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Double Deletion of Calponin 1 and Calponin 2 in Mice Decreases Systemic Blood Pressure with Blunted Length-Tension Response of Aortic Smooth Muscle

Han-Zhong Feng[1], **Hui Wang**[1], **Katsuhito Takahahashi**[2], and **J.-P. Jin**[1,*]

[1]Department of Physiology, Wayne State University School of Medicine, Detroit, Michigan 48201, USA;

[2]Department of Molecular Medicine and Pathophysiology, Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka City, Osaka 537-8511, Japan

Abstract

Calponin is a family of actin filament-associated regulatory proteins. Among its three isoforms, calponin 1 is smooth muscle specific and calponin 2 is expressed in smooth muscle and certain non-muscle cells. Previous studies showed that calponin 1 knockout mice had detectable changes in the contractility of urogenital smooth muscle whereas other smooth muscles were less affected. To investigate the possibility that calponins 1 and 2 have overlapping functions in smooth muscle, we examined the effect of double knockout of calponin 1 and calponin 2 genes (*Cnn1* and *Cnn2*) on smooth muscle functions. The results showed for the first time that calponin 1 and calponin 2 double knockout in mice does not cause lethality. The double knockout mice showed decreased systemic blood pressure, decreased force development and blunted length tension response in endothelial-removed aortic rings. A compensatory increase of calponin 1 was found in smooth muscle of $Cnn2^{-/-}$ mice but not vice versa. $Cnn1^{-/-}$ and $Cnn2^{-/-}$ double knockout aortic smooth muscle exhibits faster relaxation than that of wild type control. Double deletion or co-suppression of calponin 1 and calponin 2 in vascular smooth muscle to blunt myogenic response may present a novel approach to develop new treatment for hypertension.

Keywords

Calponin; *Cnn1* and *Cnn2* double knockout mice; smooth muscle contractility; blood pressure; myogenic response

*Corresponding author, jjin@med.wayne.edu.

Disclosures

None.

1. Introduction

Calponin is an actin filament-associated regulatory protein first found in smooth muscle [1]. Three isoforms, calponin 1, calponin 2, and calponin 3, [2–4] have evolved in vertebrates encoded by three homologous genes *Cnn1*, *Cnn2* and *Cnn3* [5]. Calponin 1 is specifically expressed in differentiated smooth muscle cells and has been extensively studied for its role in the regulation of smooth muscle contractility [6–8]. Supporting the role of calponin 1 in modulating smooth muscle contratility, *Cnn1* knockout mice showed faster contractile velocity of vas deferens smooth muscle as compared with that of wild type control [9]. High KCl-induced isometric force was lower in *Cnn1* knockout vas deferens and aortic smooth muscles [10]. *Cnn1* knockout mice exhibited the same mean arterial pressure but blunted response to phenylephrine [11], consistent with the finding in isolated aortic smooth muscle of Wistar Kyoto rats, which natually lacks calponin [12].

Calponin 2 is expressed abundantly in smooth muscles [3] and several non-muscle cell types [5]. Earlier studies suggested that calponin 2 plays a role in the organization of actin cytoskeleton [13] and inhibits cytokinesis [14]. Transfective expression of calponin 2 in cultured cells that lack endogenous calponin increased the stability of actin stress fibers. Like *Cnn1* knockout mice, *Cnn2* knockout mice survive to adulthood [15]. Calponin 2-null macrophages exhibit increased motility and phagocytosis [15] and exhibit attenuated development of inflammatory arthritis [16] and atherosclerosis [17]. Diminished calponin 2 in prostate cancer cells, fibroblasts and myeloid blood cells slows down the velocity of cell adhesion [16–18]. Calponin 2-null fibroblasts produced higher cell traction force [19]. These findings are consistent with the primary function of calponin as an inhibitor of actomyosin ATPase and motor activity [5–8, 19].

Despite the extensive data demonstrating calponin 2's role in regulating cytoskeleton and cell motility, little is known for its function in smooth muscle contraction. Passive property is an important factor in determining the contractility of smooth muscle, especially in the myogenic response of resistant blood vessels [20]. Mechanical tension transduced to the vascular smooth muscle cytoskeleton is a basis of myogenic responsiveness [21]. For the abundance of calponin 2 in smooth muscle cells and its role in regulating cytoskeleton functions, it may play a role in the sensing of passive tension in myogenic response.

Calponin 1 and calponin 2 have largely conserved primary structures [5, 22]. Since their single knockout mice exhibit minimized vascular phenotypes, calponin 1 and calponin 2 may be functionally complementary in smooth muscle cells. To investigate this hypothesis, the present study developed calponin 1 and calponin 2 double knockout mice and examined their smooth muscle phenotypes. The results showed that calponin 1 and calponin 2 double knockout in mice does not cause lethality but decreased systemic blood pressure with decreased active tension and blunted length-tension response in aortic smooth muscle. A complementary increase of calponin 1 was found in smooth muscle of $Cnn2^{-/-}$ mice. $Cnn1^{-/-}$,$Cnn2^{-/-}$ double knockout aortic smooth muscle also exhibited faster relaxation than that of wild type control. The findings suggest that double deletion or co-suppression of calponin 1 and calponin 2 may present a novel approach to develop new treatment for hypertension.

2. Materials and Methods

2.1. Cnn1$^{-/-}$ single, Cnn2$^{-/-}$ single and Cnn1$^{-/-}$,Cnn2$^{-/-}$ double knockout mice

The generation of systemic *Cnn1* gene knockout mouse line was described previously [15]. The *Cnn2* gene knockout mice were generated from a *Cnn2* gene floxed mouse line as described previously [15]. Oocyte-specific Cre-induced deletion of the exon 2 was used to produce systemic knockout of *Cnn2* gene in subsequent generations. The *Cnn1* knockout and *Cnn2* knockout mouse lines were cross-bred to produce double knockout mice. After PCR genotyping, *Cnn1*$^{-/-}$,*Cnn2*$^{-/-}$ double knockout, *Cnn1*$^{-/-}$ single knockout, *Cnn2*$^{-/-}$ single knockout and wild type offspring were selected for use in the present study. Three to 5-month-old mice of both sexes were used in the experiments.

All animal procedures were approved by the Institutional Animal Care and Use Committee of Wayne State University and were conducted in accordance with the Guiding Principles in the Care and Use of Animals, as approved by the Council of the American Physiological Society.

2.2. Blood pressure measurement using tail cuff

Mouse blood pressure was examined using a volume-pressure recording tail-cuff blood pressure measurement system (CODA-HT2 high throughput, Kent Scientific Corporation, Torrington, CT). Mouse was placed in a small acrylic restrainer with a black nosecone to mimic darkness environment as a reduction of stress. The tail was placed on a surgical platform heated at 32–35°C. The tail-cuff was gently placed around the tail at a position close to the base of the tail. Acclimation with the restrainer and tail-cuff inflation was given for 30 min a day for 2 days before the experimental measurement.

The blood pressure measurements on conscious wild type and calponin knockout mice were performed in a random and blinded manner. Each measurement was started after 15 min of acclimation and blood pressure was measured for a total of 45 times in 3 sessions. Data were collected and saved using Coda software (Kent Scientific Corporation, Torrington, CT) for later off-line analysis. The first 5 measurements were not used for analysis to avoid effect of the acclimation of the animal to tail-cuff inflations. Portions of unreliable recordings were automatically eliminated by the software. Manual discards were made only when the animal showed excessive movement that generated artificial signals.

2.3. Measurement of the contractility of mouse aortic ring

30 min after i.p. injection of 100 U of heparin, calponin knockout and wild type mice were anesthetized with pentobarbital (100 mg/kg, i.p.). The thoracic cavity was opened, the descending aorta was isolated under a dissection microscope and immediately placed in Krebs-bicarbonate buffer (NaCl, 118 mM; KCl, 4.7 mM; KH_2PO4, 1.2 mM; $CaCl_2$, 2.25 mM; $MgCl_2$, 2.25 mM; $NaHCO_3$, 25 mM; EGTA 0.32 mM; glucose 11 mM) equilibrated with 95% O_2-5% CO_2 (pH 7.4 at 37°C). After removing endothelium by gentle scribing using a spring wire with diameter similar to that of the aorta lumen, a vessel ring of ~3 mm wide was cut out from the middle thoracic region of descending aorta. A pair of stainless steel wire triangle hooks was inserted in the aorta lumen for mounting to a vertically organ

bath containing 50 mL Krebs-bicarbonate buffer continuously bubbled with 95% O_2-5% CO_2 at 37°C. One of the hooks was connected to the bottom platform and the other to an Aurora 300B force transducer (Aurora Scientific, Ontario, Canada).

