Overexpression of Myosin Phosphatase Reduces Ca^{2+} Sensitivity of Contraction and Impairs Cardiac Function

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Background: Phosphorylation of the regulatory light chain of myosin (MLC) has roles in cardiac function. In vitro, myosin phosphatase target subunit 2 (MYPT2) is a strongly suspected regulatory subunit of cardiac myosin phosphatase (MP), but there is no in-vivo evidence regarding the functions of MYPT2 in the heart.

Methods and Results: Transgenic mice (Tg) overexpressing MYPT2 were generated using the α -MHC promoter. Tg hearts showed an increased expression of MYPT2 and concomitant increase of the endogenous catalytic subunit of type 1 phosphatase (PP1c δ), resulting in an increase of the MP holoenzyme. The level of phosphorylation of ventricular MLC was reduced. The pCa-tension relationship, using β -escin permeabilized fibers, revealed decreased Ca^{2+} sensitization of contraction in the Tg heart. LV enlargement with associated impairment of function was observed in the Tg heart and ultrastructural examination showed cardiomyocyte degeneration.

Conclusions: Overexpression of MYPT2 and the increase in PP1c δ resulted in an increase of the MP holoenzyme and a decrease in the level of MLC phosphorylation. The latter induced Ca^{2+} desensitization of contraction and decreased LV contractility, resulting in LV enlargement. Thus, MYPT2 is truly the regulatory subunit of cardiac MP in-vivo and plays a significant role in modulating cardiac function. (*Circ J* 2010; **74**: 120–128)

Key Words: Ca^{2+} sensitization; Myocardial contraction; Myosin light chain; Myosin phosphatase; Transgenic mouse

Phosphorylation of the regulatory light chain of myosin II (MLC) initiates contraction of smooth muscle and the level of MLC phosphorylation primarily regulates vascular tone.¹ In striated muscle, MLC phosphorylation does not initiate contraction and is not essential for the contractile process, but rather plays a modulatory role.² In general, myosin phosphorylation in striated muscle increases force at submaximal [Ca^{2+}]; that is, an increase in Ca^{2+} sensitivity, which is most pronounced in fast-twitch skeletal muscle.

The level of MLC phosphorylation is determined by the balance between myosin light chain kinase (MLCK) and myosin phosphatase (MP). Smooth muscle MLCK and MP have been well characterized in-vitro and in-vivo,^{2–4} but less is known about cardiac-muscle-specific MLCK and MP. Recently, cardiac-muscle-specific MLCK was identified and characterized.^{5,6} We discovered a target subunit of myosin

phosphatase 2 (MYPT2), an isoform of the smooth muscle type subunit, MYPT1, which is the key regulatory subunit and has several important functions including targeting of MP to its substrate, myosin, and regulation of MP activity. Our recent in-vitro study showed that MYPT2 could form a complex with type 1 phosphatase catalytic subunit δ isoform (PP1c δ) that served as an MP holoenzyme in cardiac muscle cells.⁷ However, whether MYPT2 together with PP1c δ plays a principle role in the dephosphorylation of cardiac MLC in the living heart has remained to be investigated.

In cardiac muscle, MLC phosphorylation may be involved in determining cardiac torsion⁸ and may mediate sarcomere organization during cardiac cell hypertrophy in-vivo.⁹ However, several aspects of MLC phosphorylation in cardiac muscle remain to be investigated. In the failing heart, the level of MLC phosphorylation is altered, but in general, the status of MLC phosphorylation in cardiac muscle under path-

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Figure 1. (A) Schematic representation of the transgene construct. (B) Western blots of subunits of myosin phosphatase in wild (Wt) and transgenic (Tg) mice. Extracts (50 μ g each) from Tg and Wt hearts were applied to SDS-PAGE, followed by Western blots. The overexpressed human myosin phosphatase target subunit 2 (MYPT2) had lower mobility than the endogenous mouse MYPT2. (C) Phosphatase activity. Assays of phosphatase activity in extracts of Wt (●) and Tg line 1 (L1) (●) hearts at indicated concentrations of okadaic acid. *P<0.05 vs Wt. (D) Densitometric data for expression of each subunit in Wt, Tg(L1) and line 4 (L4). The density for each subunit in Wt was taken as 1 unit. Blue bar, Wt; red bar, Tg(L1); orange bar, Tg(L4). *P<0.05.

ological conditions has not been established.^{10,11} Recently, cardiac-muscle-specific MLCK was found to be upregulated in heart failure,^{5,6} suggesting that MLC phosphorylation in cardiac muscle may be more critical to cardiac function than thought previously. An important point is that any change in cardiac function resulting from changes in MLC phosphorylation must be evaluated over the long term.

