induced cardiomyopathy (9). Although

*Corresponding author. tanonaka@ps.toyaku.ac.jp
Published online in J-STAGE
doi: 10.1254/jphs.09208FP

degradation of DRGP complexes occurs in genetically induced or virus infection-induced cardiomyopathy, alterations in DRGP in non-genetically induced cardiomyopathy or heart failure remain to be elucidated. In a previous study, we showed that the contents of α-sarcoglycan and dystrophin were decreased in the failing rat heart following coronary artery ligation (10), indicating the contribution of the degradation of DRGP to non-genetically induced or non-virus–mediated heart failure. In addition, we showed that the protein content of the Ca^{2+}-dependent cysteine protease calpain is increased, and its proteolytic activity is also elevated, in the failing rat heart (10). This protease is present in cardiac muscles and is considered to play a role in protein turnover in them (11). Yoshida et al. (10) also reported that the degradation of α-sarcoglycan, β-sarcoglycan, and dystrophin might be mediated by m-calpain.

Matrix metalloproteinases (MMPs) are a family of over 20 endopeptidases, classified into subgroups based on substrate preference; and they act as effectors of cell migration, cytotoxicity, and tissue remodeling via the degradation of extracellular matrix components (12). Based on the results of in vitro experiments, several investigators postulated that MMP-2, a member of the gelatinase family, may be involved in the degradation of β-dystroglycan (13 – 15). However, changes in the DRGP content and content or activity of these proteolytic enzymes in the right ventricular failure remain unclear. The present study was designed to elucidate possible involvement of quantitative and qualitative alterations in the DRGP complex in the right ventricular muscle in rats with right ventricular failure following MCT-induced right ventricular hypertrophy.

Materials and Methods

Animals

Male Wistar rats (SLC, Hamamatsu) weighing 190 – 210 g were used in the present study. The rats were randomly selected to receive either a subcutaneous injection of 60 mg/kg MCT or an equal volume of saline. The animals were conditioned according to the Guide for the Care and Use of Laboratory Animals as published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The protocol of this study was approved by the Committee of Animal Use and Welfare of Tokyo University of Pharmacy and Life Sciences.

Hemodynamic parameters

Rats at the 2nd (2w-MCT), 4th (4w-MCT), 6th (6w-MCT), and 8th (8w-MCT) weeks after MCT administration and the corresponding controls (2w-CON, 4w-CON, 6w-CON, and 8w-CON rats, respectively) were anesthetized by an intraperitoneal administration of pentobarbital sodium. A polyethylene catheter (PE50) was then introduced into the right ventricle through the right jugular vein to measure the right ventricular systolic pressure and right ventricular end-diastolic pressure. Central venous pressure was measured via a catheter that was placed in the right jugular vein and advanced to the level of the right atrium. The mean arterial blood pressure and heart rate were measured by means of a pressure transducer attached to a cannula placed into the left femoral artery and a heart rate counter, respectively (16).

Tissue weight and histological study

After hemodynamic measurement, the heart was isolated and dissected free from the atria, aorta, and pulmonary artery. The free wall of the right ventricle, the left ventricle, and septum were separated and then weighed. The ratio of right ventricular weight to left ventricle + septum weight was calculated to assess right ventricular hypertrophy (17). The right ventricular and lung weights were measured, and their ratios to the body weight were also calculated.

Measurement by echocardiography

Transthoracic echocardiography was performed on rats according to the method described previously (18). In the present study, pathophysiological alterations of the rats were examined at the 2nd, 4th, 6th, and 8th weeks after administration of MCT. The animals were first anesthetized with pentobarbital sodium at 40 mg/kg, i.p., and then the hair on their chest was shaved-off before the examination. Two-dimensional and Doppler imagings were performed by using a ProSound 5500^{R} device (Aloka, Tokyo) with a 10-MHz transducer. The transthoracic echocardiographic probe was placed to obtain long-axis and apical four-chamber views. To evaluate right ventricular function, we measured cardiac output at the pulmonary artery. After determination of the pulmonary arterial flow and heart rate (HR), velocity time integral (VTI) and pulmonary arterial diameter (PAD) were measured in the long-axis view. Cardiac output (CO) and stroke volume (SV) were calculated according to the following equations (18, 19): $CO = (PAD/2)^2 \times \pi \times VTI \times HR$, $SV = CO/HR$. In addition, to characterize the pulmonary outflow, we measured the pulmonary artery flow acceleration time. Pulmonary artery flow acceleration time was estimated as the difference between the time that the increase in the systolic blood flow started and the time at which the peak velocity of the pulmonary outflow was reached (20). The right ventricular Tei index, defined below, was

assessed from Doppler recordings of right ventricular inflow and outflow. From tricuspid inflow in the apical four-chamber view, the time interval from cessation to onset of tricuspid inflow was measured (*a*-interval). Ejection time (*b*-interval) was measured from the right ventricular outflow velocity curve recorded in the long-axis view. The right ventricular Tei index, an increase in which is considered to be a sign of right ventricular dysfunction, was calculated as $(a - b) / b$ (21, 22). Then, 8 consecutive cardiac-cycle images were measured and averaged.

Western blotting

The contents of right ventricular DRGP complex proteins were determined by a modified method described previously (23). Briefly, the right ventricle was homogenized in buffer containing 250 mM sucrose, 1 mM EGTA, 1 mM dithiothreitol (DTT), 0.3 mM phenylmethanesulfonyl fluoride (PMSF), 20 mM HEPES (pH 7.40), and protease inhibitor cocktail (Roche, Mannheim, Germany). The homogenates were sampled for Western blot analysis. The samples were each boiled in the Laemmli buffer containing 125 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 0.002% bromophenol blue, and 10% β-mercaptoethanol. Then they were fractionated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% polyacrylamide gel for sarcoglycans, β-dystroglycan, m-calpain, MMP-2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and by a 4% SDS-PAGE gel for dystrophin, according to the method of Laemmli (24).

The proteins separated on the gel were transferred onto a polyvinylidene difluoride membrane (Immobilon; Millipore, Bedford, MA, USA) and then detected with their respective antibodies (10). The following antibodies were used: anti-α-sarcoglycan (NCL-a-SARC), anti-β-sarcoglycan (NCL-b-SARC), anti-γ-sarcoglycan (NCL-g-SARC), anti-dystrophin (NCL-DYS1-SARC), and anti-β-dystroglycan (NCL-b-DG) (Novocastra Lab., Newcastle upon Tyne, UK); anti-δ-sarcoglycan (H-55; SantaCruz Biochem., Santa Cruz, CA, USA); anti-MMP-2 (F-68; Daiichi Fine Chem., Toyama); and anti-GAPDH (MAB374; Millipore, Temecula, CA, USA) in Tris-buffered saline (TBS) containing 10% Block Ace (Dainippon-Sumitomo Pharmaceuticals, Osaka) and 0.1% Tween 20 and anti-m-calpain (SA-255; Biomol, Plymouth Meeting, PA, USA) in TBS containing 0.1% Tween 20. Thereafter, the proteins on the membrane were visualized by use of an ECL™ (Amersham Pharmacia Biotech, Buckinghamshire, UK); and their bands, developed on X-ray films, were semi-quantified by using NIH image software.

