Elevated calcium transients and increased myofibrillar power generation cause cardiac hypercontractility in a model of Noonan Syndrome with Multiple Lentigines

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ABSTRACT

Noonan Syndrome with Multiple Lentigines (NSML) is primarily caused by mutations in the non-receptor protein tyrosine phosphatase SHP2 and associated with congenital heart disease in form of pulmonary valve stenosis and hypertrophic cardiomyopathy (HCM). Our goal was to elucidate cellular mechanisms underlying the development of HCM caused by the Q510E mutation in SHP2. NSML patients carrying this mutation suffer from a particularly severe form of HCM. Drawing parallels to other, more common forms of HCM, we hypothesized that altered calcium homeostasis and / or sarcomeric mechanical properties play key role(s) in the pathomechanism.

We used transgenic mice with cardiomyocyte-specific expression of Q510E-SHP2 starting before birth. Mice develop neonatal-onset HCM with increased ejection fraction and fractional shortening at 4-6 weeks of age. To assess calcium handling, isolated cardiomyocytes were loaded with fluo-4. Q510E-SHP2 expression increased calcium transient amplitude during excitation-contraction coupling and increased sarcoplasmic reticulum calcium content concurrent with increased expression of the sarco/endoplasmic reticulum calcium ATPase (SERCA). In skinned cardiomyocyte preparations from Q510E-SHP2 mice, force-velocity relationships and power-load curves were shifted upward. Peak power generating capacity was increased ~2-fold. Transmission electron microscopy revealed that the relative intracellular area occupied by sarcomeres was increased in Q510E-SHP2 cardiomyocytes. Triton-X-based myofiber purification showed that Q510E-SHP2 increased the amount of sarcomeric proteins assembled into myofibers.

In summary, Q510E-SHP2 expression leads to enhanced contractile performance early in disease progression by augmenting intracellular calcium cycling and increasing the number of power-generating sarcomeres is increased. This gives important new insight into the cellular pathomechanisms of Q510E-SHP2-associated HCM.

Keywords: contractility; sarcomeric function; SERCA; cardiac hypertrophy; protein tyrosine phosphatase
Hypertrophic cardiomyopathy affects approximately 1:500 adults (31) and represents one of the most common causes of sudden cardiac death in young individuals (49). Often the disease is detected in early adolescence, when the patients develop arrhythmias, exercise intolerance, chest pain, or even sudden cardiac death. Classically, hypertrophic cardiomyopathy is characterized by concentric myocardial hypertrophy with cardiomyocyte disarray. Outflow tract obstruction resulting from segmental septal hypertrophy or systolic anterior motion of the mitral valve often augments disease. The majority of the genetic mutations reported in patients with familial hypertrophic cardiomyopathy affect components of the sarcomere, with multiple sarcomeric proteins implicated in the disease process (10, 47).

Interestingly, non-sarcomeric mutations can also induce HCM. Non-sarcomeric HCM can be clinically indistinguishable from ‘classic’ HCM caused by sarcomeric mutations, and these rare forms of HCM have therefore also been termed ‘phenocopy’ diseases (18). Examples are Fabry’s disease, Danon disease, mitochondrial cardiomyopathies, or Noonan Syndrome. Why these phenocopy diseases so closely resemble classic HCM is not known, but it suggests that different chains of molecular events may ultimately converge and trigger a common cardiomyopathic mechanism.

To address this question, we focused on elucidating the pathomechanism(s) in Noonan Syndrome with Multiple Lentigines, NSML. This syndrome also is termed LEOPARD Syndrome as an acronym for the clinical disease characteristics of multiple lentigines, electrocardiographic abnormalities, ocular telomerism, pulmonic stenosis, abnormalities of the genitalia, retardation of growth, and sensorineural deafness. Although not directly included in the acronym, HCM is a serious concern in these patients and is seen in approximately 80% of patients with NSML (26, 27, 41). In ~90% of cases, NSML is due to mutations in the non-receptor protein tyrosine phosphatase protein SHP2 which is encoded by PTPN11 (5, 24). In the remaining cases, mutations in BRAF and RAF1 have been identified (20, 32, 37, 40).

SHP2 is an essential positive or negative regulator of multiple signaling pathways (35). Using various in vitro and in vivo models, we and others previously found that NSML mutations in SHP2 result in increased stimulation of Akt and mammalian target of rapamycin (mTOR) (8, 17, 30, 43). This leads to enhanced growth signaling and thereby cardiomyocyte hypertrophy. Hyperactivation of Akt has previously been shown to increase intracellular calcium availability and enhance cardiac contractility (3, 4, 19). In classic HCM, changes in calcium handling and calcium sensitivity and/or alterations in the biophysical properties of the contractile apparatus are thought to be critical to the pathomechanism (10). Therefore, we hypothesized that altered calcium handling either alone or in combination with changes in the biomechanical characteristics of the sarcomere play a role in NSML. This could be a shared pathomechanism of classic and phenocopy forms of HCM and would explain why these disease variants are clinically similar despite very different genetic causes.
To test our hypothesis, we used a previously generated transgenic mouse model of NSML-associated HCM. In this model, cardiomyocyte-specific expression of the mutant protein Q510E-SHP2 starting before birth results in neonatal-onset HCM (43). This mouse model recapitulates the aggressive form of HCM found in patients carrying the same SHP2 mutation. Importantly, all our prior findings in the Q510E-SHP2 model are consistent with the HCM phenotype described in other NSML models based on the Y279C and T468M mutations in SHP2 (30, 45). Because of the severity and early onset of the cardiac phenotype, the Q510E-SHP2 mouse model is ideally suited for proof-of-principle studies. In this investigation, cardiac contractile function early in the course of disease progression was determined in vivo. Subsequently, isolated cardiomyocytes and skinned myofiber preparations from these mouse hearts were used to examine calcium handling and sarcomeric biomechanical properties.

