RESEARCH ARTICLE

Genetic approaches to identify pathological limitations in aortic smooth muscle contraction

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Abstract

Aortic smooth muscle contains limiting amounts of myosin light chain kinase (MLCK) for myosin regulatory light chain (RLC) phosphorylation and contraction that predisposes to thoracic aortic disease in humans containing heterozygous loss-of-function mutations in MYLK. We tested the hypothesis that thoracic aortic smooth muscle contraction may also be susceptible to variations in the smooth muscle-specific isoform of the motor protein myosin where inactivation of one Myh11 allele or the presence of one Myh11 missense variant associated with an increased risk of human aortic disease may result in a reduced force development response. Additionally, other kinds of smooth muscles may be less sensitive to the effects of mutations in one smooth muscle myosin allele, similar to results obtained with MYLK. Force development responses were reduced in aortic tissue from a conditional knockout of smooth muscle myosin heavy chain in adult mice (Myh11⁺/⁻ or Myh11⁻/⁻) with a greater reduction with homozygous vs heterozygous tissues. Similar reductions in force responses were obtained with tissues containing either a heterozygous or homozygous knockin mutation in smooth muscle myosin heavy chain (Myh11⁺/R247C or Myh11⁻/R247C mutations that cause human aortic disease) with no significant changes in RLC phosphorylation. Agonist-dependent force responses were not reduced significantly in urinary bladder, ileal, or tracheal tissues from Myh11⁺/⁻ mice while only ileal tissue showed a reduced force response in Myh11⁻/⁻ mice. Thus, heterozygous mutations in Myh11 associated with reduced myosin function result in compromised contractile function primarily in aortic smooth muscle.

Introduction

Mutations in genes encoding proteins of the contractile/signaling modules of smooth muscle cells, including ACTA2 (smooth muscle α-actin), MYH11 (smooth muscle myosin heavy chain), MYLK (myosin light chain kinase), and PRKG1 (PKG type-1) [1–4], predispose to thoracic aortic disease with aneurysms and acute ascending aortic dissections in humans [5].
identification of contractile and signaling proteins affecting force development involvement in aortic disease may indicate a lifelong role for cellular mechanotransduction to maintain aortic structure and function [6–10].

The thoracic aorta segment is exposed to the highest biomechanical forces in the circulation arising from pulsatile ejection of blood by the heart. The aortic smooth muscle cell, thus, occupies a continuously dynamic environment with imposed stresses distinct from smooth muscle cells in the gastrointestinal system, urinary bladder, airways, and other smooth muscle tissues. However, the primary smooth muscle contractile proteins α-actin, myosin (a hexamer of two each of heavy chains, essential light chain and RLC), and MLCK are expressed in all smooth muscle-dependent organs of the body [11, 12]. The physiological environment combined with loss-of-function mutations in proteins of the contractile system in aortic smooth muscle cells may contribute to the propensity of the aorta to disease [5, 13–15].

Phosphorylation of myosin RLC by MLCK is required for the cyclic interaction of myosin with actin that leads to contraction (force development) in smooth muscle [11, 15–20]. Diverse cell signaling pathways increase smooth muscle cell intracellular Ca²⁺ concentrations, [Ca²⁺], Ca²⁺ binding to calmodulin leads to MLCK activation and RLC phosphorylation to initiate smooth muscle contraction [11, 16, 21–25]. Myosin light chain phosphatase dephosphorylates RLC, and induces relaxation after a decrease in [Ca²⁺], returns MLCK to an inactive state [11, 17].

Genetic approaches have revealed essential roles for myosin and MLCK in smooth muscle contraction. Aortic tissues from homozygous Myh11R247C/R247C mice containing a rare variant that in humans is associated with increased risk of thoracic aortic aneurysms and dissections demonstrated reduced contractile responses [14]. Contractile responses of other smooth muscle tissues were not examined. In isolated neonatal bladder preparations from homozygous mice containing a conventional knockout of Myh11, there was no initial force transient and the sustained force was only 11%, presumably from nonmuscle myosin in smooth muscle cells [26–29]. Death occurred within a few days after birth. The conditional homozygous knockout of MLCK in smooth muscle cells in adult mice was also lethal because of contractile failure in different smooth muscles in vivo with greatly diminished force responses in different isolated smooth muscle tissues [15, 18–20].