Casein zymography

The right ventricular calpain activity was determined by the method of Croall et al. (25), with some modifications. Briefly, the right ventricular muscle was homogenized in buffer containing 10 mM EDTA, 10 mM EGTA, 1 mM DTT, 0.3 mM PMSF, 1 mM benzamidine hydrochloride, and 50 mM 4-morpholinepropanesulfonic acid (MOPS, pH 7.50). Leupeptin (12 μM) was added during extraction of the right ventricle. After centrifugation at $16{,}000 \times g$ for 15 min, the resultant supernatant fluids were diluted with gel loading buffer to yield sample buffer containing 150 mM Tris-HCl (pH 6.8), 5% β-mercaptoethanol, 20% glycerol, and 0.02% bromophenol blue.

Casein (0.1%, w/v, sodium salt) was copolymerized with 10% (w/v) acrylamide as the separating gel. The casein gels were pre-run with a buffer containing 35 mM HEPES and 43 mM imidazole [pH 7.4; (26)] for 30 min (125 V) at 4°C. Samples including calpain were then loaded into the wells and subsequently electrophoresed at 125 V in an ice-water bath (27, 28). Better separation of autolyzed forms was observed when electrophoresis was continued for 60 min after the bromophenol blue had migrated out of the gel. The gel was then removed and incubated in 25 mM MOPS (pH 7.5), 10 mM DTT, and 5 mM $CaCl_2$ with slow shaking for 60 min. The gel was then further incubated overnight (24 h) at room temperature in the same buffer. Gels were fixed (10% acetic acid – 8% methanol) and stained in 0.2% Coomassie blue. The destained area revealed the presence of calpain enzyme protein activity.

Gelatin zymography

Gelatin zymography for MMP-2 from the right ventricular muscle was also performed. The right ventricular muscle was homogenized in buffer containing 150 mM NaCl, 10 mM $CaCl_2$, 0.05% Briji-35 (Wako, Osaka), 0.02% NaN_3, and 50 mM Tris-HCl (pH 7.60). After centrifugation at $10{,}000 \times g$ for 15 min, the resultant supernatant fluids were diluted with gel-loading buffer to yield sample buffer containing 125 mM Tris-HCl (pH 6.8), 4% SDS, 25% glycerol, and 0.01% bromophenol blue.

The right ventricular samples were subjected to electrophoresis on 10% SDS-PAGE co-polymerized with gelatin (0.1%) as the substrate for MMP-2. After electrophoresis, the gels were renatured in 50 mM Tris-HCl (pH 7.5) containing, 5 mM $CaC1_2$, 1 μM $ZnCl_2$, 0.02% NaN_3, and 2.5% Triton X-100 at room temperature for 1 h and then incubated in the same buffer without Triton X-100 at 37°C for 24 h. The gels were stained with 0.5% Coomassie blue for 10 min and then destained with 1% acetic acid and 30% methanol in the distilled

water. Proteolytic bands in zymography were quantified by scanning densitometry (NIH image).

Statistics

The results were expressed as the means ± S.E.M. Statistical significance of differences in echocardiographic parameters, hemodynamics, tissue weight, DRGP content, and contents and enzyme activities of m-calpain and MMP-2 were estimated by using two-way analysis of variance (ANOVA) followed by Fisher's PLSD correction for multiple pairwise comparisons. Pearson's correlation coefficient was used to examine the relationship between the right ventricular Tei index measured by echocardiography and the DRGP contents of right ventricular muscles and between α-sarcoglycan and β-dystroglycan contents of right ventricular muscle. Differences with a probability of 5% or less were considered to be significant ($P<0.05$).

Results

Tissue weight and related parameters

The time course of changes in body, heart, right ventricular, and lung weights of the rats and in parameters related to them are shown in Fig. 1. The body weight of the MCT-administered rats (MCT rats) was significantly lighter at the 4th to 8th weeks after MCT administration than that of the age-matched controls at those times (Fig. 1A). The heart weight and the ratio of heart weight/body weight of the MCT rat were significantly higher at the 4th to 8th weeks after MCT administration (Fig. 1: B and C). The right ventricular weight and the ratios of right ventricular weight / body weight and right ventricular weight / (left ventricular weight + septum weight), the latter being a body weight–independent index of right ventricular hypertrophy, were significantly greater at the 4th to 8th weeks after MCT administration (Fig. 1: D, E, and F). The lung weight and the