MATERIALS AND METHODS

Animals. Generation of these transgenic mice and detailed phenotype analyses were previously reported (43). For the current studies, only 4 week-old mice of either gender were used. All protocols were in accordance with the Guiding Principles in the Care and Use of Vertebrate Animals in Research and Training of the American Physiological Society and submitted to and approved by the Animal Care and Use Committee of the University of Missouri.

Echocardiography. Echocardiograms were performed under inhalation anesthesia (1.2-1.8% isoflurane, 0.6 L flow of O2) using a Vevo 2100 ultrasound system (Visualsonics, Toronto, Canada). The echocardiographer was blinded to the mouse genotype. M-mode echocardiography was performed using the parasternal short-axis view of the left ventricle (LV). The guidelines of the American Society of Echocardiography were used for measurement of the LV end-diastolic and end-systolic diameters, and septal and posterior wall thickness. Images were captured digitally and 6 consecutive cardiac cycles were measured and averaged for each animal.

Protein analyses. For total protein extracts, flash-frozen mouse ventricles were homogenized in lysis buffer (150 mM NaCl, 10 mM Tris, pH 7.4, 1% Triton X-100, 1x HALT Protease & Phosphatase Inhibitor Cocktail (Sigma-Aldrich, St. Louis, MO). The following antibodies were used for Western blotting: Akt, phospho-Akt (Ser473), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phospholamban (PLB), and phosphoPLB (Ser16/Thr17) from Cell Signaling Technologies, Beverly, MA; sarco/endoplasmic reticulum Ca\textsuperscript{2+} ATPase-2 (SERCA), sodium-calcium exchanger (NCX), calsequestrin 2 (CSQ), ryanodine receptor (RyR), and phosphor-ryanodine receptor (pRyR Ser2808) from Abcam, Cambridge, MA; cardiac myosin binding protein C (cMYBP-C, MYBPC3) and α-myosin heavy chain (α-MHC, MYH1/2/4/6) from Santa Cruz Biotechnology, Santa Cruz, CA; α1C subunit (Ca\textsubscript{v}1.2) of the L-
Calcium channel (LTCC) from Alomone Labs, Jerusalem, Israel; cardiac Troponin I (cTnI) from Millipore, Billerica, MA. Phosphorylated and total protein bands were quantified using the Bio-Rad ChemDoc or GelDoc imaging systems (Bio-Rad, Berkeley, CA).

Calcium measurements. Hearts were excised from mice anesthetized with 60 mg/kg pentobarbital sodium and perfused via the aorta with calcium-free physiological saline solution (containing in mM: 143 NaCl, 5 KCl, 1 MgCl₂, 10 D-glucose, 10 HEPES, pH 7.4 and supplemented with 2 U/ml heparin) for 10 minutes. Hearts were then perfused for 10 minutes with a minimal essential medium (MEM)-based enzymatic isolation solution containing (in mM) 10 NaHCO₃, 2 Na-Pyruvate, 10 HEPES, 8 Taurine, 20 μM CaCl₂, 50 U/ml Penicillin-Streptomycin (Life Technologies, Grand Island, NY), and 22.5 mg/L Liberase Blendzyme TH (Roche Applied Science, Indianapolis, IN), pH 7.35, all at 37 ºC. Dissociated left ventricular cardiomyocytes were gradually adapted to calcium (from 50 μM to 500 μM calcium over 40 minutes), plated on laminin-coated coverslips, and loaded with 5 μM fluo-4/AM (Life Technologies) for 10 minutes, followed by a 20-40 minute wash. Coverslips were secured in an imaging chamber, perfused at ~2 ml/min with physiological saline solution (containing in mM: 135 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 10 D-glucose, 10 Hepes, pH 7.4 with NaOH), and imaged using a Leica SP5 confocal microscope (Leica Microsystems, Buffalo Grove, IL). Two-dimensional imaging of a central region of the cell was performed at 100 frames per second using the resonant scanhead of the Leica SP5 (HCS PL APO 40X objective: 1.25 NA. 512 x 64 pixels at Zoom 3.5, bi-directional scanning/2X line averaging, pixel size 0.22 μm), with excitation at 488 nm and emission recorded from 500-580 nm. Cardiomyocytes were electrically stimulated at 0.25, 0.5, or 1 Hz using electrical field stimulation (S48, Grass Instruments, Warwick, RI). Sarcoplasmic reticulum (SR) calcium content was assessed immediately following a 0.5 Hz pacing train by rapidly (~1 s) applying 10 mM Caffeine. All experiments were performed at room temperature (22–24°C). Example traces and summary data are presented as F/F₀, where F is the peak fluorescence in response to electrical stimulation or caffeine application and F₀ is the baseline fluorescence. Calcium transient recovery tau (τ) was determined from the decay of the normalized fluorescence signal using an exponential fit between 80% of peak and the baseline. All fluorescence values were background subtracted prior to analysis.