Interestingly, inactivation of only one MYLK allele in humans induces aortic dissections, and the partial reduction of MLCK content in smooth muscle tissues from heterozygous MLCKSM+/− mice led to a reduction of RLC phosphorylation and force responses in aortic, but not bladder, airway, or ileal smooth muscle tissues [3, 15, 18, 19]. Thus, aortic tissue showed a unique sensitivity to reduced MLCK content, probably related to the limiting amount of MLCK in aortic, but not other smooth muscles.

Mutations in one smooth muscle myosin allele may predispose to thoracic aortic disease with aneurysms and acute ascending aortic dissections in humans [5]. We considered the possibility that inactivation of one Myh11 allele or the presence of one Myh11 missense variant associated with human aortic disease may result in the reduction of force development response in thoracic aorta. Additionally, other kinds of smooth muscles may be less sensitive to the effects of mutations in one smooth muscle myosin allele. These hypotheses were tested in genetically modified mice.

**Materials and methods**

**Generation of genetically modified mice**

Myh11R247C/R247C knockin mice (denoted as Myh11R/R) containing mutated DNA within two loxP sites were previously described [14]. Mice were crossed with a transgenic SMA-Cre-ER<sup>T2</sup> mouse line in which the expression of the tamoxifen-dependent Cre-ER<sup>T2</sup> recombinase is under
the control of a large genomic DNA segment of the mouse smooth muscle α-actin (SMA) gene [30], resulting in Myh11R247C/R247C Cre+ mice. The Cre-ER<T>mediated recombination of LoxP flanked target DNA is strictly tamoxifen-dependent, and efficient in both vascular and visceral smooth muscle cells, including the aorta. Mice were bred and screened as described previously [31–33]. Male mice (8–10 weeks old) were injected intraperitoneally with tamoxifen or vehicle for five consecutive days each week for two weeks at a dose of 1 mg/day [33].

Experiments were performed in accordance with the National Institutes of Health and Institutional Animal Care and Use Guidelines. The Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center approved all procedures and protocols in compliance with recommended physiological guidelines (Drummond, 2009). Animals were administered an intraperitoneal lethal dose of tribromoethanol (250 mg/kg) for tissue collection.

**Force measurements**

Thoracic aortas isolated from anesthetized mice were dissected free of excessive adventitia, and endothelial cells were removed by gentle swabbing. Thoracic aortic segmental rings 5 mm long were mounted by triangular wires to an isometric force apparatus. Aortic rings were passively stretched to 1.8–2.0 g and remained quiescent for 60 min before being contracted with 65 mm KCl in Krebs-Ringer solution followed by treatment with 10 μM phenylephrine. Force responses were recorded isometrically by a Grass FT03 force transducer connected to Powerlab 8/SP data acquisition unit (AD Instruments, Colorado Springs, CO). Force measurements were normalized as grams of developed force per tissue wet weight. Tracheal rings of mice were isolated and connective tissue removed for force measurements as previously described [19]. Urinary bladder and ileal smooth muscle tissues were prepared for force measurements as previously described [25, 34]. Phenylephrine and carbachol were used as agonists for aortic tissue vs other smooth muscles respectively to elicit RhoA/ROCK signaling that would affect force development compared to KCl used to depolarize smooth muscle cells for Ca²⁺ influx.