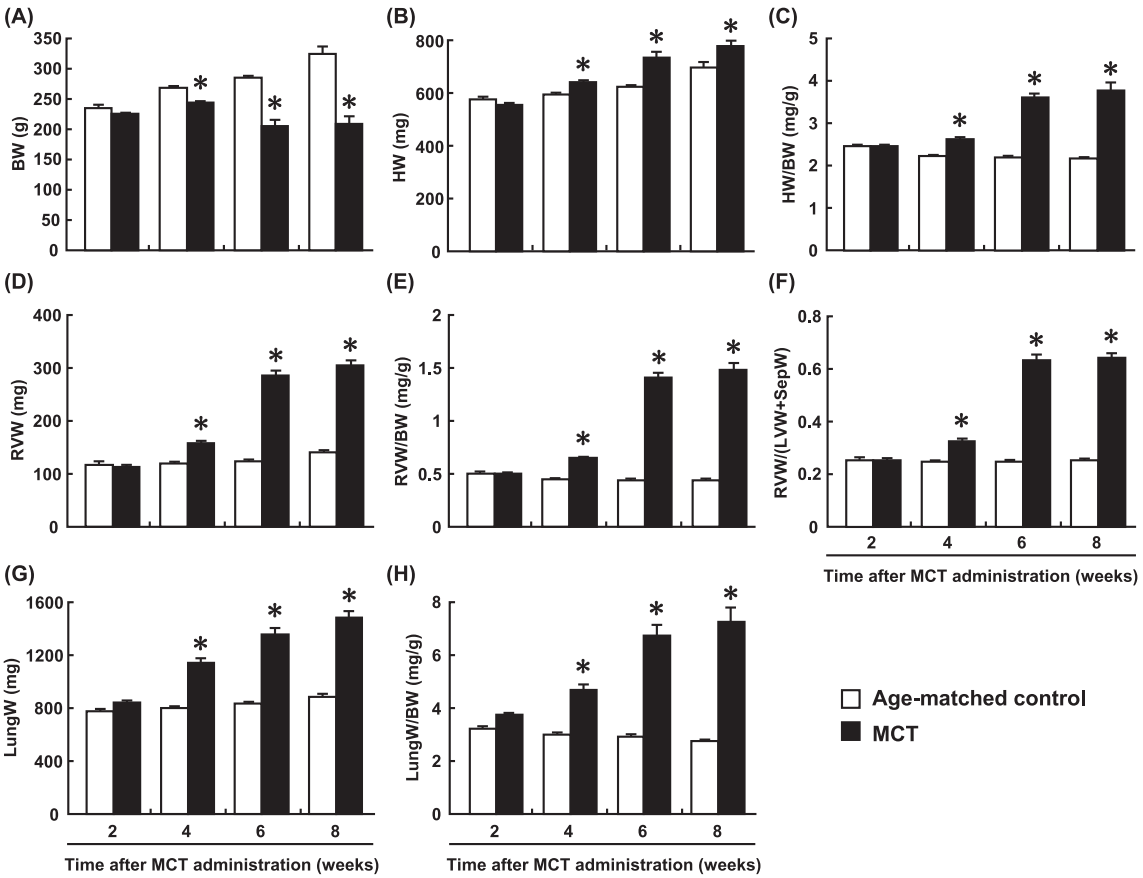

Fig. 1. Time course of changes in the tissue weight parameters of age-matched control (open columns) and MCT-administered rats (closed columns) at the 2nd, 4th, 6th, and 8th weeks after administration of saline and MCT, respectively. Each value represents the mean ± S.E.M. of 6 experiments. *$P<0.05$ vs. age-matched control group. Abbreviations: BW, body weight; HW, heart weight; HW/BW, heart weight / body weight; RVW, right ventricular weight; RVW/BW, right ventricular weight / body weight; RVW/(LVW + SepW), right ventricular weight / (left ventricular weight + septal weight); Lung W, lung weight; LungW/BW, lung weight / body weight; MCT, monocrotaline.

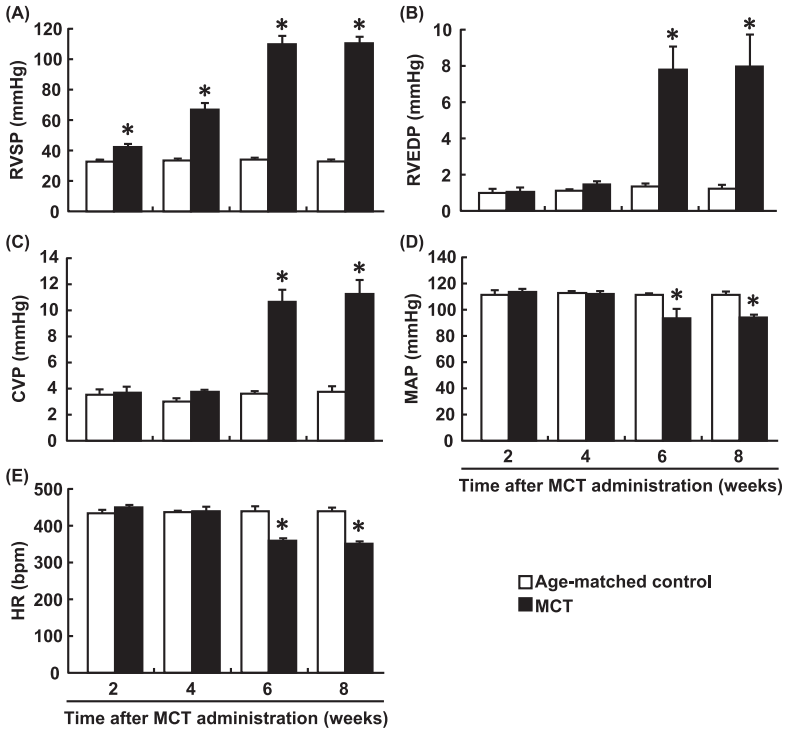

Fig. 2. Time course of changes in hemodynamic parameters of age-matched control (open columns) and MCT-administered rats (closed columns) at the 2nd, 4th, 6th, and 8th weeks after administration of saline and MCT, respectively. Each value represents the mean ± S.E.M. of 6 experiments. **P*<0.05 vs. age-matched control group. Abbreviations: RVSP, right ventricular systolic pressure; RVEDP, right ventricular end-diastolic pressure; CVP, central venous pressure; MAP, mean arterial blood pressure; HR, heart rate; MCT, monocrotaline.

ratio of lung weight/body weight of the 4w-, 6w-, and 8w-MCT rats were significantly greater than those of the controls (Fig. 1: G and H).

Hemodynamic parameters

Hemodynamic parameters of the CON and MCT rats were determined by the cannula method (Fig. 2). As compared with that of the corresponding CON rats, the right ventricular systolic pressure of the MCT rats, which is equivalent to the pulmonary arterial systolic pressure, was increased with time after MCT administration: the right ventricular systolic pressure of the 8w-MCT rats was approximately 340% of the corresponding CON value (Fig. 2A). The right ventricular end-diastolic pressure and central venous pressure were increased at the 6th and 8th weeks after MCT administration (Fig. 2: B and C). Decreases in mean arterial blood pressure and heart rate were observed at the 6th and 8th weeks after administration of the alkaloid (Fig. 2: D and E).

Echocardiographic parameters

Figure 3 shows cardiac parameters determined by the echocardiographic system to assess the progression of pulmonary artery hypertension in rats at multiple time points. Echocardiographic measurements were performed on CON and MCT rats at the 2nd, 4th, 6th, and 8th weeks after MCT administration. As compared with those of the CON rats, the cardiac output index, stroke volume index, and heart rate of the MCT rats were decreased at the 6th week (Fig. 3: A, B, and C). The pulmonary artery flow acceleration time, an estimate of the pulmonary arterial systolic pressure, was decreased at the 2nd to 8th weeks after MCT administration (Fig. 3D). The right ventricular Tei index, an increase in which indicates aggravation of the right ventricular function and provides prognostic information on a variety of myocardial conditions, was increased at the 4th to 8th weeks after MCT administration as compared with that for the corresponding CON rats (Fig. 3E).