Cardiomyocyte contractile function. Cardiac myocyte preparations were obtained by mechanical disruption of mouse hearts as described previously (33). The cardiomyocytes were subsequently skinned in 0.3% Triton X-100 (Pierce Biotechnology, Rockford, IL) in relaxing solution (in mM: 2 EGTA, 5 MgCl₂, 4 ATP, 10 imidazole, 100 KCl, pH 7.0 with the addition of protease inhibitors (Calbiochem, San Diego, CA)). The experimental apparatus for physiological measurements on myocyte preparations was similar to one previously described (33). Myocyte preparations were attached between a force transducer and torque motor by placing the ends of the myocyte preparation into stainless steel troughs (25 gauge).
The ends of the myocyte preparations were secured by overlaying a 0.5 mm length of 3-0 monofilament nylon suture (Ethicon, Somerville, NJ) onto each end of the myocyte, and then tying the suture into the troughs with two loops of 10-0 monofilament (Ethicon). The experimental apparatus was mounted on the stage of an inverted microscope (model IX-70, Olympus Instrument Co., Japan) on a pneumatic vibration isolation table. Mechanical measurements were performed using a capacitance-gauge transducer (Model 403, sensitivity of 20 mV mg⁻¹, plus a 10X amplifier) and resonant frequency of 600 Hz (Aurora Scientific, Aurora ON, Canada). Resting sarcomere length was set to ~2.30 μm in pCa 9.0 solution using an IonOptix SarcLen system (IonOptix, Milton, MA), which used a fast Fourier transform algorithm of the video image of the myocyte.

Compositions of relaxing and activating solutions used in mechanical measurements were as follows (in mM): 7 EGTA, 1 free Mg²⁺, 20 imidazole, 4 MgATP, 14.5 creatine phosphate, pH 7.0, various calcium concentrations between 10⁻⁹ M (relaxing solution) and 10⁻⁴.₅ M (maximal calcium activating solution), and sufficient KCl to adjust ionic strength to 180 mM. The final concentrations of each metal, ligand, and metal-ligand complex at 13°C were determined according to Fabiato (9). Preceding calcium activations, myocyte preparations were immersed for 30 sec in a solution of reduced calcium-EGTA buffering capacity, which was identical to normal relaxing solution except that EGTA was reduced to 0.5 mM. This protocol resulted in more rapid development of steady state force during subsequent activation and helped preserve the sarcomeric integrity during activation.

All mechanical measurements were made at 13±1°C. The protocol for force-velocity and power-load measurements has been previously described (33). Briefly, force-velocity and power-load measurements were made on each cardiomyocyte during maximal calcium activation. The cardiomyocyte was transferred into maximal calcium activating solution and after steady-state maximal force was attained, a series of force clamps was performed to determine isotonic shortening velocities. Using a servo system, force was maintained constant for a designated period of time (150 to 250 ms) while the length change was continuously monitored. After the force clamp, the cardiomyocyte preparation was slackened to reduce force to near zero to allow estimation of the relative load sustained during isotonic shortening; the cardiomyocyte was subsequently re-extended to its initial length.

Measurement of force development kinetics was accomplished as previously described (16). In short, a cardiomyocyte in activating solution was allowed to develop steady-state force after which it was rapidly slacked by 15-20% of original cardiomyocyte length (L₀) and held for 20 ms and then was rapidly re-stretched to a value slightly greater than L₀ for 2 ms before it was returned to L₀. This slack-re-stretch maneuver is thought to cause dissociation of crossbridges and redistribution to pre-force generating states, and thus force redevelopment arises from re-attachment of crossbridges to the thin filament and/or subsequent transition to force generating states.
Cyclic-AMP dependent protein kinase (PKA) enzymatic activity. PKA was obtained from whole heart cell lysate and the enzymatic activity assessed by incubation with a fluorescent peptide substrate (PepTag® assay, Promega, Madison, WI) according to the manufacturer directions. Phosphorylation by PKA alters the net charge of the substrate (L-R-R-A-S-L-G) from +1 to −1. This allows the phosphorylated and non-phosphorylated peptides to be separated on an agarose gel. The phosphorylated species migrates toward the positive electrode, while the nonphosphorylated substrate migrates toward the negative electrode. The gels were photographed using the Bio-Rad GelDoc imaging system and fluorescent bands quantified with QuantityOne software (Bio-Rad).