To address some of these concerns, we generated a cardiac-specific MYPT2 transgenic mouse (Tg) to investigate the in-vivo function of MYPT2 and the long-term effect of MLC phosphorylation on cardiac function.

Methods

Generation of Transgenic Mice

A 9.0-kb transgene, consisting of the mouse α -myosin heavy chain (MHC) promoter,¹² the human MYPT2 cDNA and the human growth hormone polyadenylation site was used for pronuclear microinjection into fertilized C57BL/6J mouse eggs (Figure 1A). Tg was identified by polymerase chain reaction (PCR) and Southern blot analysis with genomic DNA prepared from a tail sample DNA. Southern blot analysis of *EcoRI*-digested genomic DNA was performed using a ³²P-labeled 1.7-kb *EcoRI*–*EcoRI* MHC fragment as a probe that recognized the 9-kb transgenic fragment. Routine genotyping was performed by PCR using a 5' primer from the

MYPT2 cDNA (5'-CAA-GAA-AAG-ACC-TCT-GAC-CG-3') and a 3' primer from the poly A region (5'-TAT-TAG-GAC-AAG-GCT-GGT-GG-3') to amplify a 300-bp fragment spanning the junction between the MYPT2 cDNA and poly A. Three independent founder lines, namely Tg(L1), Tg(L2) and Tg(L4), were identified and mated to C57BL/6J wild-type mice (Wt) to generate pure C57BL/6J genetic background Wt and Tg offspring. Mice for the study were 15–20 weeks of age. To evaluate the survival rate, approximately 30 mice were observed for 1 year in the Tg(L1) and Wt groups, respectively. All protocols were approved by the animal protocol committee at Mie University.

Western Blot Analysis

Western blots were performed as described previously⁷ with antibodies directed against MYPT1,¹³ MYPT2,¹⁴ PP1c α (Cell Signaling), PP1c δ , PP1c γ (a gift from Dr Shima),¹⁵ leucine zipper motif of MYPT1 (found also at the C-termini of MYPT2 and HS-M₂₁, a heart specific isoform of smooth muscle M20¹⁶), phospholamban (PLB; Affinity BioReagents), phospho-PLB (Ser16; Upstate), troponin I (TnI; Cell Signaling), cardiac phospho-TnI (Ser23/24; Cell Signaling), slow skeletal TnI (Santa Cruz), smooth muscle MLCK,¹⁷ and skeletal muscle MLCK (Santa Cruz). Total protein concentration was measured using the Bradford assay (Bio-Rad) and equal amounts of protein were loaded into each well.

Phosphatase Assays

Assays of phosphatase activity were carried out using ^{32}P -labeled MLC as described previously.¹⁷ Briefly, phosphatase from the mouse heart was extracted by homogenization with lysis buffer (50 mmol/L Tris-HCl, pH 7.4, 1% NP40, 150 mmol/L NaCl, 0.25% sodium deoxycholate, 1 mmol/L ethylenediaminetetraacetic acid). To investigate the inhibitory effects of okadaic acid, the extract was pre-incubated for 4 min at 30°C with varying levels of okadaic acid in a buffer containing 30 mmol/L Tris-HCl, pH 7.4, 100 mmol/L KCl and 0.2 mg/ml bovine serum albumin, followed by measurement of phosphatase activity.