Myocardial DRGP

After the in vivo measurement of hemodynamics, the contents of myocardial sarcoglycans, dystroglycan, and dystrophin proteins in the right ventricular muscle were quantified by the Western immunoblot method (Fig. 4). The α-sarcoglycan content in the right ventricle of the 4w-, 6w-, and 8w-MCT rats was decreased to approximately 70%, 45%, and 35%, respectively, of the corresponding CON value; and the β-dystroglycan content in the right ventricle of the 4w-, 6w-, and 8w-MCT rats was decreased to approximately 655%, 305%, and 15% of this value. The β-sarcoglycan content in the right ventricle of the 6w- and 8w-MCT rats was decreased to approximately 40% of the corresponding CON value. In the right ventricle of the 8w-MCT rats, the γ-sarcoglycan content was decreased to approximately 65% of the corresponding CON value. δ-Sarcoglycan and dystrophin contents in the right ventri-

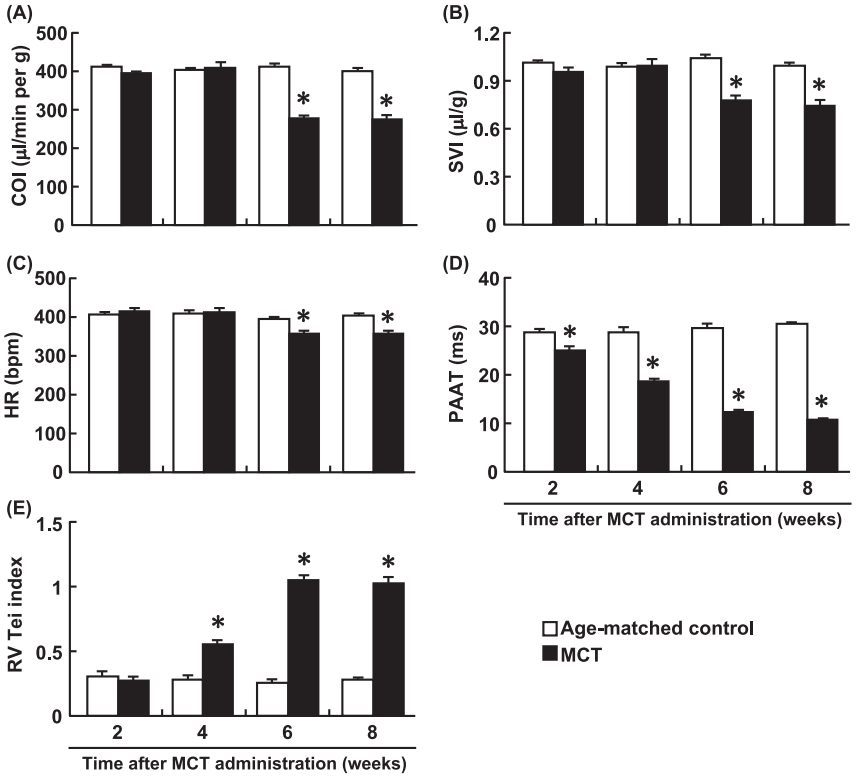

Fig. 3. Time course of changes in echocardiographic parameters of age-matched control (open columns) and MCT-administered rats (closed columns) at the 2nd, 4th, 6th, and 8th weeks after administration of saline and MCT, respectively. Each value represents the mean ± S.E.M. of 6 experiments. *P<0.05 vs. age-matched control group. Abbreviations: COI, cardiac output index; SVI, stroke volume index; HR, heart rate; PAAT, pulmonary artery flow acceleration time; RV Tei index, right ventricular Tei index; MCT, monocrotaline.

cle of the 4w-MCT rats were decreased to approximately 80% and 70%, respectively, of the corresponding CON value.

The DRGP content in the right ventricular muscle of the MCT rats was plotted against the right ventricular Tei index (Fig. 5). Each α-sarcoglycan, β-sarcoglycan, or β-dystroglycan content in the right ventricle was inversely and highly related to the right ventricular Tei index at the 2nd, 4th, 6th, and 8th weeks after administration of MCT (Fig. 5: A, B, and E). γ-Sarcoglycan and δ-sarcoglycan contents in the right ventricle were inversely related to the right ventricular Tei index (Fig. 5: C and D), whereas the dystrophin content did not correlate with this index (Fig. 5F).

When the α-sarcoglycan content in the right ventricular muscle of MCT rats was plotted against the β-dystroglycan content (Fig. 6), the former was highly related to the latter.

Myocardial calpain

Figure 7 shows the changes in the m-calpain content and its activity in the right ventricle of the 2w-, 4w-, 6w-, and 8w-MCT rats. The content of this enzyme in the right ventricle of the 4w-, 6w-, and 8w-MCT rats increased to approximately 135%, 170%, and 220%, respectively, of the corresponding CON value (Fig. 7A).

Changes in the proteolytic activity of m-calpain in the right ventricular cytosolic fraction of the CON and MCT rats were examined by using casein zymography. Casein gel, which contained the cytosolic fraction prepared from the right ventricle of the 2w-, 4w-, 6w-, or 8w-CON and MCT rats, was incubated in the presence of 5 mM $CaCl_2$ after electrophoresis. The caseinolytic activity of m-calpain in the right ventricle of the 4w-, 6w-, and 8w-MCT rats increased to approximately 160%, 240%, and 290%, respectively, of the corresponding CON value (Fig. 7B).

Myocardial MMP-2

Figure 8 shows changes in the MMP-2 content and its activity in the right ventricle of the 2w-, 4w-, 6w-, and 8w-MCT rats. The content of this metalloproteinase in the right ventricle of the 6w- and 8w-MCT rats increased to approximately 250% and 235%, respectively, of the corresponding CON value (Fig. 8A).

Changes in the proteolytic activity of MMP-2 in the right ventricle of the CON and MCT rats were examined by using gelatin zymography. Gelatin gel containing the cytosolic fraction from the right ventricle of the 2w-, 4w-, 6w-, or 8w-CON and MCT rats was incubated in the presence of 1 μM $ZnCl_2$ and 5 mM $CaCl_2$ after electrophoresis. The gelatinolytic activity of MMP-2 in the right ventricle of the 6w- and 8w-MCT rats increased to approximately 145% and 160%, respectively, of the