The aortic ring preparation was equilibrated in the organ bath for 60 min. The original length (Do) of the vessel ring was set with a small stretching to produce a detectable resting tension just above zero and measured with ruler under a dissection microscope using digital photography. A series of vessel lengths at 120%, 130%, 140%, 150% and 160% Do was applied with 3-second ramp stretching to record isometric passive tension and for length-active tension relationship studies. At each resting length, a 10 min duration was allowed for the passive viscoelastic response of the muscle to settle before the ring was induced to contract for 10 min with Krebs buffer containing 50 mM KCl as a replacement of equimolar NaCl for the measurement of active tension. The relaxation of aortic ring was measured for 10 min after refresh the organ bath with normal Krebs buffer. At 160% Do where the active tension was near the maximum, a dose response curve to norepinephrine (NE) was established with the addition of NE to accumulated concentrations from 10^{-10} to 10^{-4} M.

2.4. Polyacrylamide gel electrophoresis and Western blotting

Aorta, urinary bladder and large intestine together with representative control tissues were isolated from wild-type and calponin knockout mice immediately after euthanasia. The smooth muscle layers were obtained by removing visible connective tissues and endothelial or epithelial layer and homogenized in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing 50 mM Tris-HCl, pH 8.8, 2% SDS, 3% β-mercaptoethanol, 0.1% bromphenol blue and 10% glycerol using a high speed mechanical tissue homogenizer (PRO Scientific, Monroe, CT) to extract total proteins. After heating at 80°C for 5 min and clarification by centrifugation at top speed in a microcentrifuge, the samples were loaded on 14% gel with an acrylamide-to-bisacrylamide ratio of 180:1 or 12% gel with an acrylamide-to-bisacrylamide ratio of 29:1 prepared in a modified Laemmli discontinuous buffer system, in which the stacking and resolving gels are both at pH 8.8. After electrophoresis, the gels were fixed and stained with Coomassie Blue R250 to reveal the protein bands. The amounts of sample loading were normalized to the actin band quantified using 2-D densitometry.

The resolved protein bands in duplicate gels were electrophoretically transferred to a nitrocellulose membrane using a Bio-Rad semidry transfer apparatus at 5 mA/cm^2 for 15 min. The blotted membranes were blocked with 1% bovine serum albumin (BSA) in Tris-buffered saline (TBS,150 mM NaCl, 50mM Tris-HCl, pH 7.5) and incubated with anti-calponin primary antibodies. A rabbit polyclonal antibody RAH2 raised against purified mouse calponin 2, which strongly reacts to calponin 2 with weaker cross reaction to calponin 1 [12], a mouse monoclonal antibody (mAb) CP1 specific to calponin 1 [23], a mouse mAb 1D11 specific to calponin 2 [17], and a mouse anti-α-smooth muscle actin mAb (A5228, Sigma–Aldrich) were used in the present study. Following washes with TBS containing 0.05% Tween-20, the membranes were further incubated with alkaline phosphatase-labeled anti-rabbit IgG or anti-mouse IgG second antibody (Santa Cruz Biotechnology). After final washes as above, the expression of calponin isoforms and α-

smooth muscle actin was revealed by developing the blots in 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium chromogenic substrate solution.

2.5. Data analysis

Densitometry analysis of SDS-gel and Western blots was performed on images scanned at 600 dpi. All quantitative data are presented as mean ± SEM. Student's t test was performed for comparisons between means and ANOVA was used for comparison between curves. $P<0.05$ was used as the level of significance.

3. Results

3.1. *Cnn1*$^{-/-}$,*Cnn2*$^{-/-}$ double knockout does not cause lethality in mice

The Western blots in Fig. 1 verified the deletion of calponin 1 and/or calponin 2 in *Cnn1*$^{-/-}$ single, *Cnn2*$^{-/-}$ single and *Cnn1*$^{-/-}$,*Cnn2*$^{-/-}$ double knockout mouse tissues. *Cnn1*$^{-/-}$,*Cnn2*$^{-/-}$ double knockout mice survive, develop to adulthood and fertile. The data in Table 1 showed that the body weight of 3 – 4.5 months old WT, *Cnn1*$^{-/-}$, *Cnn2*$^{-/-}$ and *Cnn1*$^{-/-}$,*Cnn2*$^{-/-}$ double knockout mice had no significant difference.

3.2. *Cnn1*$^{-/-}$,*Cnn2*$^{-/-}$ double knockout lowered blood pressure in mice

Blood pressure measurement using tail-cuff in conscious mice detected significantly lower systolic, diastolic and mean arterial blood pressures in young adult *Cnn1*$^{-/-}$,*Cnn2*$^{-/-}$ double knockout mice in comparison with wild type control (Fig. 2). *Cnn1*$^{-/-}$ and *Cnn2*$^{-/-}$ single knockout mice exhibited trends of decreases in blood pressure, more visible for the diastolic pressure, but no statistical significance was established (Fig. 2).

3.3. *Cnn1*$^{-/-}$,*Cnn2*$^{-/-}$ double knockout decreased contractile force in mouse aortic rings

To demonstrate the effect of deleting both calponin 1 and calponin 2 on arterial smooth muscle contractility, the *ex vivo* contraction of endothelial-free mouse aortic rings showed lower maximum tension development normalized vessel size in *Cnn1*$^{-/-}$,*Cnn2*$^{-/-}$ double knockout mouse aortae upon norepinephrine activation than that of wild type control (Fig. 3A). The dose response curves normalized to maximum tension were not different between *Cnn1*$^{-/-}$,*Cnn2*$^{-/-}$ double knockout and wild type groups, indicating no change in the sensitivity to norepinephrine activation (Fig. 3B).

3.4. *Cnn1*$^{-/-}$,*Cnn2*$^{-/-}$ double knockout mouse aortic smooth muscle exhibits faster relaxation

The times for developing peak or 50% of peak force after the induction of contraction by adding Krebs solution containing 50 mM KCl were not different in wild type and *Cnn1*$^{-/-}$,*Cnn2*$^{-/-}$ double knockout mouse aortic rings (data not shown). The force decay during the relaxation of endothelium-free *Cnn1*$^{-/-}$,*Cnn2*$^{-/-}$ double knockout mouse aortic rings after replacing the KCl bathing media with normal Krebs solution was significantly faster than that of wild type control (Fig. 4A). Quantitative analysis further showed that *Cnn1*$^{-/-}$,*Cnn2*$^{-/-}$ double knockout aortic rings had similar early relaxation to that of wild type control as reflected by the time for reaching 25% and 50% relaxation (TR25 and

TR50). However, *Cnn1*$^{-/-}$,*Cnn2*$^{-/-}$ double knockout aortic rings had shorter time reaching 75% and 90% relaxation (TR75 and TR90) than that of wild type control (Fig. 4B), indicating increased later phase relaxation.

3.5. *Cnn1*$^{-/-}$ *single but not Cnn2*$^{-/-}$ single knockout contributed to the lower contractile force and faster relaxation of mouse aortic smooth muscle

Contractility studies further showed that the lower maximum force produced by *Cnn1*$^{-/-}$,*Cnn2*$^{-/-}$ double knockout mouse aortic rings (Fig. 3A) was also seen in *Cnn1*$^{-/-}$ single but not *Cnn2*$^{-/-}$ single knockout mouse aortae (Fig. 5A), indicating that the deletion of calponin 1, but not calponin 2, predominantly contributed to the reduction of force in aortic smooth muscle.

Analysis of relaxation time parameters also showed that *Cnn1*$^{-/-}$ single but not *Cnn2*$^{-/-}$ single knockout mouse aortic rings exhibited shorter relaxation time similar to that of *Cnn1*$^{-/-}$,*Cnn2*$^{-/-}$ double knockout group (Fig. 5B). *Cnn2*$^{-/-}$ single knockout mouse aortae showed a slower early phase relaxation as reflected by the longer time for reaching 25% relaxation (TR25, Fig. 5B). Significance of this effect of *Cnn2*$^{-/-}$ single knockout remains to be investigated.