**Protein analyses**

At indicated times after specific treatments, tissues were quick frozen by clamps pre-chilled in liquid nitrogen. Frozen muscles were processed as described previously by immersion in a frozen slurry of 10% trichloroacetic acid in acetone containing 10 mM dithiothreitol for 30 min, then thawed slowly at room temperature and transferred to microcentrifuge tubes. Tissues were rinsed three times with diethylether, briefly exposed to air for dispersion of residual diethylether, and suspended in urea sample buffer containing 8 M urea, 18.5 mM Tris (pH 8.6), 20.4 mM glycine, 10 mM dithiothreitol, 4 mM EDTA, and 5% sucrose. Proteins were then solubilized in a Bullet Blender (Next Advance, Inc., Averill Park, NY) (with 2-mm zirconium oxide beads, four spins × 3-min each at setting 9) and protein measured by Bradford assay with bovine serum albumin as the standard. Bromophenol blue was added to 0.004% and the sample was stored at -80°C. Aortic samples were quick frozen at 15 secs for maximal phosphorylation response which precedes the early maximal force response [15].

Protein samples were subjected to electrophoresis (4–15% SDS-PAGE) followed by staining 0.1% Coomassie Blue R250 in 10% acetic acid, 50% methanol, and 40% H₂O with shaking overnight. Gels were destained by rocking for 3 to 4 hours in 10% acetic acid, 50% methanol, and 40% H₂O with solution changes, then stored in 10% acetic acid. Myosin heavy chain and actin contents were determined by quantitative densitometry using the ImageQuantTL software package (GE). Data were normalized to the amount of protein in samples from wildtype mice for each tissue.
RLC phosphorylation was measured by urea/glycerol-PAGE as previously described [15, 35]. Muscle proteins in 8 M urea sample buffer were subjected to urea/glycerol-PAGE to separate nonphosphorylated and monophosphorylated RLC. Because of the reduction in RLC amount in tissues from Myh11<sup>-/-</sup> or Myh11<sup>+/+</sup> mice, additional protein was loaded for electrophoresis to improve the sensitivity of the phosphorylation measurements. Following electrophoresis, proteins were transferred to PVDF membranes, and probed with rabbit polyclonal antibodies against smooth muscle RLC [36]. The urea/glycerol-PAGE system separates non-phosphorylated from the monophosphorylated RLC so direct quantitative measures of RLC phosphorylation is obtained as mol of phosphate per mol of RLC. Diphosphorylation results in additional migration of RLC in the urea-PAGE system but there is normally little diphosphorylated RCL formed in response to KCl or agonists in isolated smooth muscle tissues, including herein. Quantitative measurements were processed on a Storm PhosphorImager and analyzed by ImageQuant software.

**Statistical analyses**

All data are presented as mean ± S.E.M. Statistical comparisons were performed by Student’s t test for force development and RLC phosphorylation. One-way ANOVA was performed followed by Newman-Keuls post hoc test for multiple comparisons. Data analyses were performed with statistical software (GraphPad Prism Software, San Diego, CA, USA). P values less than 0.05 were considered statistically significant.

**Results**

**Myosin heavy chain expression is reduced in different mouse smooth muscle tissues with Myh11 gene ablation, but not with the R247C knockin mutation**

The conditional inactivation of both Myh11 alleles decreased smooth muscle myosin heavy chain expression in bladder, aortic, and ileal tissues (Fig 1A). Tissues were harvested 18–20 days from these animals after starting tamoxifen injections before myosin heavy chain protein was fully extinguished because of impending death related to the essential role of myosin in maintenance of homeostasis by smooth muscle organ systems [26, 27]. With the removal of different tissues, the intestines appeared flaccid and empty while bladders were typically full, presumably due to the inability to empty [27]. These results suggest weak contractions consistent with observations described below for isolated tissues in vitro.

In contrast to the results reported for neonatal bladders containing a conventional gene ablation in smooth muscle Myh11<sup>-/-</sup> mice [26], the bladder, aortic, and ileal tissues from the conditional knockout in Myh11<sup>+/+</sup> mice showed reductions in myosin heavy chain content (Fig 1A). In tissues from Myh11<sup>-/-</sup> mice, myosin heavy chain content in bladder, aorta and ileum decreased by 87±6%, 92±1% and 92±2%, respectively. Consistent with knockout of one allele in tissues from Myh11<sup>+/+</sup> mice, the myosin heavy chain content in the bladder, aorta and ileum decreased by 39±6%, 37±5% and 34±7%, respectively.