PKA backphosphorylation assay. PKA-induced phosphate incorporation into myofibrillar substrates was determined as described previously (14, 34). Briefly, skinned cardiac myocytes (10 μg) were incubated with the catalytic subunit of PKA (5 μg/mL) and 50 μCi [γ-32P] ATP at room temperature (21–23°C) for 45 min. The reaction was stopped by the addition of electrophoresis sample buffer and heating at 95°C for 3 min. The samples were then separated by SDS-PAGE for 2.5 hrs at 12 mA, silver stained to control for loading, and subsequently exposed to X-ray film for visualization.

Transmission electron microscopy. Hearts were perfused with phosphate-buffered saline containing 25 mM KCl and 5% dextrose, fixed in 2% paraformaldehyde and 2% glutaraldehyde in 0.1 sodium cacodylate, and processed for thin sectioning. Photomicrographs were obtained using a JEOL 1400 transmission electron microscope (JEOL, Peabody, MA). Photos were taken from each section at 3000X magnification in random fashion. To determine sarcomere length, 4-5 sarcomeres per heart were measured using Image J (NIH, Bethesda, MD) (n = 3 NTG, n = 3 TG). For assessment of the relative area per visual field occupied by sarcomeres, 30 – 35 randomly chosen, non-overlapping photos were evaluated using Image J.

Quantitative real time polymerase chain reaction (qRT-PCR). Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) for first-strand DNA synthesis (Superscript® III First-Strand Synthesis System, Invitrogen). SYBR Green-based quantitative real time PCR was carried out on a Bio-Rad MyiQ iCycler. Primer sequences were obtained from Roche’s Universal ProbeLibrary: α-MHC left cgc atc aag gag ctc acc, right cct gca gcc gca tta agt, cTnI left gca ggt gaa gaa gga gga ca, right cga tat tct tgc gec aet c, cMyBP-C left gca tga agc agg atg aaa aga, right tct tgt ggc cct tgt tta cc, GAPDH left agc ttg tca tca acg gga ag right ttt gat gtt agt ggg gtc tcg. The relative expression levels were determined using the 2-ΔΔCT method with GAPDH as the housekeeping gene (28).

Cardiac myofiber purification. Equal amounts of ventricular tissue (by weight) from NTG and TG mice were used for extraction of cardiac myofibers as described (22, 44). Tissue was homogenized in low-salt F-60 buffer (60 mM KCl, 30 mM imidazole, 2 mM MgCl₂, pH 7.4) and soluble proteins separated by low-speed centrifugation. F-60 washes were repeated without and with 1 mM EGTA, then
with 1% Triton X-100, followed by more F-60 washes. The resulting sarcomeric protein pellets were
dissolved in equal amounts of high-salt buffer (0.5 M NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.76 mM
KH₂PO₄, pH 7.4) and equal volumes loaded onto SDS-page gels. Protein bands were stained with
GelCode Blue Safe Protein Stain (Thermo Fisher Scientific, Rockford, IL) and the total stain intensity of
the entire lane across all molecular weights quantified (GelDoc imaging systems, Bio-Rad).

Statistical analysis. Skinned myocyte preparation length traces, force-velocity curves, power-load
curves, and rate constants of force redevelopment were analyzed as previously described (16, 33).
Frequency-dependent measurements of calcium transients and recovery rates were analyzed using
repeated measures analysis of variance (ANOVA) with Bonferroni post hoc analysis. All other statistical
comparisons were made using unpaired Student t tests (non-transgenic versus Q510E-SHP2), with
summary data presented as mean ± standard error of the mean. P<0.05 was considered significant.

RESULTS

Q510E-SHP2 expression induces hyperdynamic ventricular function in young mice. We previously
generated a transgenic mouse model that recapitulates NSML-associated HCM with neonatal onset (43).
In short, newborn mice exhibit increased cardiomyocyte cross-sectional areas, heart-to-body weight
ratios, interventricular septum thickness, and cardiomyocyte disarray. In adult mice, interstitial fibrosis
can be detected and contractile function is depressed.

To quantify the degree of cardiac hypertrophy in TG hearts early in the course of disease, we obtained
detailed gravimetric data at 4 weeks of age for this study. As shown in Fig. 1 A and B, absolute heart
weights as well as the heart-to-body weight ratio were significantly increased both in male and female
TGs compared to NTG mice. Since we had noted a small variation in body weight between groups (NTG
female 18.55 ± 0.26, TG female 17.93 ± 0.36, NTG male 20.35 ± 0.56, TG male 19.41 ± 0.8 g) and since
atrial enlargement could have substantially contributed to the difference in heart-to-body weight ratio, we
also calculated the ventricular weight-to-tibia length ratios (Fig. 1C). Again, this index was significantly
increased both in male and female TG mice. Furthermore, we measured isolated cardiomyocyte sizes after
enzymatic digestion. Cardiomyocytes obtained from 4 week-old TG mice exhibited cellular hypertrophy
with a significant 18% increase in cell length and a 49% increase in cell width compared to
cardiomyocytes from NTG mice (Fig. 1, H and I).