3.6. *Effects of Cnn1*$^{-/-}$ *and/or Cnn2*$^{-/-}$ knockout on myofilament contents in mouse aortic smooth muscle

Total protein extracts from wild type, *Cnn1*$^{-/-}$ single, *Cnn2*$^{-/-}$ single, and *Cnn1*$^{-/-}$,*Cnn2*$^{-/-}$ double knockout mouse aortae were analyzed using SDS-PAGE and Western blotting for the level of myosin heavy chain (MHC) and smooth muscle actin (SMA) (Fig. 6A). Densitometry quantification showed that the level of MHC vs. total protein was not different between knockout and wild type groups (Fig. 6B). However, the level of SMA vs. total actin was decreased in *Cnn1*$^{-/-}$ single knockout mouse aortae with no such ratio change detected in *Cnn2*$^{-/-}$ single and *Cnn1*$^{-/-}$,*Cnn2*$^{-/-}$ double knockout groups (Fig. 6C).

Since the level of SMA is measured relative to that of total actin, the explanation may be that a) *Cnn1* KO specifically affects the contractile machinery that contains SMA whereas *Cnn2* KO mainly affects cytoskeleton actins; and b) cytoskeleton actin and SMA may both decrease in double KO smooth muscle to mask the relative change in SMA. This notion also suggests a decrease in myofilament contents in *Cnn1*$^{-/-}$ single knockout mouse aortic smooth muscle corresponding to the decreased contractile force (Fig. 5A).

3.7. *Cnn1*$^{-/-}$,*Cnn2*$^{-/-}$ double knockout mouse aortic rings had passive tension similar to wild type controls but a blunted length-active tension response

Normalized to vessel size, the passive tension in resting endothelial-free *Cnn1*$^{-/-}$,*Cnn2*$^{-/-}$ double knockout mouse aortic rings was similar to wild type control (Fig. 7A). However, length-active tension relationship studies on 50 mM KCl-activated contractions of endothelial-free mouse aortic rings showed that in contrast to wild type control, *Cnn1*$^{-/-}$,*Cnn2*$^{-/-}$ double knockout group had a blunted response to the stretching of resting length to 130–160% Do while the anticipated positive responses were maintained in *Cnn1*$^{-/-}$ and *Cnn2*$^{-/-}$ single knockout groups (Fig. 7B). In the meantime, *Cnn1*$^{-/-}$ single knockout

mouse aortic rings produced significantly lower active tension across the muscle lengths tested whereas *Cnn2*$^{-/-}$ knockout group showed a trend of decreases (Fig. 7B).

3.8. Compensatory increase of calponin 1 in *Cnn2*$^{-/-}$ mouse aortic smooth muscle

Densitometry analysis of SDS-PAGE gels and Western blots showed that normalized to the level of actin or myosin, the amount of calponin 1 was significantly increased in *Cnn2*$^{-/-}$ mouse aortae from that of the wild type level (Fig. 8A), indicating a compensation for the loss of calponin 2 via increased protein expression or accumulation. No compensatory increase of calponin 2 was detected in *Cnn1*$^{-/-}$ mouse aortic smooth muscle (Fig. 8B).

Considering that mouse aortic smooth muscle normally expresses a relatively low level of calponin 1 in comparison with other smooth muscles (Fig. 1), this unidirectional compensation between calponin 1 and calponin 2 in aortic smooth muscle was verified in other types of smooth muscle. Urinary bladder and large intestinal were examined as representatives that express calponin 1 at higher levels vs. that of calponin 2 than in aortic smooth muscle in wild type mice (Fig. 9A). SDS-PAGE gel and Western blot densitometry quantification showed significant increases of calponin 1 in the bladder (Fig. 9B) and large intestine (Fig. 9C) of *Cnn2*$^{-/-}$ mouse as compared to wild type controls, whereas the trends of calponin 2 increase in *Cnn1*$^{-/-}$ bladder and large intestine did not establish statistical significance. The compensatory increase of *Cnn1* in *Cnn2* knockout my play a role in the blood pressure phenotype in *Cnn2* knockout mice. In contrast to *Cnn1* or *Cnn2* single knockout, the lower blood pressure of *Cnn1*,*Cnn2* double knockout mice suggests that *Cnn1* and *Cnn2* have a combined role in blood pressure regulation.

4. Discussion

Calponin is an abundant protein in smooth muscle and many non-muscle cell types. Three decades after its discovery, its physiological function remains not fully understood. Extensive biochemical studies have shown that calponin 1 is an actin filament associated regulatory protein that inhibits myosin ATPase [6]. The role of calponin 1 in modulating smooth muscle contractility has been demonstrated in previous studies [5]. Smooth muscles also contain abundant calponin 2 [14] of which the functional significance has not been established. Our present study generated *Cnn1*$^{-/-}$,*Cnn2*$^{-/-}$ double knockout mice and showed for the first time that mice systemically lacking both calponin 1 and calponin 2 survive and have a normal life span under standard cage conditions. Using this novel animal model, we demonstrated that the deletion of both calponin 1 and calponin 2 in mice significantly decreased blood pressure with reduced contractile force and blunted myogenic response of vascular smooth muscle. Several new findings are discussed below for their mechanistic insights.

4.1. Loss of calponin 1 but not calponin 2 decreases the contractile force and increases relaxation velocity of vascular smooth muscle

Our data showed that in the absence of compensation in *Cnn1*$^{-/-}$,*Cnn2*$^{-/-}$ double knockout mice, blood pressure had more changes than that in single knockout mice, suggesting both calponin 1 and 2 contribute to blood pressure regulation. In the meantime, *Cnn1*$^{-/-}$ single

knockout showed more effect than that of calponin 2 single knockout, consistent with the fact that calponin1 is the major calponin in normal smooth muscles. The results in Fig. 5 suggest that deletion of calponin 1 altered aortic smooth muscle contraction and relaxation. Similar to our data in Table 1, a previous study did not see difference in aortic smooth muscle thickness, cell shape and size between *Cnn1*$^{-/-}$ and wild type mice, but found the reduced active tension development of in aorta and vas deference in *Cnn1*$^{-/-}$ mice with no alteration of the length-resting tension relationship [10]. Previous studies of *Cnn1*$^{-/-}$ mice reported impaired blood pressure responses during exercise due to increased vasodilation [24, 25]. Increased vascular smooth muscle relaxation [26] and vasodilation [27, 28] have been found to reduce blood pressure, whereas impaired relaxation of arterial smooth muscle causes hypertension [29]. Despite the importance of smooth muscle relaxation in blood pressure regulation, both systolic and diastolic blood pressure, and thus the mean blood pressure, are determined by vessel tone produced by tonic contraction of vascular smooth muscle. While the faster relaxation in calponin KO muscle reflects altered contractility and vascular tone in the regulation of blood pressure, it does not directly relate to diastolic blood pressure.

Another previous study showed no difference in force production and Ca^{2+} sensitivity but a faster shortening velocity of *Cnn1*$^{-/-}$ vas deference smooth muscle [30]. The same study found a decrease in actin but not myosin in vas deferens and bladder smooth muscles [30] also similar to our finding in Fig. 6. Therefore, deletion of calponin 1 may specifically reduce the contractile myofilament content as indicated by the decreased level of SMA.

The significantly shortened time of later phase relaxation in *Cnn1*$^{-/-}$ knockout aortic smooth muscle suggests a role of calponin 1 in the regulation smooth muscle relaxation, a potential mechanism remains to be investigated. Mouse aortic smooth muscle expresses a relatively low level of calponin 1 as compared with that of bladder (Fig. 1), which may render the moderate effect of *Cnn1*$^{-/-}$ single knockout on the contractility of aortic rings as compared to that of urogenital smooth muscles. Consistently, altered smooth muscle contractility of *Cnn1*$^{-/-}$ single knockout mice was more clearly detected in urogenital smooth muscles that naturally express high levels of calponin 1 [30].