Quantification of MLC Phosphorylation

Cardiac muscle that had been quick-frozen in liquid nitrogen was placed in a frozen slurry of TCA (10% wt/vol) in acetone plus dithiothreitol (10 mmol/L) and allowed to thaw. Next, the levels of MLC phosphorylation were measured as described previously with an antibody specific for cardiac regulatory MLC (BioCytex, Marseille).⁷

Real-Time PCR

Real-time quantitative RT-PCR was performed on cDNA generated from 1 μg of total RNA using Multiscribe reverse transcriptase (Applied Biosystems) and random hexamers according to the manufacturer's protocol. For PCR, we used Assay-on-Demand Gene Expression Products as the pre-designed sense and antisense primers (Applied Biosystems), TaqMan probe (Applied Biosystems), the TaqMan Universal PCR Master Mix (Applied Biosystems) final volume of 50 μl and an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). The data were normalized with GAPDH.

Transthoracic Echocardiography and LV Pressure Loop

The 16-week-old mice were anesthetized with sodium pentobarbital (30 mg/kg IP) and examined with M-mode echocardiography in the short-axis view using a 15-MHz transducer (TOSHIBA Power Vision 6000). In the conscious state, systolic blood pressure and heart rate were assessed by the tail cuff method. Hemodynamic measurement by catheterization was done by Medical Microtechnology Inc Japan as described.¹⁸

Measurement of Ca^{2+} Sensitization

Ca^{2+} sensitization of ventricular fibers was measured using β -escin-permeabilized cardiac muscle fibers.¹⁹ The permeabilized fibers were transferred to the TCA solution immediately after exposure at pCa 5, 6 and 7 and the MLC phosphorylation levels were measured.

Histology

Paraffin-embedded longitudinal sections of heart fixed in 4% paraformaldehyde were stained with hematoxylin-eosin (H&E). For ultrastructural analysis, hearts were fixed in 2.5% glutaraldehyde. After washing with dextrose in phosphate-buffered saline and post-fixation in 2% OsO_4 , the sample were dehydrated using ethanol, and embedded in epoxy resin. Ultrathin sections were double-stained with uranyl acetate and lead citrate, followed by examination under the electron microscope.

Statistical Analysis

Data are reported as the mean $\pm$ SEM and were compared by Student's *t*-tests. Comparisons between multiple groups were performed by 1-way ANOVA followed by Fisher's protected

least significant difference tests. Survival rate was analyzed using the Kaplan-Meier method with the log-rank test. A value of $P < 0.05$ was considered statistically significant.

Results

Transgene Expression

Three independent Tg lines, designated Tg(L1), Tg(L2) and Tg(L4), were obtained. Overexpression of MYPT2 was confirmed by Western blots using a polyclonal antibody against MYPT2 and which also cross-reacts with MYPT1. The molecular weight of MYPT1 is higher than that of MYPT2 and the 2 isoforms can be identified easily on Western blots (data not shown). Increased expression of MYPT2 was detected in the Tg hearts (**Figures 1B, D**) and Tg(L1) showed higher expression of MYPT2 (6.19 ± 0.48 -fold vs Wt, $P < 0.05$) compared with Tg(L4) (2.09 ± 0.40 -fold vs Wt, $P < 0.05$). The expression level of MYPT2 in Tg(L2) was similar to that in Tg(L1) (data not shown). MYPT2 overexpression was restricted to the heart and was not detected in other tissues, including liver and brain (data not shown). The expression of endogenous MYPT2 did not change significantly among the 3 lines (**Figure 1B**), and the expression of MYPT1 was unchanged in Wt and Tg(L1) (data not shown).

A surprising finding was that the endogenous catalytic subunit of PP1c δ was increased concomitantly with MYPT2 in Tg(L1) (1.71 ± 0.10 -fold vs Wt, $P < 0.05$), whereas the other isoforms of PP1 (PP1c α and PP1c γ) were unchanged (**Figure 1D**). Real-time PCR showed an increase in the mRNA for PP1c δ (1.63 ± 0.16), indicating that synthesis of PP1c δ , not reduced proteolysis, accounted for the increased level (data not shown). The catalytic subunit of type 2A phosphatase (PP2A) was unchanged (data not shown). The expression of endogenous PP1c δ in Tg(L4) was not significantly altered. Total phosphatase activity in Tg(L1) hearts was markedly increased (≈ 3.7 -fold) compared with Wt (**Figure 1C**). The increased phosphatase activity in Tg(L1) was inhibited only at 1,000 nmol/L okadaic acid, suggesting that the increased activity in Tg(L1) was because of PP1 not PP2A. The absolute concentrations of the overexpressed MYPT2 and the upregulated PP1c δ measured by Western blot using purified MYPT2 and PP1c δ as standards were 11.3 and 10.4 $\mu\text{mol/mg}$ extracted protein, respectively. Thus, the overexpressed MYPT2 and the upregulated PP1c δ formed a complex at a molar ratio of 1:1. In addition, immunoprecipitation of MYPT2 from the Tg(L1) lysate reduced the level of MYPT2 by approximately 95%, with a corresponding decrease in PP1c δ (data not shown). Thus, all the overexpressed MYPT2 was considered to be bound with all of the increased PP1c δ to form MP and thus there would not be uncomplexed (free) MYPT2 or PP1c δ . As a consequence, the overall level of MP was increased in the Tg hearts. The expression of HS-M21 was unchanged in Wt and the Tg lines (**Figures 1B, D**).