In many cases of HCM, early hypercontractile cardiac function precedes the transition to overt
contractile dysfunction (51). To evaluate in vivo cardiac function in early stage disease, we therefore
performed echocardiography in 4-6 week-old mice under isoflurane anesthesia. This time point was
chosen on the basis of pilot data indicating that contractile function is increased in 4-6 week-old mice and normal in 8 week-old mice, whereas contractile function is impaired in mice at 11 weeks and thereafter (43). Representative M-mode scans obtained in the parasternal short axis at the mid-papillary plane are shown in Fig. 1, D and E. In contrast to our previous data obtained in 3 month-old mice (43), 4-6 week-old TG hearts had enhanced contractile function with significantly increased ejection fraction and fractional shortening compared to NTG littermates (Fig. 1, F and G), indicating hypercontractile function. Next, we confirmed that signaling through Akt is increased in TG mice at this age. Western blotting using ventricular tissue showed a 2.6-fold increase of relative Akt phosphorylation over baseline (Fig. 1, J and K).

**Q510E-SHP2 expression enhances intracellular calcium cycling.** The positive inotropic effects on the heart are primarily mediated via modulation of the amplitude of the systolic intracellular calcium transient during excitation-contraction coupling (ECC). We therefore examined calcium transients during ECC in enzymatically isolated cardiomyocytes of 4 week-old TG and NTG mice. Cardiomyocytes loaded with the calcium-sensitive indicator dye fluo-4/AM were electrically-stimulated at 0.25, 0.5, and 1 Hz stimulation frequencies. Shown in Fig. 2 are example images and F/F₀ profiles of cardiomyocytes from a NTG (Fig. 2A) and TG (Fig. 2B) heart prior to (diastole) and immediately following (systole) action-potential stimulation. Calcium transient amplitude was significantly higher in TG cardiomyocytes at all stimulation frequencies examined (Fig. 2C). Recovery of the calcium transient (i.e., transient τ) trended towards shorter times in cardiomyocytes of TG mice although this finding did not reach statistical significance (p = 0.06) (Fig. 2D). SR calcium content was assessed by rapid application of 10 mM caffeine following cessation of a 0.5 Hz stimulation train, and was significantly elevated in cardiomyocytes from TG mice (Fig. 2E).

To further investigate the ability of the SR to sequester calcium we monitored the protein expression of SERCA and its regulatory accessory protein PLB. While SERCA protein expression was significantly increased in TG, the expression of PLB was unchanged between TG and NTG hearts (Fig. 3). Further, the ratio between phospho-PLB and PLB, was unchanged comparing TG and NTG hearts (Fig. 3). Protein expression of RyR and the phospho-RyR/RyR ratio were unchanged between TG and NTG hearts. Similarly, protein expression of CSQ, Caᵥ1.2, and NCX were also not altered by Q51E-SHP2 expression (Fig. 3).

**Q510E-SHP2 expression increases sarcomeric contractile function.** Next, we considered the possibility of other, calcium-independent effects of Q510E-SHP2 expression that may enhance contractile function. In particular, we focused on the biomechanical characteristics of the contractile apparatus. Using skinned cardiomyocyte preparations from NTG and TG hearts, the force-generating capability was measured at a fixed calcium concentration. Fig. 4 A - D shows representative recordings of shortening and
force over time during a series of force clamps followed by slackening. Fig. 4 E and F show the respective force-velocity relationships and power-load curves. In skinned cardiomyocytes from TG hearts, both curves were shifted upwards compared to preparations from NTG hearts. Furthermore, peak power generating capacity was increased ~2-fold in cardiomyocytes from Q510E-SHP2 mice compared to cardiomyocytes from NTG mice; this effect was mostly due to the increase in tension-generating capacity of the cardiomyocyte preparations. The rate of force development was measured using a slack restretch maneuver during maximal calcium activation. Cardiomyocyte preparations from Q510E-SHP2 mice exhibited significantly greater rate constants of force development ($k_{tr}$) than cardiomyocytes from NTG mice (Q510E-SHP2- 10.5 ± 0.9 s$^{-1}$ versus NTG- 8.0 ± 0.7 s$^{-1}$ p < 0.05) (Fig. 4, G and H). Fig. 4 I - K shows the summary data of the tension and power measurements, indicating that sarcomeric function is substantially increased in TG cardiomyocytes, which is consistent with the hypercontractility seen in echocardiography at the same age.