4.2. Blunted length-active tension response in Cnn1$^{-/-}$,Cnn2$^{-/-}$ double knockout mouse aortic smooth muscle

Myogenic response is a fundamental mechanism in the function of arterial smooth muscle, in which a positive length-tension relationship is a determining factor [31]. Our data in Fig. 7B showed that while the resting tension was unchanged, the length-active tension response of *Cnn1*$^{-/-}$,*Cnn2*$^{-/-}$ double knockout mouse aortic smooth muscle was blunted. This observation suggests that calponin plays a novel role in myogenic response of vascular smooth muscle.

The blunted length-active tension response was detected in *Cnn1*$^{-/-}$,*Cnn2*$^{-/-}$ double knockout mouse aortae but not either of the single knockout models (Fig. 7B). This finding suggests that in addition to calponin 1's function in directly tuning contractility, calponin 2's function in regulating cytoskeleton myosin motors [19, 32] may also contribute to the myogenic response of smooth muscle. Deletion of calponin 1 lowers the length-dependent

force generation and deletion of calponin 2 would increase cytoskeleton myosin motor activity which may increase the stiffness of the cytoskeleton and decrease the sensitivity of contractile machinery to stretching signals. This hypothesis requires further investigation to determine the underlying mechanisms for *Cnn1*$^{-/-}$,*Cnn2*$^{-/-}$ double knockout to blunt myogenic response of resistant blood vessel and lower blood pressure.

Although the similar contractility of *Cnn1*$^{-/-}$ single and *Cnn1*$^{-/-}$,*Cnn2*$^{-/-}$ double knockout mouse aortae suggests that calponin 2 has no dominant effect on isometric contractile force and relaxation time of aortic smooth muscle, the decreased blood pressure in *Cnn1*$^{-/-}$,*Cnn2*$^{-/-}$ double knockout but not in the *Cnn1*$^{-/-}$ single knockout mice shows a physiological function of calponin 2 in regulating vascular smooth muscle contractility *in vivo*. Aortic smooth muscle expresses much higher level of calponin 2 relative to calponin 1 than that in bladder (Fig. 1) and large intestine (Fig. 9), supporting the proposed role of calponin 2 in aortic smooth muscle contractility by regulating cytoskeleton stiffness and myogenic response providing a novel target for the treatment of hypertension.

4.3. Unidirectional calponin 1 compensation for the loss of calponin 2 in smooth muscles

It is interesting that the level of calponin 1 increases as a potential complementation for the loss of calponin 2 in *Cnn2*$^{-/-}$ mouse smooth muscles but not vice versa. The expression of calponin 2 in mouse aortic smooth muscle is rather low in comparison to that of calponin 1 and a compensatory increase of calponin 2 in *Cnn1*$^{-/-}$ aortic mouse aorta would only add a small portion of the total calponin 2 and might not be readily detectable. Supporting this observation, the unidirectional calponin 1 compensation for calponin 2 deficiency is also seen in bladder and large intestinal smooth muscles (Fig. 9). The calponin 1 compensation for the loss of calponin 2 in smooth muscle of *Cnn2*$^{-/-}$ single knockout mice may have functional contributions to modify contractile phenotypes via secondary adaptions and is, therefore, worth further investigating.

4.4. Functional diversity and exchangeability between calponin isoforms

The three calponin isoforms are highly conserved in the N-terminal and middle regions that contain all known functional sites [5]. While the isoform-specific C-terminal variable region of calponin 1 and calponin 2 may determine their subcellular distribution and functional diversities, the two isoforms may be functionally exchangeable, at least partially. The compensatory increase of calponin 1 in calponin 2 deficient mouse smooth muscle supports functional exchangeability of these two calponin isoforms. On the other hand, the different ratios of calponin 1 and calponin 2 in aorta, bladder and large intestine are apparent, indicating different contents of the two isoforms in different smooth muscle types corresponding to diverged structure and function.

The other isoform of calponin, calponin 3, is expressed in non-muscle cells [33, 34], skeletal myoblasts [35] and smooth muscle at low levels [4]. The physiological function of calponin 3 is much less understood than that of calponins 1 and 2. The *Cnn1*$^{-/-}$,*Cnn2*$^{-/-}$ double knockout mice provide an informative experimental system to examine the functional contribution and exchangeability of calponin 3 in smooth muscle, in line of the exploration of double deletion or suppression of calponins 1 and 2 for the treatment of hypertension.

Our results also implicate that *in vitro* contractility of isolated blood vessels may not fully explain the vessel tone and blood pressure regulation *in vivo*. The indirect effects from nonvascular elements need to be considered when study the global knockout mouse models, especially since calponin 2 is widely expressed in multiple tissue and cell types. Further studies using integrative approaches to understand the role of calponin in regulating blood pressure may develop an effective molecular target for the treatment of hypertension.

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Abbreviations:

BSA	bovine serum albumin
mAb	monoclonal antibody
MHC	myosin heavy chain
PAGE	polyacrylamide gel electrophoresis
SMA	smooth muscle actin
TBS	Tris-buffered saline

References

[1]. Takahashi K, Hiwada K, Kokubu T, Isolation and characterization of a 34,000-dalton calmodulin- and F-actin-binding protein from chicken gizzard smooth muscle, Biochem Biophys Res Commun 141(1) (1986) 20–6. [PubMed: 3606745]

[2]. Takahashi K, Nadal-Ginard B, Molecular cloning and sequence analysis of smooth muscle calponin, J Biol Chem 266(20) (1991) 13284–8. [PubMed: 2071603]

[3]. Strasser P, Gimona M, Moessler H, Herzog M, Small JV, Mammalian calponin. Identification and expression of genetic variants, FEBS Lett 330(1) (1993) 13–8. [PubMed: 8370452]

[4]. Applegate D, Feng W, Green RS, Taubman MB, Cloning and expression of a novel acidic calponin isoform from rat aortic vascular smooth muscle, J Biol Chem 269(14) (1994) 10683–90. [PubMed: 8144658]

[5]. Liu R, Jin JP, Calponin isoforms CNN1, CNN2 and CNN3: Regulators for actin cytoskeleton functions in smooth muscle and non-muscle cells, Gene 585(1) (2016) 143–153. [PubMed: 26970176]

[6]. Winder SJ, Allen BG, Clement-Chomienne O, Walsh MP, Regulation of smooth muscle actin-myosin interaction and force by calponin, Acta physiologica Scandinavica 164(4) (1998) 415–26. [PubMed: 9887965]

[7]. Small JV, Gimona M, The cytoskeleton of the vertebrate smooth muscle cell, Acta physiologica Scandinavica 164(4) (1998) 341–8. [PubMed: 9887957]

[8]. Morgan KG, Gangopadhyay SS, Invited review: cross-bridge regulation by thin filament-associated proteins, Journal of applied physiology 91(2) (2001) 953–62. [PubMed: 11457814]

[9]. Takahashi K, Yoshimoto R, Fuchibe K, Fujishige A, Mitsui-Saito M, Hori M, Ozaki H, Yamamura H, Awata N, Taniguchi S, Katsuki M, Tsuchiya T, Karaki H, Regulation of shortening velocity by calponin in intact contracting smooth muscles, Biochem Biophys Res Commun 279(1) (2000) 150–7. [PubMed: 11112431]

[10]. Fujishige A, Takahashi K, Tsuchiya T, Altered mechanical properties in smooth muscle of mice with a mutated calponin locus, Zoolog Sci 19(2) (2002) 167–74. [PubMed: 12012779]

[11]. Masuki S, Nose H, Arterial baroreflex control of muscle blood flow at the onset of voluntary locomotion in mice, The Journal of physiology 553(Pt 1) (2003) 191–201. [PubMed: 12937292]

[12]. Nigam R, Triggle CR, Jin JP, h1- and h2-calponins are not essential for norepinephrine- or sodium fluoride-induced contraction of rat aortic smooth muscle, Journal of muscle research and cell motility 19(6) (1998) 695–703. [PubMed: 9742453]

[13]. Danninger C, Gimona M, Live dynamics of GFP-calponin: isoform-specific modulation of the actin cytoskeleton and autoregulation by C-terminal sequences, J Cell Sci113 Pt 21 (2000) 3725–36. [PubMed: 11034901]

[14]. Hossain MM, Hwang DY, Huang QQ, Sasaki Y, Jin JP, Developmentally regulated expression of calponin isoforms and the effect of h2-calponin on cell proliferation, American journal of physiology. Cell physiology 284(1) (2003) C156–67. [PubMed: 12388067]

[15]. Huang QQ, Hossain MM, Wu K, Parai K, Pope RM, Jin JP, Role of H2-calponin in regulating macrophage motility and phagocytosis, J Biol Chem 283(38) (2008) 25887–99. [PubMed: 18617524]

[16]. Huang QQ, Hossain MM, Sun W, Xing L, Pope RM, Jin JP, Deletion of calponin 2 in macrophages attenuates the severity of inflammatory arthritis in mice, American journal of physiology. Cell physiology 311(4) (2016) C673–C685. [PubMed: 27488671]

[17]. Liu R, Jin JP, Deletion of calponin 2 in macrophages alters cytoskeleton-based functions and attenuates the development of atherosclerosis, Journal of molecular and cellular cardiology 99 (2016) 87–99. [PubMed: 27575021]

[18]. Hossain MM, Wang X, Bergan RC, Jin JP, Diminished expression of h2-calponin in prostate cancer cells promotes cell proliferation, migration and the dependence of cell adhesion on substrate stiffness, Febs Open Bio 4 (2014) 627–636.