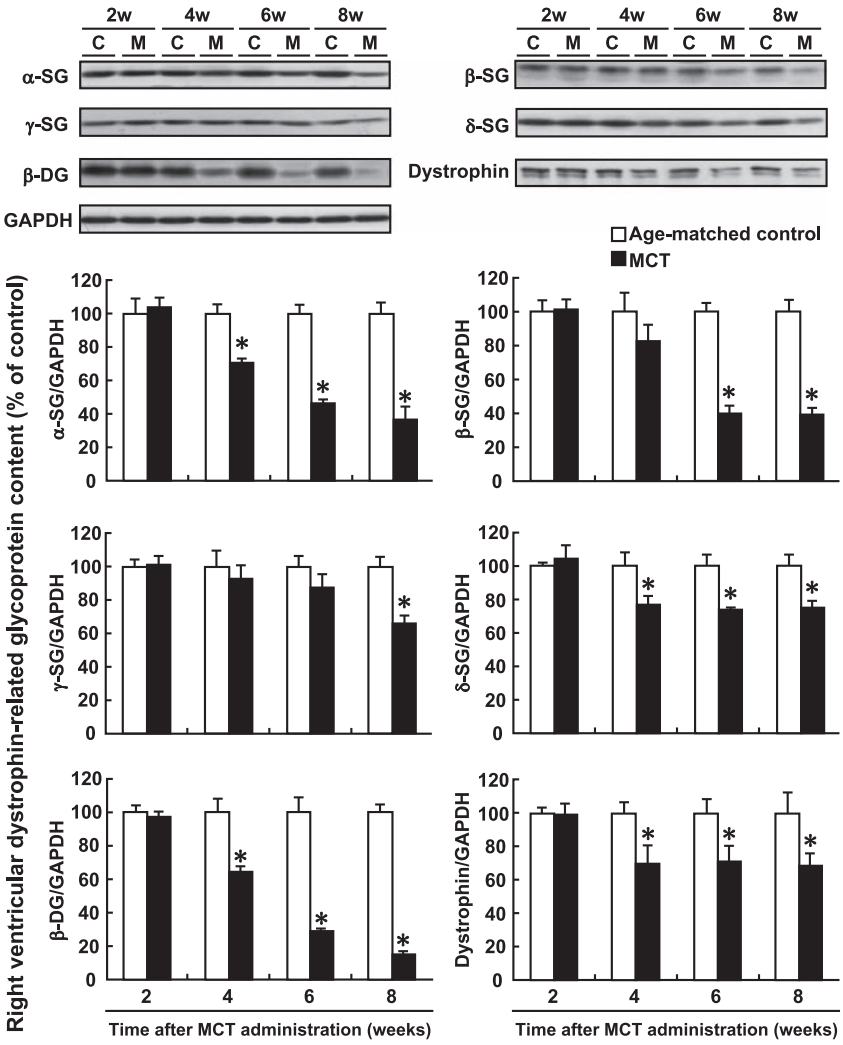

Fig. 4. Time course of changes in the DRGP contents including *α*-, *β*-, *γ*-, and *δ*-sarcoglycan (SG); *β*-dystroglycan (DG); and dystrophin in the right ventricular muscle of age-matched control (open columns) and MCT-administered rats (closed columns) at the 2nd, 4th, 6th, and 8th weeks after administration of saline and MCT, respectively. Upper panels show representative Western blots (A) and lower panels, semi quantified data (B). "C" and "M" indicate the age-matched control and MCT group, respectively. Each value represents the mean ± S.E.M. of 6 experiments. **P*<0.05 vs. age-matched control group.

corresponding CON values (Fig. 8B), whereas that of MMP-9, another member of the gelatinase family, was not detected (data not shown).

Discussion

Genetic defects of sarcoglycans, dystroglycan, and/or dystrophin are believed to play an important role in the development of muscular diseases such as cardiomyopathy and muscular dystrophies (6, 7, 29, 30). However, whether or not there is a substantial contribution of alterations in the DRGP complex of the heart without genetic mutation to the development of heart failure remains unclear. Therefore, the present study focused on this problem by using the failing right ventricular muscle following MCT administration to rats.

Determination of tissue weights revealed greater increases in the heart weight, heart weight / body weight, right ventricular weight, right ventricular weight / body weight, lung weight, and lung weight / body weight of the MCT rats at the 4th to 8th weeks. The alterations in the RVW and the ratios related to them suggest the genesis of right ventricular hypertrophy in this model. Since the ratio of right ventricular weight / (left ventricular weight + septum weight) is considered to be a marker of right ventricular hypertrophy (17, 20, 31), augmentation of this ratio for the MCT rats clearly represents the genesis of right ventricular hypertrophy.

The hemodynamic parameters of MCT rats, as determined by the cannula method, showed an increase in right ventricular systolic pressure at the 2nd to 8th week and increases in right ventricular end-diastolic pressure and central venous pressure at the 6th and 8th weeks after the MCT administration. Decreases in heart rate and mean arterial blood pressure were also observed at the 6th and 8th weeks after the MCT administration. In contrast, we found that the left ventricular end-diastolic pressure of the animals at the 8th week after the MCT administration was slightly increased (data not shown), which is a convenient marker of the left ventricular

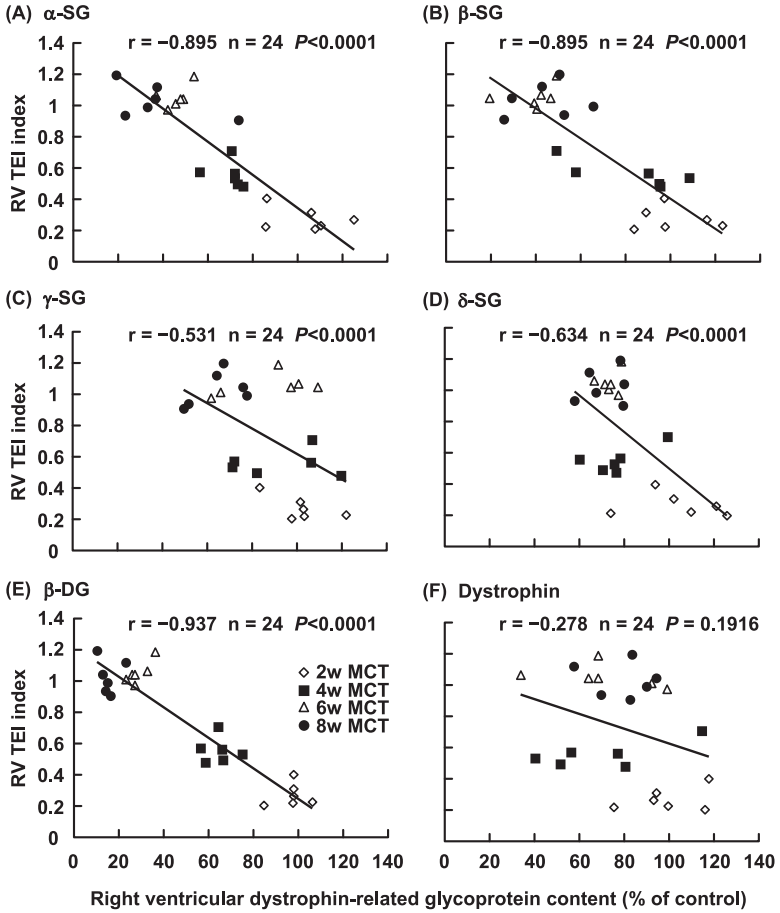

Fig. 5. Relationship between the right ventricular Tei index and DRGP contents of α- (A), β- (B), γ- (C), and δ-sarcoglycan (SG) (D); β-dystroglycan (DG) (E); or dystrophin (F) of the 2w- (open diamonds), 4w- (closed squares), 6w- (open triangles), and 8w-MCT rats (closed circles). Significant relationships between the right ventricular Tei index and the α-sarcoglycan content (n = 24, P<0.0001), between the right ventricular Tei index and β-sarcoglycan content (n = 24, P<0.0001) and between the right ventricular Tei index and the β-dystroglycan content (n = 24, P<0.0001) were observed.