Morphological Abnormalities

The Tg mice appeared normal, without any marked change in appearance or behavior. The 1-year survival rates were 87.5% in Tg(L1) and 86.2% in Wt ($P = \text{NS}$). The heart rates, blood pressures and body weights were not significantly altered in Wt, Tg(L1) or Tg(L4) (**Table 1**). However, the heart-to-body weight ratio for Tg(L1) was significantly higher than those for Wt and Tg(L4) (**Table 1**), indicating an increase in heart weight.

As shown in **Figure 2A**, morphometric analysis of H&E-stained myocardium showed increases in the LV and RV

	Wt	Tg	
		L1	L4
Heart rate (beats/min)	627±18	610±24	650±16
Blood pressure (mmHg)	108±3	108±4	100±3
Body weight (g)	29.1±0.3	28.7±0.7	29.0±0.4
Heart weight/body weight (%)	0.48±0.01	0.57±0.02*	0.45±0.01
Ventricular dimensions (mm)			
IVST	0.99±0.02	0.88±0.04	0.91±0.03
PWT	0.97±0.02	0.88±0.03	0.92±0.04
LVDd	2.98±0.12	3.87±0.14*	3.19±0.12
LVDs	1.48±0.13	2.69±0.19*	1.60±0.08
Fractional shortening	0.50±0.03	0.31±0.03*	0.48±0.01

Values are mean±SEM. *P<0.05 vs Wt.

Wt, wild-type; Tg, transgenic mice; IVST, intraventricular septum thickness; PWT, posterior wall thickness; LVDd, left ventricular end-diastolic diameter; LVDs, left ventricular end-systolic diameter.

Figure 2. (A) Hematoxylin and eosin-stained longitudinal sections. (B) M-mode echocardiography from wild-type (Wt) and transgenic (Tg) (L1 and L4).

dimensions of Tg(L1) as compared with Wt and Tg(L4). Echocardiography was used to assess chamber size and cardiac function. Significant increases in LV end-diastolic and end-systolic dimensions and significant decreases in fractional shortening were observed in Tg(L1) compared with Wt (**Figure 2B**, **Table 1**). These tendencies were noted in Tg(L4), but the results were not significant. Hearts from Tg(L2), expressing a similar level of MYPT2 as Tg(L1), showed similar gross morphological changes and biventricular dilation (data not shown), indicating that the change in phenotype was not related to an insertional mutagenic event.

Expression and Phosphorylation Levels of the Contractile Proteins

The expression level of ventricular regulatory MLC was not significantly different in Tg(L1) and Wt (data not shown). However, urea-glycerol-PAGE showed that the level of

phosphorylation of ventricular regulatory MLC in Tg(L1) ($29.1\pm3.4\%$) was significantly lower than in Wt ($44.3\pm1.6\%$) (**Figure 3A**). Expression of the smooth muscle MLCK isoform was unchanged in the Tg hearts (**Figure 3B**). The skeletal muscle MLCK isoform was not detected in Wt or Tg hearts (data not shown). PLB and TnI play important roles in Ca^{2+} handling and Ca^{2+} sensitivity and thus their phosphorylation status in Tg(L1) was investigated. The level of phosphorylation of PLB was higher than in Wt (1.53 ± 0.17 -fold, $P<0.05$), although the total level of PLB was unchanged (**Figure 3C**). The expression and phosphorylation levels of TnI were not altered (**Figure 3D**). The expression of slow skeletal TnI, possibly expressed in the failing human heart, was not detected in either Tg(L1) or Wt (data not shown).