[19]. Hossain MM, Zhao G, Woo MS, Wang JH, Jin JP, Deletion of Calponin 2 in Mouse Fibroblasts Increases Myosin II-Dependent Cell Traction Force, Biochemistry 55(43) (2016) 6046–6055. [PubMed: 27733037]

[20]. Folkow B, Myogenic mechanisms in the control of systemic resistance. Introduction and historical background, J Hypertens Suppl 7(4) (1989) S1–4.

[21]. Davis MJ, Wu X, Nurkiewicz TR, Kawasaki J, Davis GE, Hill MA, Meininger GA, Integrins and mechanotransduction of the vascular myogenic response, American journal of physiology. Heart and circulatory physiology 280(4) (2001) H1427–33. [PubMed: 11247750]

[22]. Jin JP, Zhang Z, Bautista JA, Isoform diversity, regulation, and functional adaptation of troponin and calponin, Critical reviews in eukaryotic gene expression 18(2) (2008) 93–124. [PubMed: 18304026]

[23]. Jin JP, Walsh MP, Resek ME, McMartin GA, Expression and epitopic conservation of calponin in different smooth muscles and during development, Biochemistry and cell biology = Biochimie et biologie cellulaire 74(2) (1996) 187–96. [PubMed: 9213427]

[24]. Masuki S, Takeoka M, Taniguchi S, Nose H, Enhanced baroreflex sensitivity in free-moving calponin knockout mice, American journal of physiology. Heart and circulatory physiology 284(3) (2003) H939–46. [PubMed: 12433658]

[25]. Masuki S, Takeoka M, Taniguchi S, Yokoyama M, Nose H, Impaired arterial pressure regulation during exercise due to enhanced muscular vasodilatation in calponin knockout mice, The Journal of physiology 553(Pt 1) (2003) 203–12. [PubMed: 12963798]

[26]. Rinaldi GJ, Blood pressure fall and increased relaxation of aortic smooth muscle in diabetic rats, Diabetes Metab 31(5) (2005) 487–95. [PubMed: 16357793]

[27]. Ferrari AU, Radaelli A, Mori T, Mircoli L, Perlini S, Meregalli P, Fedele L, Mancia G, Nitric oxide-dependent vasodilation and the regulation of arterial blood pressure, J Cardiovasc Pharmacol 38 Suppl 2 (2001) S19–22. [PubMed: 11811370]

[28]. Fritz M, Rinaldi G, Influence of nitric oxide-mediated vasodilation on the blood pressure measured with the tail-cuff method in the rat, J Biomed Sci 14(6) (2007) 757–65. [PubMed: 17634759]

[29]. Qiao YN, He WQ, Chen CP, Zhang CH, Zhao W, Wang P, Zhang L, Wu YZ, Yang X, Peng YJ, Gao JM, Kamm KE, Stull JT, Zhu MS, Myosin phosphatase target subunit 1 (MYPT1) regulates

the contraction and relaxation of vascular smooth muscle and maintains blood pressure, J Biol Chem 289(32) (2014) 22512–23. [PubMed: 24951589]

[30]. Matthew JD, Khromov AS, McDuffie MJ, Somlyo AV, Somlyo AP, Taniguchi S, Takahashi K, Contractile properties and proteins of smooth muscles of a calponin knockout mouse, The Journal of physiology 529 Pt 3 (2000) 811–24. [PubMed: 11118508]

[31]. Walsh MP, Cole WC, The role of actin filament dynamics in the myogenic response of cerebral resistance arteries, Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism 33(1) (2013) 1–12.

[32]. Hossain MM, Crish JF, Eckert RL, Lin JJ, Jin JP, h2-Calponin is regulated by mechanical tension and modifies the function of actin cytoskeleton, J Biol Chem 280(51) (2005) 42442–53. [PubMed: 16236705]

[33]. Ferhat L, Charton G, Represa A, Ben-Ari Y, der Terrossian E, Khrestchatisky M, Acidic calponin cloned from neural cells is differentially expressed during rat brain development, The European journal of neuroscience 8(7) (1996) 1501–9. [PubMed: 8758957]

[34]. Ferhat L, Rami G, Medina I, Ben-Ari Y, Represa A, Process formation results from the imbalance between motor-mediated forces, J Cell Sci 114(Pt 21) (2001) 3899–904. [PubMed: 11719556]

[35]. Shibukawa Y, Yamazaki N, Daimon E, Wada Y, Rock-dependent calponin 3 phosphorylation regulates myoblast fusion, Experimental cell research 319(5) (2013) 633–48. [PubMed: 23276748]

Highlights:

- Calponin is an actin filament-associated regulatory protein in smooth muscle
- Double KO of calponins 1 and 2 decreased systemic blood pressure in mice
- Double KO of calponins 1 and 2 blunted length tension response of aortic ring
- Calponins 1 and 2 may present novel targets for the treatment of hypertension