**Q510E-SHP2 expression does not affect PKA activity.** Post-translational modification of sarcomeric proteins is a major mechanism for fine-tuning myofibrillar function. Importantly, PKA-mediated phosphorylation of myofibrillar proteins increases power generation (12). Furthermore, interaction of SHP2 and PKA in a signalosome complex induced by shear stress has been reported (7), suggesting that similar interactions could also play a role in cardiomyocytes expressing Q510E-SHP2. Therefore, we tested whether PKA-dependent posttranslational modification of the sarcomeric proteins is responsible for the increase in contractile function. PKA activity assays were conducted using myocardial tissue samples from NTG and TG hearts. No significant difference was noted in PKA activity as assessed by the relative degrees of phosphorylation of the fluorescently tagged PKA specific peptide ($p = 0.45$, Fig. 5, A and B). For further confirmation, a backphosphorylation assay was used. Sarcomeric proteins from skinned cardiomyocytes were incubated with active PKA in the presence of radiolabeled ATP, and subsequently separated by gel electrophoresis. The degree of radiolabel incorporation in the various sarcomeric proteins did not differ between cardiomyocytes from NTG versus TG hearts (Fig. 5, C and D).

**Q510E-SHP2 expression increases the contractile apparatus, but not sarcomeric protein mRNA.** Electron microscopy was utilized to examine ultrastructural morphological differences in NTG and TG cardiomyocytes that could explain the functional differences. Representative electron microscopy images prepared from 4 week-old hearts are shown in Fig. 6A. There were no significant differences in sarcomere organization or sarcomere length between the NTG and TG hearts ($p = 0.22$, Fig. 6B). However, the relative area per visual field occupied by sarcomeres was significantly increased in TG compared to NTG myocardium (Fig. 6C). This indicates that more thin and thick filaments per cardiomyocyte width may be available for force generation, which is consistent with the increased power developed by the skinned TG cardiomyocyte preparations.
This led to the hypothesis that transcription of sarcomeric proteins may be increased by expression of Q510E-SHP2. To quantify mRNA levels of α-MHC, cTnI, and cMyBP-C, qRT-PCR was used. There were no significant differences in mRNA expression of α-MHC, cTnI, or cMyBP-C when comparing ventricular tissue samples from NTG (n = 5) and TG (n = 4) mice (Fig. 6D), although there was a trend toward significance with increased cMyBP-C mRNA expression in TG (p = 0.073). GAPDH expression was used as a reference and did not differ between groups. To determine sarcomeric protein levels, total proteins from ventricular tissue samples underwent Western blotting. Again, GAPDH was used as a housekeeping gene to correct for protein loading. As shown in Fig. 6 E and F, there were no significant differences in total α-MHC, cTnI, or cMyBP-C expression.

Since the TEM data had revealed an increase in assembled contractile myofibers, we hypothesized that Q510E-SHP2 does not change total sarcomeric protein content, but alters the relative amount of incorporated contractile proteins versus those remaining in a free pool. To test this, we purified cardiac myofibers from equal amounts of TG and NTG ventricular tissue and quantified the total protein yield of the extraction using gel electrophoresis. In TG samples, the total amount of extracted proteins was increased (Fig. 6, G and H).

DISCUSSION

The goal of our study was to investigate the cellular mechanism(s) underlying the development of HCM in Q510E-SHP2-induced NSML. Cardiac-specific Q510E-SHP2 expression induces a hyperdynamic contractile state in hypertrophied young mouse hearts. Mechanistically, we found two independent mechanisms that synergistically promote hypercontractility. First, intracellular calcium transients were increased in TG cardiomyocytes concurrent with increased SERCA expression and SR calcium content. Secondly, TG cardiomyocytes exhibited an enlarged contractile apparatus, resulting in increased cardiac myofibrillar force and power generation.

In this NSML model, the early disease stage is characterized by cardiomyocyte hypertrophy with enhanced ventricular function. In the literature, a hyperdynamic contractile state has not yet been described in patients carrying the Q510E mutation in SHP, a finding likely attributed to clinical diagnosis after the onset of heart failure (6, 27, 46). Importantly, normal or hypercontractile function is a common characteristic in early stages of ‘classic’ HCM due to sarcomeric mutations (51). For example, hypercontractility identified as an increased ejection fraction and enhanced LV twist was noted in patients with familial forms of HCM (38, 48) as well as in mice (11). This suggests that hypercontractility early in disease progression could be a common denominator of various forms of HCM, regardless of etiology.
We previously determined and now again confirmed that overexpression of Q510E-SHP2 leads to increased Akt activation in cardiomyocytes (42, 43). Since Akt signaling is known to control calcium handling and thereby contractile function, we assessed calcium transients following action-potential stimulation. At all stimulation frequencies examined, calcium transients were elevated by Q510E-SHP2 expression. In mice, the amplitude of the intracellular calcium transient is primarily due to SR calcium release (~90% release versus 10% entry, (25)), which in turn is governed by the content of calcium within the SR (1). Consistent with the increase in calcium transient amplitude, SR calcium content was significantly elevated in Q510E-SHP2 mice. Mechanistically, the increased ability of the SR to sequester calcium was due to an increase in protein expression of SERCA, and not due to changes in expression or phosphorylation status of the SERCA inhibitory protein PLB (21). These data are in agreement with studies utilizing adenoviral or transgenic overexpression of active Akt in rodents, which increased SERCA expression and enhanced contractile function (3, 19). Notably, cardiac overexpression of SERCA alone results in enhanced calcium transients and hypercontractility (13). SERCA overexpression also accelerates relaxation (13), which is consistent with the trend towards decreased $\tau$ that we observed.