Ca^{2+} Sensitivity of Ventricular Fibers

In striated muscle the phosphorylation of MLC regulates the

Figure 3. (A) Phosphorylation levels of cardiac myosin light chain (MLC) determined by urea-glycerol-PAGE. Non-phosphorylated (non P) and phosphorylated (P) species are indicated. The ratio (%) of phosphorylated MLC vs total MLC was calculated and summarized. (B) Expression of smooth muscle MLC kinase (MLCK). The expression of smooth muscle MLCK in Wt was taken as 1 unit. (C) Phosphorylation (Ser16) and expression of phospholamban (PLB). (D) Phosphorylation (Ser23/24) and expression of troponin I (Tnl). Upper panels show representative Western blots and the lower panels summarize the densitometric analyses. In panels C and D, the relative ratio of phosphorylated (P) to total protein of PLB and Tnl in respectively. *P<0.05. Wt, wild-type; Tg(L1), transgenic line 1.

Figure 4. (A) pCa-tension relationship of isolated ventricular fibers. Permeabilized ventricular fibers for Wt (●) and Tg(L1) (●). Force development is expressed as percentage of maximum tension. (B) MLC phosphorylation levels of each fiber are shown at pCa 5, 6 and 7. Upper panels show representative Western blots of urea-glycerol-PAGE and the lower panels summarize the densitometric analyses. *P<0.05. Wt, wild-type; Tg(L1), transgenic line 1.

Figure 5. In-vivo assessment of cardiac function and pressure-volume relationship. (A) Representative examples of left ventricular (LV) pressure vs volume loops. (B) LV pressure curve. (C) dP/dt curve. Wt, wild-type; Tg(L1), transgenic line 1.

Ca^{2+} sensitivity of contraction, hence the pCa-tension relationship was investigated using ventricular fibers permeabilized by β -escin. As shown in Figure 4A, there was a slight, but significant, rightward shift of the tension- $[\text{Ca}^{2+}]$ curve for Tg(L1) ventricular muscle fibers (Tg, $\text{pCa}_{50}=6.02\pm0.04$; Wt, $\text{pCa}_{50}=6.13\pm0.02$, $P<0.05$), indicating Ca^{2+} desensitization in the Tg heart compared with Wt. In the permeabilized fibers the level of phosphorylation of the regulatory MLC at different Ca^{2+} concentrations was markedly reduced in Tg(L1) compared with Wt (Figure 4B).

Hemodynamic Measurements

The LV pressure-volume relationships were obtained by catheterization (Figure 5). End-systolic and end-diastolic volumes (ESV and EDV) were increased in Tg(L1) accompanied by a reduced ejection fraction compared with Wt. There was also a trend toward reduction in the cardiac index and the stroke volume index in the Tg hearts (Table 2). The end-systolic pressure-volume relationship in Tg(L1) was characterized by a displacement of the line to the right with a reduced slope compared with Wt (Figure 5A), and $\text{dP/dt}_{\text{max}}$ was decreased (Figure 5C). The normalized end-systolic elastance (NL E_{es}), preload recruitable stroke work (PRSW) and $(\text{dP/dt}_{\text{max}})/\text{EDV}$ in Tg(L1) were significantly lower than in Wt (Table 2), indicating depressed LV contractility in Tg(L1). In Tg(L1), an index of LV relaxation, τ , was significantly prolonged, without significant changes in heart rate or end-systolic pressure (Figure 5B, Table 2), and $\text{dP/dt}_{\text{min}}$ was decreased (Figure 5C). Increased end-diastolic pressure was also observed in Tg(L1) (Figure 5A). Furthermore, the markedly steep slope of the end-diastolic pressure-volume relationship and the normalized end-diastolic elastance (NL E_{ed}) in Tg(L1) was significantly greater than in Wt (Table 2), indicating depressed LV diastolic function in Tg(L1). These findings suggest that the LV was enlarged and LV systolic and diastolic performance was impaired in Tg(L1).