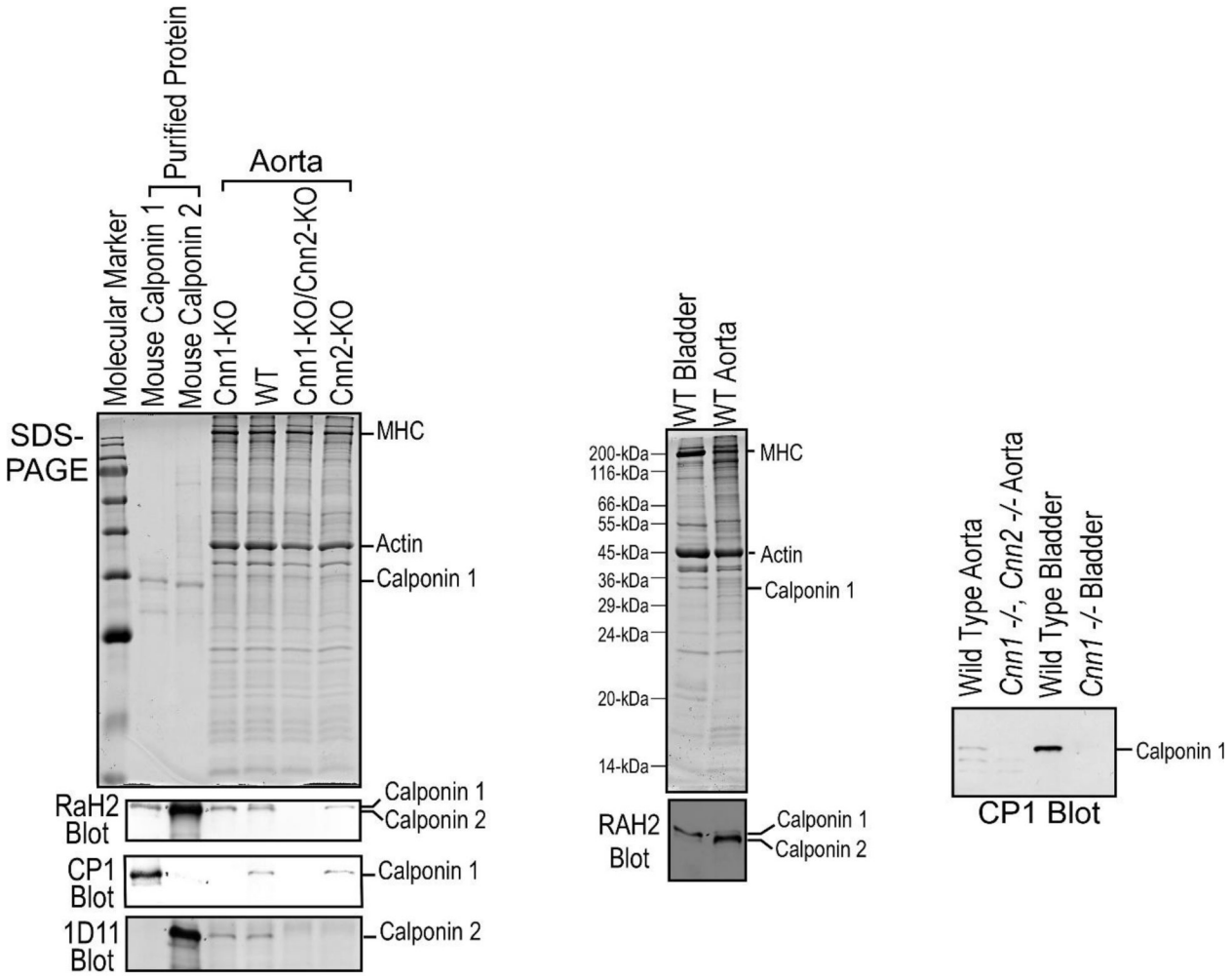

Figure 1. Confirmation of *Cnn1*$^{-/-}$ single, *Cnn2*$^{-/-}$ single and *Cnn1*$^{-/-}$,*Cnn2*$^{-/-}$ double knockout mice.

SDS-PAGE and Western blot using calponin 1-specific mAb CP1, calponin 2-specific mAb 1D11, and anti-calponin 2 polyclonal antibody RAH2 that cross-reacts with calponin 1 showed the expression of both isoforms in wild type (WT) mouse aortic smooth muscle. The deletion of calponin 1 or calponin 2 in the single knockout mouse smooth muscles was confirmed in aorta and bladder samples. Both isoforms became undetectable in *Cnn1*$^{-/-}$,*Cnn2*$^{-/-}$ double knockout mouse aortic smooth muscle. It is worth noting that the affinity of RAH2 antibody to its immunogen calponin 2 [12] is higher than the cross reaction to calponin 1 when the same loading of purified mouse calponin 1 and calponin 2 was tested in Western blot. Therefore, the actual level of calponin 1 relative to that of calponin 2 is higher than that seen in the RAH2 Western blots, as shown in the Western blot using anti-calponin 1 mAb CP1 although the level of calponin 1 in aorta is much lower than that in bladder.

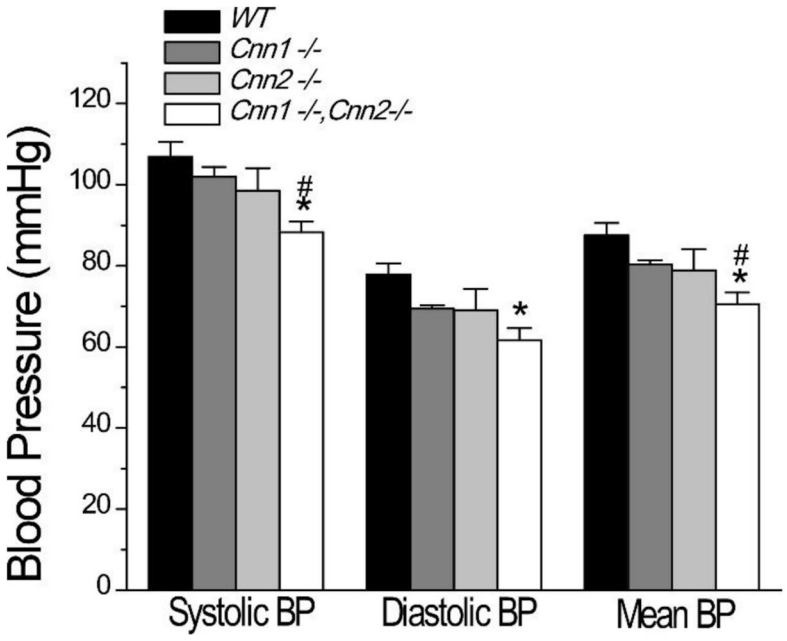

Figure 2. *Cnn1*$^{-/-}$,*Cnn2*$^{-/-}$ double knockout mice had decreased blood pressure.

Measurements using tail cuff in conscious mice detected significantly lower systolic, diastolic and mean arterial blood pressures in *Cnn1*$^{-/-}$,*Cnn2*$^{-/-}$ double knockout mice as compared with wild type (WT) control while such trends were also seen in *Cnn1*$^{-/-}$ and *Cnn2*$^{-/-}$ single knockout mice. N = 9 in WT, n = 3 in *Cnn1*$^{-/-}$ single, n = 5 in *Cnn2*$^{-/-}$ single, and n = 5 in *Cnn1*$^{-/-}$,*Cnn2*$^{-/-}$ double knockout groups. Values are presented as Mean ± SEM. *P<0.05 and **P<0.01 vs. WT; #P<0.05 vs. *Cnn1*$^{-/-}$ single knockout in Student's *t* test.

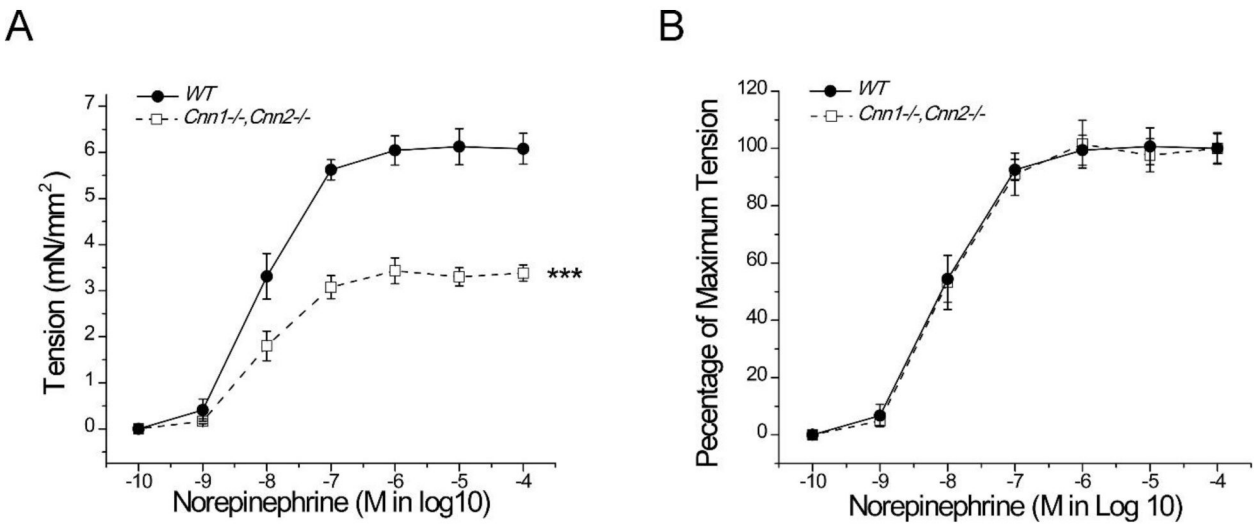

Figure 3. Decreased contractile force of *Cnn1*$^{-/-}$,*Cnn2*$^{-/-}$ double knockout mouse aortic smooth muscle with no change in the sensitivity to norepinephrine.

(A) *Ex vivo* contractility showed that the norepinephrine-activated, vessel size-normalized maximum tension was lower in *Cnn1*$^{-/-}$,*Cnn2*$^{-/-}$ double knockout aortic rings than that of wild type (WT) control. (B) Activation curves normalized to the maximum tension showed nearly identical sensitivities of *Cnn1*$^{-/-}$,*Cnn2*−/− double knockout aortic rings and WT control. N = 4 mice in WT and 5 mice in *Cnn1*$^{-/-}$,*Cnn2*$^{-/-}$ groups. Values are presented as Mean ± SEM. ***P<0.001 in two-way ANOVA.