As expected, myosin heavy chain content did not change in these different smooth muscle tissues from either Myh11<sup>R247C/+</sup> or Myh11<sup>R247C/R247C</sup> mice (Fig 1B).

**Aortic force development is reduced with Myh11 gene ablation**

The initial one-minute force development or sustained five-minute force responses to KCl or phenylephrine treatments were reduced to 15% in aortas from Myh11<sup>-/-</sup> mice (Fig 2A and 2B), consistent with the marked reduction in smooth muscle myosin heavy chain content. We also
**Fig 1. Quantification of myosin heavy chain content.** A) Representative gel image of tissue homogenates from bladder, aortic and ileal tissues from Myh11 knockout mice. Proteins separated by SDS-PAGE were stained with Coomassie blue, and myosin content was normalized to actin. Amount measured in Myh11+/+ (+/+, dark gray bars) and Myh11−/− (−/−, black bars) tissues were normalized to the average of Myh11+/+ (+/+, open bars). B indicates bladder; A, aorta; and I, ileum; MHC, myosin heavy chain. B) Myosin heavy chain content in tissue homogenates of bladder, aorta and ileum from Myh11 R257C mutation knockin mice. Values from Myh11 R257C/+ (R/+, light gray bars) or Myh11 R257C/R257C mice (R/R, hatched bars) were compared with values for Myh11++/+ (+/+, open bars); *p<0.05, **p<0.01, N=3 by Student’s t test.

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**Fig 2. Developed force responses in aortic rings from smooth muscle myosin heavy chain mutant mice.** A) KCl (65 mM) stimulated initial force responses at 1 min and sustained force responses at 5 min. B) Phenylephrine (PE)(10 μM) stimulated initial force responses at 1 min and sustained force responses at 5 minutes. Sample groups are follows: Myh11++/+ (+/+, open bars), Myh11+/− (+/−, dark gray bars), Myh11−/− (−/−, black bars), Myh11 R257C/+ (R/+, light gray bars) and Myh11 R257C/R257C (R/R, hatched bars). Significance was determined by comparisons to Myh11++/+ average at 1 min or 5 min. *p<0.05, **p<0.01, N≥8 by One-way ANOVA followed by Newman-Kuels post hoc test for multiple comparisons.

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found that force responses were reduced 42% in aortic tissues from Myh11<sup>−/−</sup> mice (Fig 2A and 2B), consistent with the partial reduction in myosin heavy chain content (Fig 1A and 1B).

The extents of RLC phosphorylation obtained with KCl or phenylephrine treatments in aortas from Myh11<sup>−/−</sup> or Myh11<sup>+/−</sup> were not changed compared to tissues from wildtype animals (Fig 3A). Thus, the reduced force responses appear to be proportional to the reduced myosin content and its associated RLC subunit with a greater reduction in aortic tissue from Myh11<sup>−/−</sup> mice compared to Myh11<sup>+/−</sup> mice. The western blot quantification of RLC phosphorylation does not distinguish between smooth vs nonmuscle RLC. However, the homozygous

![Graph showing RLC phosphorylation in aortic rings from smooth muscle myosin heavy chain mutant mice.](https://doi.org/10.1371/journal.pone.0193769.g003)
knockout of the smooth muscle myosin heavy chain resulted in 8–13% of myosin remaining in the smooth muscle tissues. Thus, the amount of nonmuscle RLC is substantially less than smooth muscle RLC. Although there were no changes in the extent of RLC phosphorylation, the total amount of phosphorylated RLC would be reduced relative to the reduction in myosin heavy chain in Myh11+/− and Myh11−/− mice. Signaling mechanisms leading to MLCK activation and RLC phosphorylation appear not to be affected with the myosin heavy chain knockout.