Table 2. Hemodynamics

	Wt	Tg (L1)
Heart rate (beats/min)	392 $\pm$ 7	373 $\pm$ 5
ESP (mmHg)	94.7 $\pm$ 3.7	87.7 $\pm$ 2.9
EDP (mmHg)	3.0 $\pm$ 0.2	5.8 $\pm$ 1.2*
ESV (μ l)	8.4 $\pm$ 0.8	40.1 $\pm$ 5.6*
EDV (μ l)	26.1 $\pm$ 1.1	49.7 $\pm$ 3.2*
EF	0.67 $\pm$ 0.04	0.21 $\pm$ 0.07*
SI (μ l/g)	0.66 $\pm$ 0.07	0.38 $\pm$ 0.12
CI ($\text{ml}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$)	262 $\pm$ 30	145 $\pm$ 49
Systolic function		
$\text{dP/dt}_{\text{max}}$ (mmHg/s)	7,413 $\pm$ 475	4,774 $\pm$ 371*
NL E_{es} ($\text{mmHg}\cdot\mu\text{l}^{-1}\cdot 100\text{mg}^{-1}$)	11.9 $\pm$ 1.0	6.2 $\pm$ 0.9*
PRSW (mmHg)	78.8 $\pm$ 2.4	43.6 $\pm$ 2.5*
$(\text{dP/dt}_{\text{max}})/\text{EDV}$ ($\text{mmHg}\cdot\text{s}^{-1}\cdot\mu\text{l}^{-1}$)	260.1 $\pm$ 12.71	129.0 $\pm$ 15.7*
Diastolic function		
$\text{dP/dt}_{\text{min}}$ (mmHg/s)	-6,793 $\pm$ 498	-4,024 $\pm$ 370*
NL E_{ed} ($\text{mmHg}\cdot\mu\text{l}^{-1}\cdot 100\text{mg}^{-1}$)	0.19 $\pm$ 0.05	0.57 $\pm$ 0.08*
τ (ms)	9.2 $\pm$ 0.5	12.5 $\pm$ 0.2*

Values are mean $\pm$ SEM. * $P<0.05$ vs Wt.

ESP, end-systolic pressure; EDP, end-diastolic pressure; ESV, end-systolic volume; EDV, end-diastolic volume; EF, ejection fraction; SI, stroke volume index; CI, cardiac index; NL E_{es} , normalized end-systolic volume elastance; PRSW, preload recruitable stroke work; NL E_{ed} , normalized end-diastolic volume elastance; τ , monoexponential time constant of relaxation. Other abbreviations see in Table 1.

Changes in Hypertrophic Molecular Markers

Molecular markers of cardiac hypertrophy were analyzed by real-time RT-PCR. As shown in Figure 6A, transcriptional levels of brain natriuretic peptide (BNP) and β -MHC, but not α -MHC, were significantly elevated in Tg(L1) compared with Wt. In Tg(L4), only the BNP message was significantly

Figure 6. (A) Changes in hypertrophic and failure response gene expression. Density of each marker in wild (Wt) taken as 1 unit. Blue bar, Wt; red bar, transgenic (Tg) line 1 (L1); orange bar, Tg line 4 (L4). * $P < 0.05$. (B) Histological analysis by electron microscopy. Upper panel shows Wt and lower panel shows Tg(L1). Left ventricular cross sections were used from Wt and Tg(L1). BNP, brain natriuretic peptide; MHC, myosin heavy chain; PLB, phospholamban; SERCA, sarcoplasmic reticulum calcium ATPase.

upregulated. The magnitude of these responses seems to be consistent with a relationship linking increased MYPT2 expression to BNP and β -MHC expression. Although the transcriptional level of β -MHC was increased in Tg(L1), the expression level of total MHC was not significantly different between Tg(L1) and Wt (data not shown). The gene expression of PLB and sarcoplasmic reticulum calcium ATPase (SERCA) were unchanged in Tg(L1).

Histological and Ultrastructural Analyses

Histological examination using H&E and Masson's trichrome staining revealed no obvious differences. However, electron microscopy indicated degeneration of the sarcomeric assembly, degeneration (vacuolization) of the sarcoplasmic reticulum (SR) and disarray of the myofibrillar structure in Tg(L1) (Figure 6B). These results indicate that fine tuning of the phosphorylation of MLC is necessary for maintaining the normal structure of the sarcomere in-vivo.