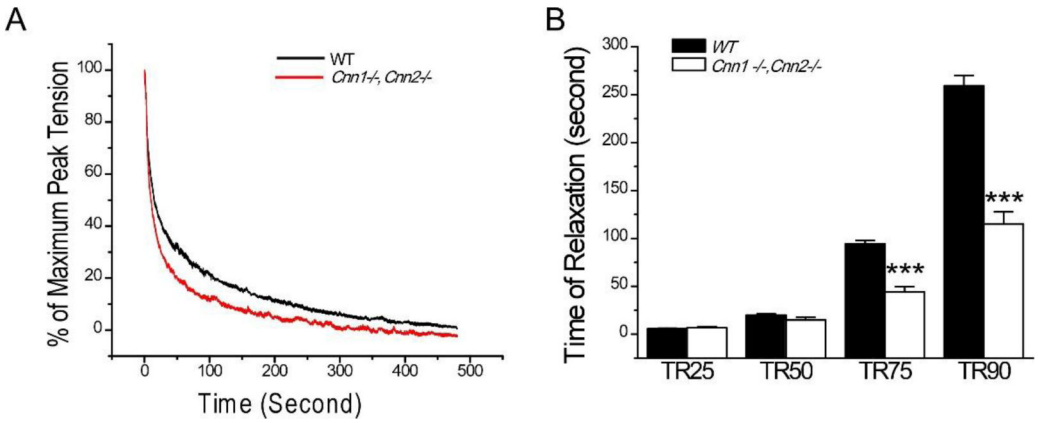

Figure 4. Faster relaxation of *Cnn1*$^{-/-}$,*Cnn2*$^{-/-}$ double knockout mouse aortic rings.

(A) Representative relaxation curves of aortic rings of *Cnn1*$^{-/-}$,*Cnn2*$^{-/-}$ double knockout mice showed a faster relaxation than that of wild type (WT) control after replacing the 50 mM KCl buffer with normal Krebs solution. (B) Quantitative analysis of relaxation time showed significantly shorter TR75 and TR90 in *Cnn1*$^{-/-}$,*Cnn2*$^{-/-}$ double knockout aortic ring than that of WT control, indicating increased later phase relaxation. N = 4 mice in WT and 5 mice in *Cnn1*$^{-/-}$,*Cnn2*$^{-/-}$ double knockout groups. Values are presented as Mean ± SEM. ***P<0.001 in Student's *t* test.

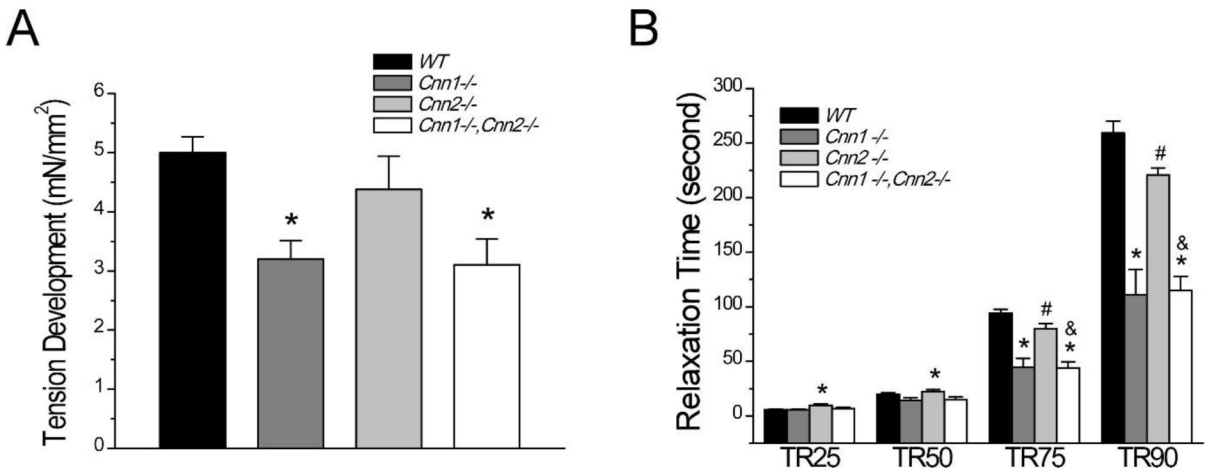

Figure 5. *Cnn1* single knockout produced lower contractile force and faster relaxation in mouse aortic smooth muscle.

(A) *Cnn1*$^{-/-}$ single but not *Cnn2*$^{-/-}$ single knockout mouse aortic rings produced lower maximum force than wild type (WT) control, similar to that of *Cnn1*$^{-/-}$,*Cnn2*$^{-/-}$ double knockout aortae. (B) Analysis of relaxation time parameters showed that *Cnn1*$^{-/-}$ single knockout mouse aortic rings produced shorter TR50, TR75 and TR90 than WT control, similar to the effect of *Cnn1*$^{-/-}$,*Cnn2*$^{-/-}$ double knockout. No change in relaxation time was found for *Cnn2*$^{-/-}$ single knockout aortae except a longer TR25 indicating slower early relaxation. Values are presented as Mean ± SEM. N = 10 mice in WT, 5 mice in *Cnn1*$^{-/-}$,*Cnn2*$^{-/-}$ double knockout, 5 mice in *Cnn1*$^{-/-}$ single knockout and 5 mice in *Cnn2*$^{-/-}$ single knockout groups. * P<0.05 vs. WT; #P<0.05 vs. *Cnn1*$^{-/-}$,*Cnn2*$^{-/-}$ double knockout; $^{\&}$P<0.05 vs. *Cnn1*$^{-/-}$ single knockout in Student's *t* test.

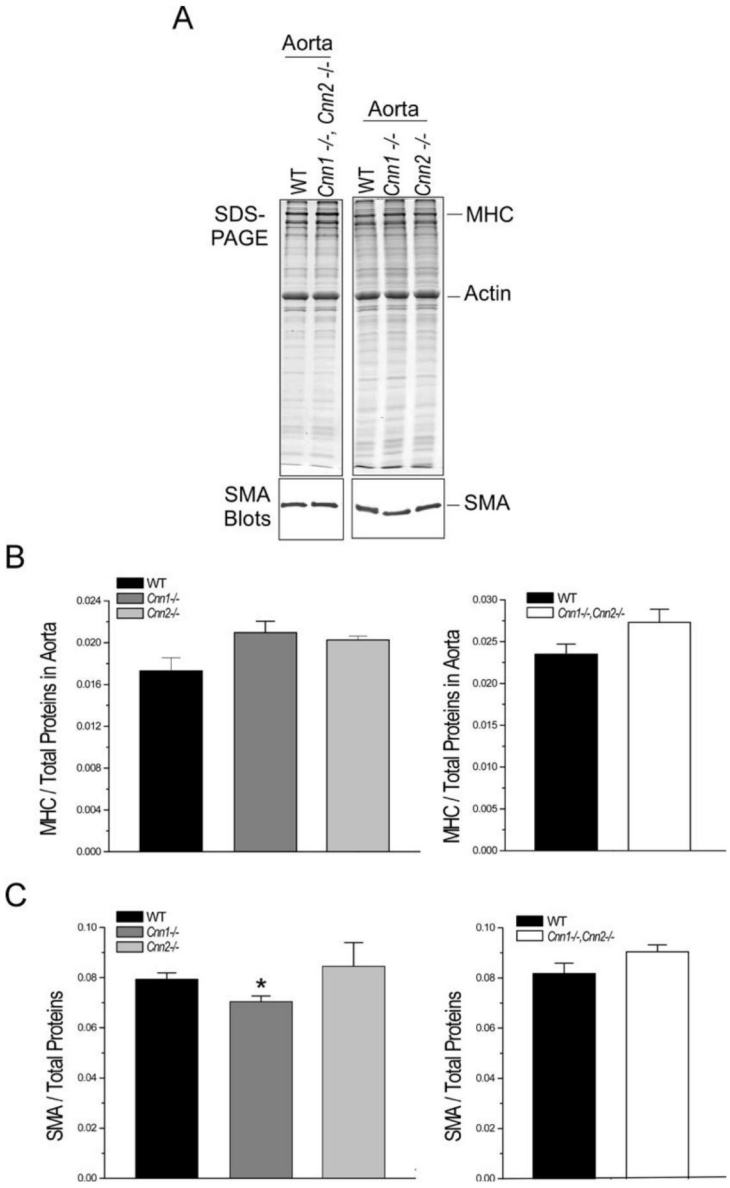

Figure 6. Decreases of myofilament contents in aorta of *Cnn1*$^{-/-}$ single but not *Cnn2*$^{-/-}$ single or *Cnn1*$^{-/-}$,*Cnn2*$^{-/-}$ double knockout mouse aortae.