Therefore, the increase in SERCA expression in the Q510E-SHP2 model is most likely mediated by Akt. In addition to regulating SERCA expression, Akt has been shown to regulate LTCC activity (2, 19). Similar to the findings of Kim et al. (19), LTCC expression was unchanged between TG and NTG in our study. We cannot completely rule out that LTCC-mediated calcium influx may be increased, but in the absence of changes in LTCC expression functional activity is primarily regulated by PKA-dependent phosphorylation. Importantly, we were not able to detect any changes in PKA activity or the phosphorylation status of other target proteins such as sarcomeric proteins. Furthermore, intracellular calcium transport in mice is dominated by SR calcium cycling (25). Therefore, the increase in SERCA expression likely represents the mechanisms by which calcium transients are increased in Q510E-SHP2 mice.

Independent of all changes in calcium handling, we found that Q510E-SHP2 increases contractile function of skinned cardiac myofibers. Our initial hypothesis had been that the increase in power generation could be due to altered posttranslational modification of sarcomeric proteins. The biomechanical properties of the contractile proteins are fine-tuned by post-translational modifications such as phosphorylation by PKA. However, our data demonstrate that PKA-induced posttranslational modifications are unlikely to have contributed to the hypercontractile phenotype observed in this NSML model. But at this point, other modifications for example induced by protein kinase C or calcium-calmodulin-dependent protein kinase II cannot be excluded.

Having excluded increased PKA activity as the responsible mechanism, we used electron microscopy to quantify the amount of contractile fibers in the TG myocardium and found that Q510E-SHP2
expression increased the contractile machinery. Consistent with this, we previously reported that Q510E-SHP2 increases sarcomeric organization as well as overall protein synthesis in neonatal rat cardiomyocytes (42). Our current data are also consistent with the electron microscopic findings in a different NSML mouse model based on ubiquitous expression of Y279C-SHP2 (30). As sarcomeres are assembled, strict stoichiometry between the different components appears to be preserved (36). Importantly, sarcomeres are dynamic structures with ongoing incorporation and turnover of the contractile proteins via exchange from a free pool (29, 39, 50). As we could not detect any changes in total sarcomeric protein levels, but found increased amounts of myofibers that could be extracted from ventricular tissue, we suspect that Q510E-SHP2 expression leads to alterations in the kinetics of sarcomere assembly. This would be consistent with a recent *Xenopus* study demonstrating that SHP2 regulates formation and polarity of cardiac actin fibers during development (23). On the other hand, it is possible that Q510E-SHP2 expression reduces contractile fiber degradation and turnover. To date, there is no evidence that SHP2 participates in proteasomal pathways, but this remains to be explored. It is possible that isoform switches of various contractile proteins contribute to the increase in power output in transgenic hearts. We previously found that $\alpha$-skeletal actin mRNA was increased in transgenic hearts. Interestingly, expression of this isoform has been shown to be associated with increased contractility compared to hearts primarily expressing $\alpha$-cardiac actin (15). Therefore, this could be another contributing factor enhancing contractility in our NSML model.

Understanding the molecular and cellular mechanisms that induce the NSML phenotype is critical for improving current therapeutic approaches. The mechanistic overlap with classic HCM identified in this study is intriguing, and raises the question whether or not NSML-associated and classic HCM should be treated with the same pharmacological compounds. We recently showed that rapamycin and various other pathway-specific inhibitors are effective against cardiomyocyte hypertrophy induced by Q510E-SHP2 expression (42, 43). This argues for a custom-tailored therapeutic approach for NSML-associated HCM. However, rapamycin treatment started late in the disease process after contractile function had deteriorated did not improve cardiac function in our model (43). Our new data suggest that initiating treatment during the early, hypercontractile stage might be more effective.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

REFERENCES


FIGURE LEGENDS

Fig. 1. TG Q510E-SHP2 expression induces hypertrophy and increases cardiac contractile function in young mice. A-C: Heart weights, heart-to-body weight ratios, and left + right ventricular (LV + RV) weight-to-tibia length ratios were significantly increased both in female and in male TG mice at 4 weeks of age. N = 22-26 mice per group. In these and all following panels: white bars, NTG; black bars, TG; *P < 0.05 vs. NTG. D and E: Echocardiographic end-systolic and end-diastolic LV diameter measurements are indicated by arrows in representative M-mode scans. F and G: LV ejection fraction and fractional shortening were significantly increased in TG mice. N = 4 mice per group. H and I: Cardiomyocyte length and width were significantly increased in isolates from 4 week-old TG mice. N = 59 cells from 3 NTG mice, 53 cells from 3 TG mice. J and K: Ventricular tissue homogenates from 4 week-old mice underwent Western blotting to quantify the Akt phosphorylation status. Expression of Q510E-SHP2 increased the relative level of Akt phosphorylation ~2.6 fold compared to NTG samples. N= 4 per group.