Discussion

The main findings of this study are: (1) overexpression of MYPT2 was coincident with increased expression of PP1c δ at molar stoichiometry, leading to increased expression of the MP holoenzyme; (2) overexpression of MP resulted in a decrease in the level of phosphorylation of regulatory MLC and of Ca^{2+} desensitization of contraction; (3) long-term decrease in MLC phosphorylation caused LV enlargement with systolic and diastolic dysfunction and partial disruption of myofibrillar structure without any change in the survival rate, suggesting that MLC phosphorylation plays a modulatory role in cardiac function.

Three genes encode the PP1c isoforms, α , δ (also termed

β) and γ .²⁰ These isoforms are specifically associated with a variety of targeting/regulatory components that localize the catalytic subunits to distinct subcellular locales and/or substrates, and thus may confer specificity and regulate activity. Our recent in-vitro data indicated that MYPT2 specifically interacts with PP1c δ and to a lesser extent with PP1c α , but not with PP1c γ .⁷ There exists another cardiac type 1 phosphatase holoenzyme, PP1c complex with another type of regulatory subunit (RGL or G_M), which catalyzes dephosphorylation of PLB²¹ and in turn reduces the calcium uptake by SERCA.²² In our study, endogenous PP1c δ was increased, but this was not associated with dephosphorylation of PLB (the phosphorylation level of PLB actually increased in Tg(L1)). These results support the contention that the overexpressed MYPT2 is associated stoichiometrically with increased PP1c δ , forming MP, and thus the increased phosphatase activity would target the substrates of MP, that is, predominantly phosphorylated myosin. This scenario is consistent with a reduction in the level of phosphorylated MLC in Tg hearts. Ours is the first study to demonstrate that MYPT2 is truly the key target subunit of cardiac MP and, in association with PP1c δ , dramatically dephosphorylates cardiac MLC in-vivo. Interestingly, a similar increase in endogenous PP1c was found in the skeletal muscle of Tg mice overexpressing the glycogen target subunit (RGL) and the increase was suggested to reflect stabilization of PP1c.²³ The dynamic states of MYPT2 under various physiological and/or pathological conditions of the heart have not been investigated and remain to be determined. In contrast, the level of the putative third subunit of MP⁷, HS-M₂₁,¹⁶ was unchanged, which questions a functional role for HS-M₂₁ in the MP holoenzyme.

In the Tg model, expression of the smooth muscle MLCK isoform, known to be present in heart,²⁴ was unchanged.

Considering the level of MLC phosphorylation is primarily regulated by MLCK and MP, an increase in MP activity would be predicted to decrease the level of MLC phosphorylation. As the primary responsive kinase for MLC phosphorylation seems to be the newly identified cardiac-muscle-specific MLCK, its expression level in our model remains to be examined.

Several reports propose a modulatory role for myosin II phosphorylation in cardiac muscle. In rat ventricular cardiomyocytes, α -adrenergic stimulation induced an increase in the level of phosphorylation of MLC and increased Ca^{2+} sensitivity to tension.²⁵ Olsson et al²⁶ also showed that myosin phosphorylation is a positive modulator of Ca^{2+} sensitivity and the kinetics of myocardial force development. A slight decrease in Ca^{2+} sensitivity was associated with reduced levels of phosphorylation in β -escin skinned cardiac fibers. Although the level of MLC phosphorylation was to some extent decreased by permeabilization in both Wt and Tg(L1) hearts, a significant difference between them in MLC phosphorylation was still observed after permeabilization. Altered levels of MLC phosphorylation also may be linked to several pathological conditions, such as reduced MLC phosphorylation in end-stage human heart failure,¹⁰ and increased MLC phosphorylation in the induced failing myocardium of dogs.¹¹ Additionally, some patients with hypertrophic cardiomyopathy possibly associated with some change in Ca^{2+} sensitivity have mutations in cardiac ventricular MLC.²⁷

With regard to cardiac function, MYPT2 Tg showed an increase in the heart weight/body weight ratio, dilation of the LV, impaired LV function, and an increase in both β -MHC (a marker for cardiac hypertrophy)²⁸ and BNP (a marker for heart failure).²⁹ Considered together, the evidence suggests that the Tg hearts are in a state of pre-heart failure. Although the level of phosphorylation of PLB was increased, it may be a compensating reaction preliminary to heart failure. A quasi-temporal relationship has been observed between postexercise peak LV pressure potentiation and MLC phosphorylation³⁰ and thus MLC phosphorylation might respond to increased cardiovascular load.