**Aortic force development is reduced with the Myh11 R247C mutation**

Aortic tissues homozygous for the myosin heavy chain knockin mutation R247C showed reduced force responses to KCl and phenylephrine, consistent with our previous report [14]. However, force responses in aortic rings from heterozygous Myh11R247C/+ mice were also similarly reduced (Fig 2A and 2B). These findings are particularly relevant to understanding compromised force development since this heterozygous MYH11 R247C rare variant has been identified in patients with thoracic aortic disease.

There was also no attenuation in RLC phosphorylation in aortic rings from Myh11R247C/+ or Myh11R247C/R247C mice (Fig 3B). Therefore, the defective contractile responses did not result from changes to signaling modules acting on RLC phosphorylation, but appear to be intrinsic to the properties of the mutated myosin.

**Force responses with Myh11 gene ablation or myosin containing the R247C mutation in different smooth muscles**

Considering the attenuation of force responses in aortic but not other kinds of smooth muscles from MLCKSM−/− mice [15, 18, 19], we determined force responses in bladder, ileum, and trachea from Myh11+/− and Myh11R247C/R247C mice (Fig 4). Similar forces were obtained in response to agonist with no significant attenuation compared to wildtype mice for bladder, ileal, and tracheal tissues from Myh11+/− mice while there was a significant force reduction response in aorta from Myh11+/− mice. There was also no reduction in force responses in

![Relative force responses in different smooth muscles from myosin heavy chain mutant mice.](https://doi.org/10.1371/journal.pone.0193769.g004)
bladder or tracheal tissues from Myh11 R247C/R247C mice compared to responses from wildtype tissues. However, there was a reduction in the force response for ileal tissues. Collectively, the aortic smooth muscle cell appears particularly sensitive to compromised force responses with mutations in Myh11 genes, similar to observations with MLCK.

**Discussion**

Through genetic approaches we investigated the properties of aortic smooth muscle contraction associated with changes in smooth muscle myosin. The conditional homozygous knockout of smooth muscle myosin heavy chain in adult mice led to a profound reduction in myosin content as well as force responses in aortic, bladder, and ileal smooth muscle similar to results obtained with neonatal bladders from a conventional myosin heavy chain knockout [26, 27]. The conditional heterozygous myosin heavy chain knockout partially reduced myosin heavy chain content with a proportional decrease in the force response without a change in the extent of RLC phosphorylation. Thus, there appear to be no changes in Ca$^{2+}$ signaling pathways to RLC phosphorylation, and the force reduction may be explained by the reduction in the amount of myosin in aortic smooth muscle cells. These results would be consistent with heterozygous loss-of-function myosin mutations potentially leading to compromised force development in humans that may predispose to aortic disease [5, 7, 14].

Force production may also be affected by mutations in myosin heavy chain that alter its biochemical properties. The myosin mutation R247C located in the head region of the myosin heavy chain decreased maximal actin-activated myosin ATPase activity with a reduction in release of ATP hydrolysis products ADP and phosphate when RLC was phosphorylated [14]. The mutant myosin had no activity in the absence of RLC phosphorylation as expected for regulation by phosphorylation. These biochemical properties are consistent with the reduction in aortic force for tissues from Myh11 R247C/R247C mice presented herein and previously published [14]. Importantly, force development was also reduced for aortas from Myh11 R247C/+ mice. Because the extent of RLC phosphorylation was similar in tissues from wildtype and mutant mice, it probably followed a random phosphorylation process without a selective phosphorylation of RLC on the mutant or wildtype heavy chain [36]. Also, the mutation was in the head region and similar amounts of myosin heavy chain were expressed in tissues from wildtype and mutant mice so thick filaments probably form from the random polymerization of mutant and wildtype myosin heavy chains. Thus, the altered biochemical properties of the mutated myosin appear to have a dominant negative effect on wildtype myosin in smooth muscle cells. These results predict heterozygous dysfunctional myosin mutations in humans may result in reduced force production that predisposes to thoracic aortic disease [5].