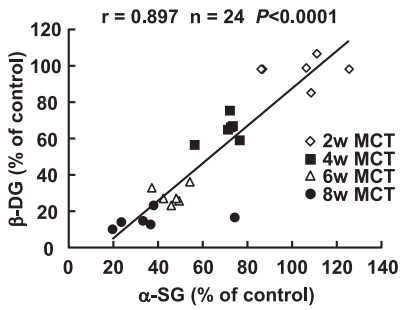

Fig. 6. Relationship between α-sarcoglycan (SG) and β-dystroglycan (DG) contents in the right ventricle of the 2w- (open diamonds), 4w- (closed squares), 6w- (open triangles), and 8w-MCT rats (closed circles). A significant relationship between α-sarcoglycan and the β-dystroglycan contents (n = 24, P<0.0001) was observed.

dysfunction. Therefore, these results suggest that MCT induces pulmonary artery hypertension, which is followed by an increase in right ventricular pressure overload that leads to right, but not left, ventricular dysfunction.

We utilized the images of the pulmonary arterial flow by using the color Doppler method of echocardiography to further assess the detailed profile of cardiac function and hemodynamics of the pulmonary hypertensive animal. Since the pulmonary artery flow acceleration time is the measure that is most closely correlated with an increase in the pulmonary arterial systolic pressure, pulmonary artery flow acceleration time can be used for estimation of the pulmonary arterial systolic pressure in this model (20). The cardiac output index and stroke volume index of the 6w- and 8w-MCT rats were decreased, suggesting the genesis of heart failure in this model. An increase in the right ventricular Tei index is used for the prognostic sign of right ventricular dysfunction in patients with pulmonary artery hypertension (22, 32, 33). The right ventricular Tei indices of the 6w- and 8w-MCT rats were markedly increased, suggesting that the right ventricular function was maintained until the 4th week, but became aggravated thereafter. These results suggest that the right ventricular failure occurs, at the latest, by 6 weeks after administration of MCT.

In the present study, we found diverse changes in DRGP complex in the right ventricular muscle during the development of the right ventricular failure

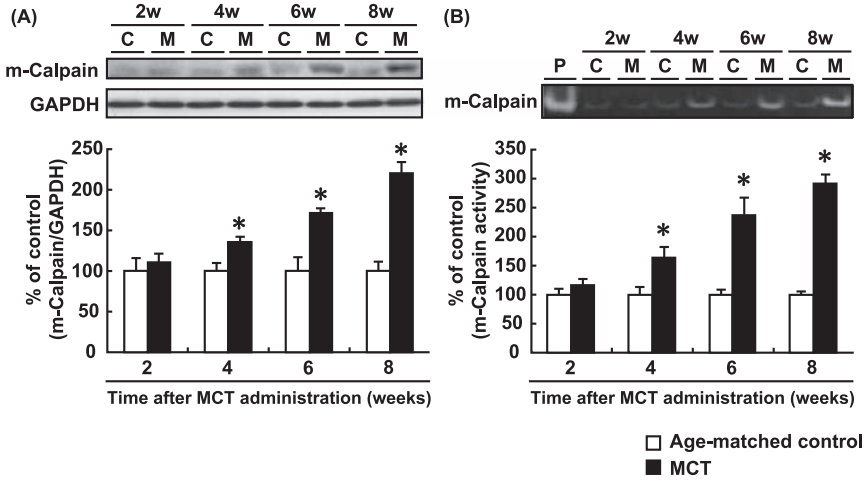

Fig. 7. Time course of changes in the m-calpain content (A) and activity (B) in the right ventricular muscle of age-matched control (open columns) and MCT-administered rats (closed columns) at the 2nd, 4th, 6th, and 8th weeks after administration of saline and MCT, respectively. Upper panels show a representative Western blot (A) or casein zymogram (B) and lower panels, semi quantified data. "C" and "M" indicate the age-matched control and MCT group, respectively. "P" indicates the positive control (rat recombinant m-calpain). Each value represents the mean ± S.E.M. of 6 experiments. **P*<0.05 vs. age-matched control group.

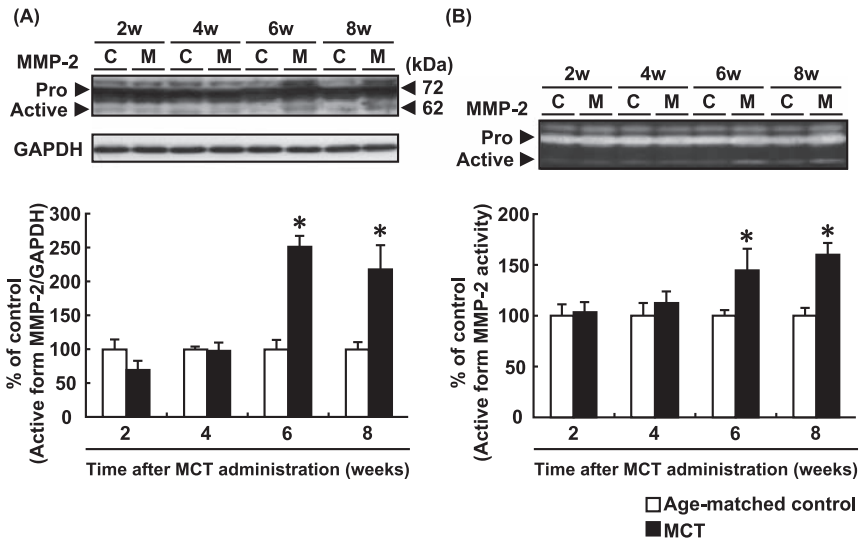

Fig. 8. Time course of changes in the content of the active form of metalloproteinase-2 (A) and activity of this enzyme (B) in the right ventricular muscle of age-matched control (open columns) and MCT-administered rats (closed columns) at the 2nd, 4th, 6th, and 8th weeks after administration of saline and MCT, respectively. Upper panels show a representative Western blot (A) or gelatin zymogram (B) and lower panels, semi quantified data. "C" and "M" indicate the age-matched control and MCT group, respectively. Each value represents the mean ± S.E.M. of 6 experiments. **P*<0.05 vs. age-matched control group.

following MCT administration. The right ventricular α-sarcoglycan and β-dystroglycan contents of the 4w-, 6w-, and 8w-MCT rats markedly decreased in a post-administration time-dependent manner, and the β-sarcoglycan content of the 6w- and 8w-MCT rats also became lower. The right ventricular γ-sarcoglycan content of the 8w-MCT rats decreased as well. Both δ-sarcoglycan and dystrophin contents in the right ventricle of the 4w-MCT rats were decreased. Thereafter, further decreases in the proteins were not observed. These findings suggest that α-sarcoglycan content is most sensitively altered among the sarcoglycans and that alterations in α-sarcoglycan, β-sarcoglycan, and β-dystroglycan are likely to result in the genesis of right ventricular failure. Furthermore, we found that there was a significant and inverse relationship between the right ventricular Tei index and α-sarcoglycan, β-sarcoglycan, or β-dystroglycan content, suggesting that decreases in these DRGP complexes may be, at least in part, related to the right ventricular contractile dysfunction of MCT rats.