Mice expressing non-phosphorylatable MLC in the heart do not show Ca^{2+} desensitization and do not develop ventricular hypertrophy or reduce cardiac function; however, ultrastructural abnormalities (ie, cardiomyocyte degeneration in the ventricle) and tricuspid regurgitation accompanying atrial dilation are observed.³¹ Interestingly, decreased phosphorylation of TnI and myosin-binding protein-C were also observed in that model.³² In contrast, our MYPT2 Tg showed a decreased level of phosphorylation of MLC and decreased Ca^{2+} sensitization, leading to 4-chamber dilation and cardiac dysfunction, accompanied by similar ultrastructural cardiomyocyte degeneration. Importantly, Ca^{2+} sensitization of contraction also can be regulated by phosphorylation of TnI at Ser23/Ser24 and this reduces myofibrillar ATPase activity.^{33,34} Thus, the overall cardiac Ca^{2+} sensitivity depends primarily on the balance between the phosphorylation states of MLC and TnI.¹⁰ In our study, there was no increase in either TnI expression or phosphorylation and only a decrease in MLC phosphorylation was observed, which explain why MYPT2 Tg showed a slight but significant Ca^{2+} desensitization, whereas non-phosphorylated MLC Tg does not. In addition, cardiac MLCK knockout in zebrafish results in dilated cardiac ventricles and immature sarcomere structures.^{5,6} Overall, these animal models illustrate the importance of myosin phosphorylation in long-term cardiac function and structure, although there are differences in experimental design.

Based on clinical trials, Ca^{2+} sensitizing agents, such as levosimendan, which augments Ca^{2+} binding to troponin C, are expected to improve cardiac function in both acute and chronic heart failure with appropriate treatment.³⁵ There are still conflicting results regarding changes in the level of MLC phosphorylation in heart failure models;^{10,11} however, in human endstage heart failure there could be a tendency toward lower levels compared with the normal heart.³⁶ As Ca^{2+} sensitivity is thought to be controlled not only by phosphorylation of TnI but also by MLC phosphorylation, an interesting option for future studies is to explore the possibility of therapeutic intervention for heart failure in terms of modifying the level of MLC phosphorylation in order to change Ca^{2+} sensitivity.

Some ultrastructural changes were observed in Tg(L1) hearts, including myofibrillar disarray and vacuolization of parts of the SR. It is not known if these changes and cardiac dilatation are directly caused by Ca^{2+} desensitization because of decreased MLC phosphorylation or are the results of remodeling because of cardiac dysfunction.³⁷ It has been suggested that the level of myosin phosphorylation is an important factor in both myofibril organization and myocardial function.^{9,38} It also has been shown that a mutated troponin T knock-in results in some tissue damage accompanied by a decrease in Ca^{2+} sensitivity and cardiac dysfunction.³⁹ Intuitively, it seems that the first option is most likely, but further studies are required to distinguish between both possibilities.

In summary, this is the first report demonstrating the function of cardiac MP and MYPT2, and the role of cardiac MLC phosphorylation in-vivo. Overexpression of MYPT2 associated with endogenous PP1c δ , leads to increased formation of the cardiac MP holoenzyme. A higher level of MP activity caused LV dysfunction and LV enlargement, possibly via decreased Ca^{2+} sensitivity, and also some deterioration of myofibrillar structure.

Study Limitations

Based on previous studies, it was assumed that the primary substrate for MP is cardiac regulatory MLC. However, we cannot exclude the possibility that the phenotypes observed in our model might be mediated in part by other functions of MP, including additional substrates for MP or interaction/targeting with other proteins. We have no evidence to support these possibilities, which have to be resolved by future studies.

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