Different cellular mechanisms may be associated with aortic force reduction responses. Reduction of MLCK content by half did not significantly reduce forces in bladder, tracheal, or ileal smooth muscles [15, 18]. These results were thought to be related to differences in the amount of free Ca$^{2+}$/calmodulin in smooth muscle cells. A genetically encoded biosensor MLCK was not saturated with Ca$^{2+}$/calmodulin after maximal increases in [Ca$^{2+}$], in bladder tissues [23, 24, 37] and the reduction of MLCK to half the normal amount did not affect RLC phosphorylation [15]. The normal MLCK content was less in aortic tissue, and RLC phosphorylation and force responses were reduced when MLCK was reduced 50%, suggesting in aortic smooth muscle cells, MLCK, not Ca$^{2+}$/calmodulin, was limiting. Thus, the aortic smooth muscle cell appears particularly sensitive to the partial loss of MLCK.

Other smooth muscle tissues containing half of the normal amount of myosin or a mutated myosin R247C had no or little reduction in force development compared to responses from aorta. Thus, the aortic smooth muscle cell also appears particularly sensitive to the disrupting
effects of mutations in myosin. Force development is not only dependent on smooth muscle myosin and RLC phosphorylation, but also requires the polymerization of submembranous cytoskeletal actin filaments to strengthen membrane adhesion complexes involved in transmitting force generated in actin–myosin filaments to extracellular structures [29, 38]. The integrin-containing membrane adhesome complexes on the cell surface involve many dynamic processes that couple the contractile actin–myosin system to the extracellular matrix for force transmission. Stronger adhesome complexes combined with the high elastin and collagen content as well as tissue-specific stroma structures for aorta vs other smooth muscles [39] may produce less compliant structures for force transmission by the contractile actin–myosin system where compromised myosin function is more readily revealed [5, 7]. Additionally, contractions that lead to shortening of smooth muscle cells reduce blood flow in small arteries as well as emptying the hollow organs (bladder and intestines), but do not play this role in the aorta. Rather, aortic smooth muscle cell contraction potentially functions as a mechanosensor [5, 40]. Mutations in proteins affecting the actin–myosin system of aortic smooth muscle cells independently converge to disrupt the ability of the smooth muscle cell to generate force through the elastin–contractile units in response to pulsatile blood flow. Thus, the vulnerability of the thoracic aorta to aneurysms and dissections may result from the compromised ability of the smooth muscle cells to sense their mechanical environment, and maintain and regulate the mechanical state of the extracellular matrix [5, 7].

Mutations that reduce aortic force production in mice do not produce aortic aneurysms or dissections similar to human disease for up to 10 months of age [3, 14, 15]. Myh11\textsuperscript{R247C/R247C} mice exhibited normal growth, reproduction, and aortic histology but decreased aortic contraction. In response to vascular injury, Myh11\textsuperscript{R247C/R247C} mice showed significantly increased neointimal formation due to increased SMC proliferation when compared with the wild-type mice. Although additional studies have not been performed on Myh11\textsuperscript{+/−} or Myh11\textsuperscript{R247C/+} mice we note that they live for at least six months and appear normal. In the absence of other disease modifiers, these mutations may be tolerated because the smooth muscle cells can compensate physiologically \textit{in vivo} for the force output necessary to maintain aorta structure and function. In terms of induction of aortic disease in mice, additional studies are needed to identify additional genetic or environmental modifiers.

Conclusion

In summary, studies with genetically modified mice show that modifications of MLCK and myosin heavy chain independently converge to disrupt the ability of the aortic smooth muscle cell to generate force. Heterozygous mutations in Myh11 associated with reduced myosin function without changing the extent of RLC phosphorylation result in compromised contractile function in aortic smooth muscle, similar to reported results for decreased MLCK function which acts to reduce myosin function by reducing the extent of RLC phosphorylation. The aorta is particularly sensitive to compromised contractile function compared to other kinds of smooth muscles which may disrupt mechanosensing mechanisms and predispose to thoracic aorta disease.

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