To elucidate the possible mechanism underlying the decreases in the right ventricular DRGP content after MCT administration, we focused on the proteolytic ability of endogenous enzymes including m-calpain and MMP-2. In previous studies we found increases in the content and activity of m-calpain, a Ca^{2+}-dependent neutral cysteine protease present in all mammalian cells, in the failing heart following coronary artery ligation (10, 16, 34). Yoshida et al. (10) reported that incubation of a sarcolemmal membrane fraction in the presence of m-calpain under in vitro experimental conditions resulted in decreases in α-sarcoglycan, β-sarcoglycan, and dystrophin contents. In a preliminary study, we found that there were no changes in calpastatin content of the MCT-administered animals throughout the experi-

ment, which is a regulator of proteolysis activity of calpain (data not shown). These results suggest that an increase in m-calpain levels in the failing right ventricular muscle contributes to the decrease in the content of DRGP complexes during the development of right ventricular failure.

In the present study, we found a greater degree of decrease in α-sarcoglycan content in the failing right ventricular muscle than that in β-sarcoglycan and dystrophin contents. The 3 other sarcoglycans, that is, β-, γ-, and δ-sarcoglycan, have their N-terminal part in the intracellular space and a large extracellular domain including a cysteine-rich cluster near the C-terminal region, whereas α-sarcoglycan has its C-terminal amino acid residue in the intracellular space and a cysteine-rich cluster near the transmembrane domain (4, 35). As shown in previous studies (10, 16), these findings suggest that, owing to its structure, α-sarcoglycan in the sarcolemma has a higher affinity for intracellular proteases such as calpain than do other sarcoglycans and dystrophin. The greater degradation of α-sarcoglycan may be attributed to its high affinity for the protease.

By determining the right ventricular MMP-2 content and activity of MCT rats, we found that both were increased in the 6w- and 8w-MCT rats, but that those of 2w- and 4w-MCT rats were not altered. Based on the results of an in vitro experiment, Yamada et al. (13) postulated that the sarcoglycan complex masks the MMP cleavage site on β-dystroglycan in normal skeletal muscle and that a deficiency in the sarcoglycan complex in sarcoglycanopathy results in an MMP-induced reduction in β-dystroglycan, causing disruption of the link between the basement membrane and sarcolemma. In fact, degradation of β-dystroglycan was elevated in the skeletal and cardiac muscles of the cardiomyopathic hamster, a model of sarcoglycanopathy (36). Furthermore, several in vitro studies have shown that MMP-2 degrades β-dystroglycan in neurons, brain samples, Schwannoma cells, and cancer cells (14, 15, 37, 38). We found that there was the close relationship between α-sarcoglycan and β-dystroglycan contents in the right ventricle following MCT administration. These findings suggest that further decreases in the right ventricular β-dystroglycan content of 6w- and 8w-MCT rats were associated with increases in the MMP-2 level and that decreases in α-sarcoglycan contents may enhance the MMP-2–mediated degradation of β-dystroglycan.

Furthermore, γ-and δ-sarcoglycans have high tolerance to calpain-induced proteolysis (10). In the present study, we found that γ-sarcoglycan was significantly decreased at the 8th week after MCT administration. These results indicate the possibility that a rise in MMP-2 activity in the right ventricular failing heart may contribute to decreases in γ-sarcoglycan. Sakamoto et al. (29) and Barresi et al. (39) suggested that a decrease in any one of sarcoglycan complex proteins may lead to either total or partial loss of that sarcoglycan. Additionally, decreases in γ- and δ-sarcoglycan contents may also contribute to decreases in α-sarcoglycan and/or β-sarcoglycan contents of right ventricular muscle in MCT rats.

In summary, we found that, as is the case in the failing heart following coronary artery ligation, the DRGP complex in the failing right ventricular muscle after MCT administration was altered in rats without genetic defects. The decreases in α- and β-sarcoglycans and β-dystroglycan may result in a reduction in the amount of functional DRGP complex in the sarcolemma, suggesting that a decrease in the content of this complex may play an important role in the genesis of contractile dysfunction in right ventricular failure. The advanced right ventricular failure after MCT administration may develop as a consequence of decreases in multiple DRGP proteins, not by a decrease in a single protein. Our results suggest that m-calpain and MMP-2 are involved in the reduction in the content of DRGP complexes, whose loss eventually leads to the development of right ventricular failure.

References

1 Meyrick B, Gamble W, Reid L. Development of Crotalaria pulmonary hypertension: hemodynamic and structural study. Am J Physiol. 1980;239:H692–H702.

2 Rosenberg HC, Rabinovitch M. Endothelial injury and vascular reactivity in monocrotaline pulmonary hypertension. Am J Physiol. 1988;255:H1484–H1491.

3 Seyfarth T, Gerbershagen HP, Giessler C, Leineweber K, Heinroth-Hoffmann I, Ponicke K, et al. The cardiac beta-adrenoceptor-G-protein(s)-adenylyl cyclase system in monocrotaline-treated rats. J Mol Cell Cardiol. 2000;32:2315–2326.

4 Ozawa E, Noguchi S, Mizuno Y, Hagiwara Y, Yoshida M. From dystrophinopathy to sarcoglycanopathy: evolution of a concept of muscular dystrophy. Muscle Nerve. 1998;21:421–438.

5 Vatta M, Stetson SJ, Perez-Verdia A, Entman ML, Noon GP, Torre-Amione G, et al. Molecular remodelling of dystrophin in patients with end-stage cardiomyopathies and reversal in patients on assistance-device therapy. Lancet. 2002;359:936–941.

6 Kunkel LM, Hejtmancik JF, Caskey CT, Speer A, Monaco AP, Middlesworth W, et al. Analysis of deletions in DNA from patients with Becker and Duchenne muscular dystrophy. Nature. 1986;322:73–77.

7 Melacini P, Fanin M, Duggan DJ, Freda MP, Berardinelli A, Danieli GA, et al. Heart involvement in muscular dystrophies due to sarcoglycan gene mutations. Muscle Nerve. 1999;22:473–479.