Fig. 2. Cardiomyocytes of Q510E-SHP2 TG mice exhibit enhanced calcium signaling during excitation-contraction coupling. A and B: example 2-D laser-scanning confocal fluorescence images (fluo-4/AM, upper) and F/F₀ profiles (lower) of calcium responses prior to (cellular diastole) and immediately following 0.5 Hz action-potential stimulation (cellular systole, stimuli marked with black dots in profiles) in cardiomyocytes from 4 week-old NTG and TG mice. C: summary data showing significantly enhanced calcium transients during excitation-contraction coupling in TG cardiomyocytes (black bars, n = 9 cells from 4 mice) compared to NTG (white bars, n = 11 cells from 4 mice). D: Recovery of the calcium transient (transient τ) trended towards shorter times in cardiomyocytes of TG mice (black bars) although this finding did not reach statistical significance (p = 0.06). E: sarcoplasmic reticulum (SR) calcium content as assessed by rapid application of 10 mM caffeine (at arrow) following cessation of a 0.5 Hz pacing train (black dots) in NTG and TG cardiomyocytes. Bar graphs show summary data demonstrating elevated SR calcium content in TG cardiomyocytes (black bars; n = 7-8 cells from 4 mice per group). * P<0.05 vs. NTG.

Fig. 3. Cardiac Q510E-SHP2 expression increases SERCA protein levels in 4 week-old mice. A and B: Ventricular tissue samples underwent Western blotting to assess total proteins levels and respective phosphorylation status. GAPDH was used as a loading control, n = 4 per group. The relative expression level of SERCA was significantly increased in TG samples (*P<0.05). In contrast, expression and phosphorylation levels of PLB, RyR, CSQ, Caᵥ1.2, and NCX were not significantly different between NTG and TG samples.

Fig. 4. Q510E-SHP2 expression increases force and power generation in skinned cardiac myofibers. A-D: force clamps (for 150 to 250 ms) and length traces during maximal calcium activations of a cardiomyocyte from NTG and TG mice, respectively. E-F: force-velocity and power-load curves of a myocyte (at maximal calcium activation) from a NTG mouse heart (open circles) and a TG heart (closed circles). G: force redevelopment traces after a slack-re-stretch maneuver of an NTG cardiomyocyte (gray trace) and a TG cardiomyocyte (black trace) during maximal calcium activation. TG cardiomyocyte preparations exhibited faster force redevelopment kinetics than compared to NTG. H-K: bar plots summarizing rate constants, tension, and power (both absolute and normalized for cardiomyocyte size) generated by cardiomyocyte preparations from NTG and TG hearts. In all bar graphs, * P<0.05 vs. NTG, n = 9.

Fig. 5. PKA activity is normal in Q510E-SHP2-expressing cardiomyocytes. A: ventricular tissue lysates were incubated with a PKA-specific peptide substrate and subsequently the degree of substrate phosphorylation assessed by gel electrophoresis. The phosphorylated species migrates toward the positive electrode, while the nonphosphorylated substrate migrates toward the negative electrode. B: quantification
of the relative degree of PKA substrate phosphorylation, n = 11 per group. C and D: representative autoradiograms (C) and silver-stained protein gel (D) following PKA backphosphorylation. After normalization for total protein load, the degree of radiolabel incorporation in the various sarcomeric proteins did not differ between cardiomyocytes from NTG versus TG hearts. N = 3 per group.

Fig. 6. The contractile apparatus is expanded Q510E-SHP2-expressing hearts. A: representative electron micrographs showing sarcomeres in NTG and TG hearts. B and C: quantification of sarcomere length and relative area per random visual field occupied by contractile fibers. Q510E-SHP2 expression significantly increased the relative area per visual field occupied by sarcomeres. N = 14 images from 3 hearts per group. Both in NTG and TG, each image contained 5-7 sarcomeres as counted in the longitudinal direction of the myofibers. D: mRNA levels of α-MHC, cTnI, and cMyBP-C were quantified using qRT-PCR with GAPDH as a housekeeping gene. There were no significant differences in mRNA expression, although a trend towards increased cMyBP-C mRNA expression in TG compared to NTG was noted. N = 8-10 in all groups. E and F: Homogenized ventricular tissue from NTG and TG hearts underwent Western blotting. GAPDH was used as loading control. Samples were probed for total α-MHC, cTnI, and cMyBP-C. Signal quantification did not show any difference in relative protein expression levels between NTG and TG. N = 4 per group. (§, of note, the two GAPDH blots shown are the same as in Fig. 3 because the Western blots were run at the same time from the same tissue lysates.) G and H: Cardiac myofibers were extracted from equal amounts of NTG and TG ventricular tissue. The total protein yield of the extraction procedure was quantified via gel electrophoresis followed by Coomassie Blue staining. The amount of sarcomeric proteins obtained from the TG samples was significantly increased compared to NTG. N = 8 per group. * P<0.05 vs. NTG in all panels.