8 Kawada T, Nakazawa M, Nakauchi S, Yamazaki K, Shimamoto R, Urabe M, et al. Rescue of hereditary form of dilated cardiomyopathy by rAAV-mediated somatic gene therapy: ameliora-

tion of morphological findings, sarcolemmal permeability, cardiac performances, and the prognosis of TO-2 hamsters. Proc Natl Acad Sci U S A. 2002;99:901–906.
9 Badorff C, Lee GH, Lamphear BJ, Martone ME, Campbell KP, Rhoads RE, et al. Enteroviral protease 2A cleaves dystrophin: evidence of cytoskeletal disruption in an acquired cardiomyopathy. Nat Med. 1999;5:320–326.
10 Yoshida H, Takahashi M, Koshimizu M, Tanonaka K, Oikawa R, Toyo-oka T, et al. Decrease in sarcoglycans and dystrophin in failing heart following acute myocardial infarction. Cardiovasc Res. 2003;59:419–427.
11 Toyo-Oka T, Shimizu T, Masaki T. Inhibition of proteolytic activity of calcium activated neutral protease by leupeptin and antipain. Biochem Biophys Res Commun. 1978;82:484–491.
12 Tomasek JJ, Halliday NL, Updike DL, Ahern-Moore JS, Vu TK, Liu RW, et al. Gelatinase A activation is regulated by the organization of the polymerized actin cytoskeleton. J Biol Chem. 1997;272:7482–7487.
13 Yamada H, Saito F, Fukuta-Ohi H, Zhong D, Hase A, Arai K, et al. Processing of beta-dystroglycan by matrix metalloproteinase disrupts the link between the extracellular matrix and cell membrane via the dystroglycan complex. Hum Mol Genet. 2001;10:1563–1569.
14 Leone L, De Stefano ME, Del Signore A, Petrucci TC, Paggi P. Axotomy of sympathetic neurons activates the metalloproteinase-2 enzymatic pathway. J Neuropathol Exp Neurol. 2005;64:1007–1017.
15 Shang ZJ, Ethunandan M, Gorecki DC, Brennan PA. Aberrant expression of beta-dystroglycan may be due to processing by matrix metalloproteinases-2 and -9 in oral squamous cell carcinoma. Oral Oncol. 2008;44:1139–1146.
16 Takahashi M, Tanonaka K, Yoshida H, Oikawa R, Koshimizu M, Daicho T, et al. Effects of ACE inhibitor and AT1 blocker on dystrophin-related proteins and calpain in failing heart. Cardiovasc Res. 2005;65:356–365.
17 Werchan PM, Summer WR, Gerdes AM, McDonough KH. Right ventricular performance after monocrotaline-induced pulmonary hypertension. Am J Physiol. 1989;256:H1328–H1336.
18 Kawahara Y, Tanonaka K, Daicho T, Nawa M, Oikawa R, Nasa Y, et al. Preferable anesthetic conditions for echocardiographic determination of murine cardiac function. J Pharmacol Sci. 2005;99:95–104.
19 Yang XP, Liu YH, Rhaleb NE, Kurihara N, Kim HE, Carretero OA. Echocardiographic assessment of cardiac function in conscious and anesthetized mice. Am J Physiol. 1999;277:H1967–H1974.
20 Jones JE, Mendes L, Rudd MA, Russo G, Loscalzo J, Zhang YY. Serial noninvasive assessment of progressive pulmonary hypertension in a rat model. Am J Physiol Heart Circ Physiol. 2002;283:H364–H367.
21 Tei C. New non-invasive index for combined systolic and diastolic ventricular function. J Cardiol. 1995;26:135–136.
22 Yeo TC, Dujardin KS, Tei C, Mahoney DW, McGoon MD, Seward JB. Value of a Doppler-derived index combining systolic and diastolic time intervals in predicting outcome in primary pulmonary hypertension. Am J Cardiol. 1998;81:1157–1161.
23 Tanonaka K, Furuhama KI, Yoshida H, Kakuta K, Miyamoto Y, Toga W, et al. Protective effect of heat shock protein 72 on contractile function of perfused failing heart. Am J Physiol Heart Circ Physiol. 2001;281:H215–H222.
24 Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970;227:680–685.
25 Croall DE, Moffett K, Hatch H. Casein zymography of calpains using a 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-imidazole buffer. Anal Biochem. 2002;304:129–132.
26 McLellan T. Electrophoresis buffers for polyacrylamide gels at various pH. Anal Biochem. 1982;126:94–99.
27 Raser KJ, Posner A, Wang KK. Casein zymography: a method to study mu-calpain, m-calpain, and their inhibitory agents. Arch Biochem Biophys. 1995;319:211–216.
28 Arthur JS, Mykles DL. Calpain zymography with casein or fluorescein isothiocyanate casein. Methods Mol Biol. 2000;144:109–116.
29 Sakamoto A, Ono K, Abe M, Jasmin G, Eki T, Murakami Y, et al. Both hypertrophic and dilated cardiomyopathies are caused by mutation of the same gene, delta-sarcoglycan, in hamster: an animal model of disrupted dystrophin-associated glycoprotein complex. Proc Natl Acad Sci U S A. 1997;94:13873–13878.
30 Megeney LA, Kablar B, Perry RL, Ying C, May L, Rudnicki MA. Severe cardiomyopathy in mice lacking dystrophin and MyoD. Proc Natl Acad Sci U S A. 1999;96:220–225.
31 Boissiere J, Gautier M, Machet MC, Hanton G, Bonnet P, Eder V. Doppler tissue imaging in assessment of pulmonary hypertension-induced right ventricle dysfunction. Am J Physiol. 2005;289:H2450–H2455.
32 Tei C, Dujardin KS, Hodge DO, Bailey KR, McGoon MD, Tajik AJ, et al. Doppler echocardiographic index for assessment of global right ventricular function. J Am Soc Echocardiogr. 1996;9:838–847.
33 Sugiura T, Suzuki S, Hussein MH, Kato T, Togari H. Usefulness of a new Doppler index for assessing both ventricular functions and pulmonary circulation in newborn piglet with hypoxic pulmonary hypertension. Pediatr Res. 2003;53:927–932.
34 Takahashi M, Tanonaka K, Yoshida H, Koshimizu M, Daicho T, Oikawa R, et al. Possible involvement of calpain activation in pathogenesis of chronic heart failure after acute myocardial infarction. J Cardiovasc Pharmacol. 2006;47:413–421.
35 Lapidos KA, Kakkar R, McNally EM. The dystrophin glycoprotein complex: signaling strength and integrity for the sarcolemma. Circ Res. 2004;94:1023–1031.
36 Matsumura K, Zhong D, Saito F, Arai K, Adachi K, Kawai H, et al. Proteolysis of beta-dystroglycan in muscular diseases. Neuromuscul Disord. 2005;15:336–341.
37 Agrawal S, Anderson P, Durbeej M, van Rooijen N, Ivars F, Opdenakker G, et al. Dystroglycan is selectively cleaved at the parenchymal basement membrane at sites of leukocyte extravasation in experimental autoimmune encephalomyelitis. J Exp Med. 2006;203:1007–1019.
38 Zhong D, Saito F, Saito Y, Nakamura A, Shimizu T, Matsumura K. Characterization of the protease activity that cleaves the extracellular domain of beta-dystroglycan. Biochem Biophys Res Commun. 2006;345:867–871.
39 Barresi R, Confalonieri V, Lanfossi M, Di Blasi C, Torchiana E, Mantegazza R, et al. Concomitant deficiency of beta- and gamma-sarcoglycans in 20 alpha-sarcoglycan (adhalin)-deficient patients: immunohistochemical analysis and clinical aspects. Acta Neuropathol. 1997;94:28–35.