(A) Representative SDS-PAGE and Western blots using anti-SMA mAb of aortae of wild type (WT), *Cnn1*$^{-/-}$ single, *Cnn2*$^{-/-}$ single and *Cnn1*$^{-/-}$,*Cnn2*$^{-/-}$ double knockout mice. (B) Densitometry quantification of SDS-gel bands showed no change in the level of MHC vs. total muscle protein in the calponin knockout groups as compared to WT control. (C) Normalized to total protein, SMA was decreased in the aortae of *Cnn1*$^{-/-}$ single knockout, but not *Cnn2*$^{-/-}$ single or *Cnn1*−/−,*Cnn2*−/− double knockout mouse aortae. Values are presented as Mean ± SEM. N = 7 in WT *vs.* n=3 in *Cnn2*$^{-/-}$ groups and n = 5 mice in *Cnn1*$^{-/-}$ group. N=4 in WT *vs.* n=4 in *Cnn1*$^{-/-}$,*Cnn2*$^{-/-}$ group. *P<0.05 *vs.* WT in Student's *t* test.

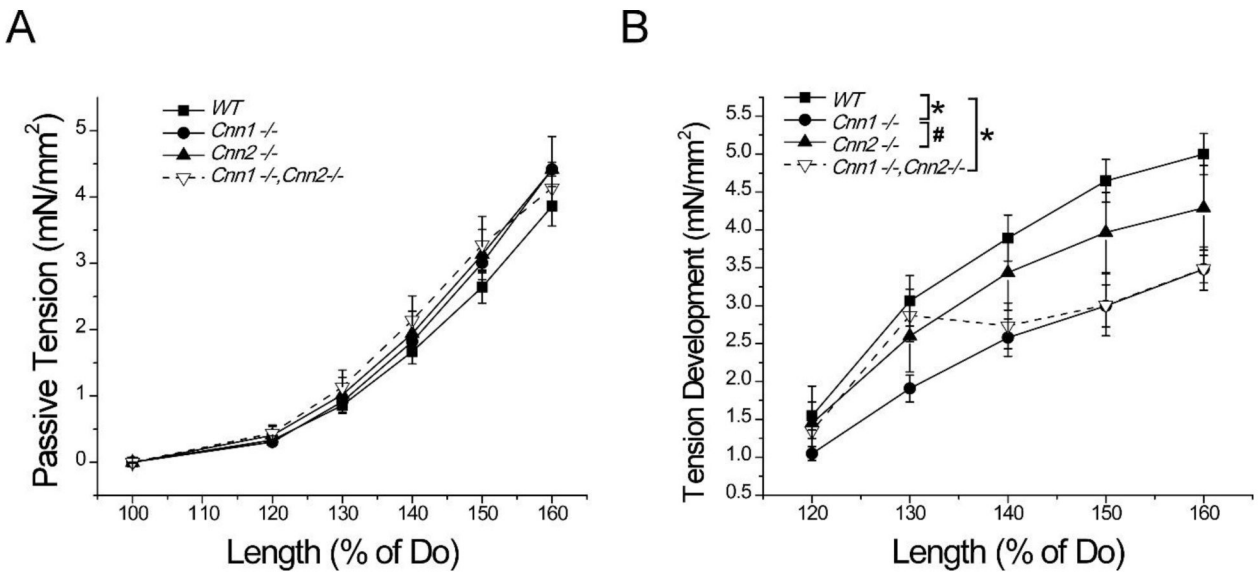

Figure 7. Resting tension and length-dependence of active tension development of mouse aortic smooth muscle.

(A) The resting tension-muscle length relationship were similar in the calponin knockout and wild type (WT) mouse aortic rings. (B) The active tension-muscle length relationship derived from total tension subtracting resting tension showed lower maximum force development in *Cnn1*$^{-/-}$ single and *Cnn1*$^{-/-}$,*Cnn2*$^{-/-}$ double knockout aortic smooth muscle in which the double knockout group had blunted responses to the increase in muscle length. N = 11 mice in WT, 5 mice in *Cnn1*$^{-/-}$, 5 mice in *Cnn2*$^{-/-}$ and 4 mice in *Cnn1*$^{-/-}$,*Cnn2*$^{-/-}$ double knockout groups. Values are presented as Mean ± SEM. ***P<0.001 in *Cnn1*$^{-/-}$ vs. WT. #P<0.05 in *Cnn1*$^{-/-}$,*Cnn2*$^{-/-}$ double knockout vs. WT. Statistics was performed by two-way ANOVA with Bonferroni test for mean comparisons.

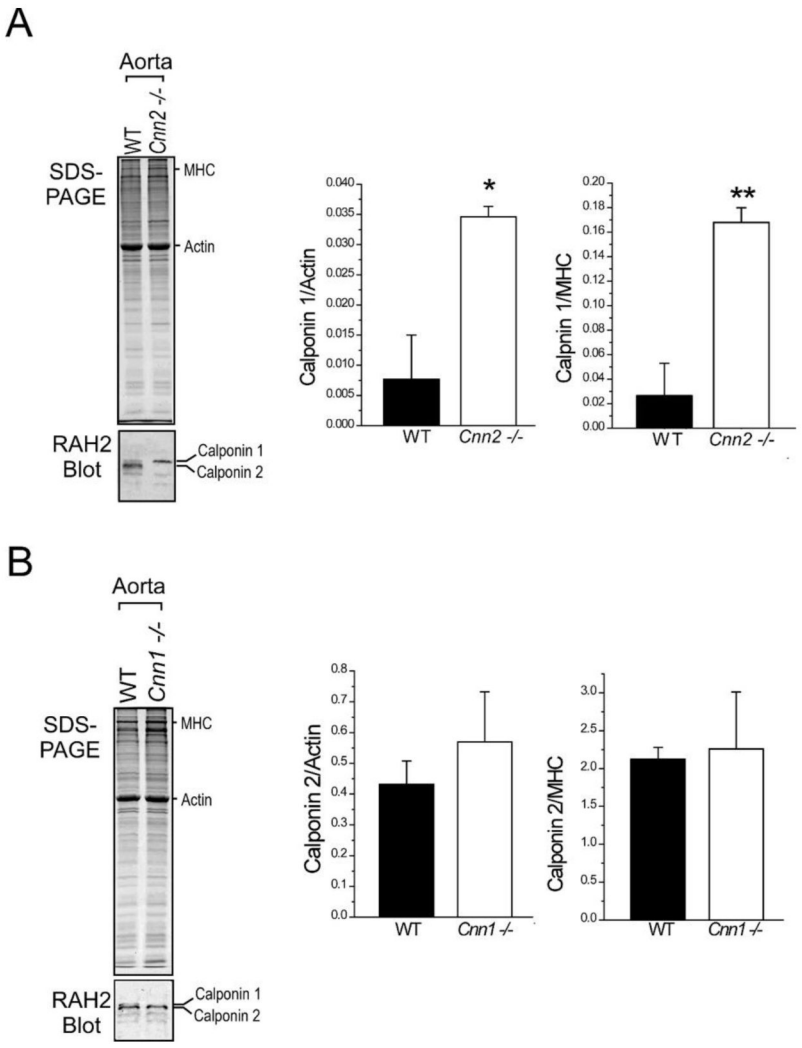

Figure 8. Compensatory increase of calponin 1 in *Cnn2*$^{-/-}$ mouse aortic smooth muscle.

(A) SDS-gel/Western blot and densitometry quantification normalized to actin or MHC showed significantly increased level of calponin 1 in *Cnn2*$^{-/-}$ mouse aortic smooth muscle. (B) No significant increase of calponin 2 was found in *Cnn1*$^{-/-}$ mouse aortic smooth muscle. Values are presented as Mean ± SEM. N = 4 mice each in WT and *Cnn2*$^{-/-}$ groups, n = 3 mice in *Cnn1*$^{-/-}$ group. *P<0.05 and **P<0.01 vs. WT in Student's *t* test.

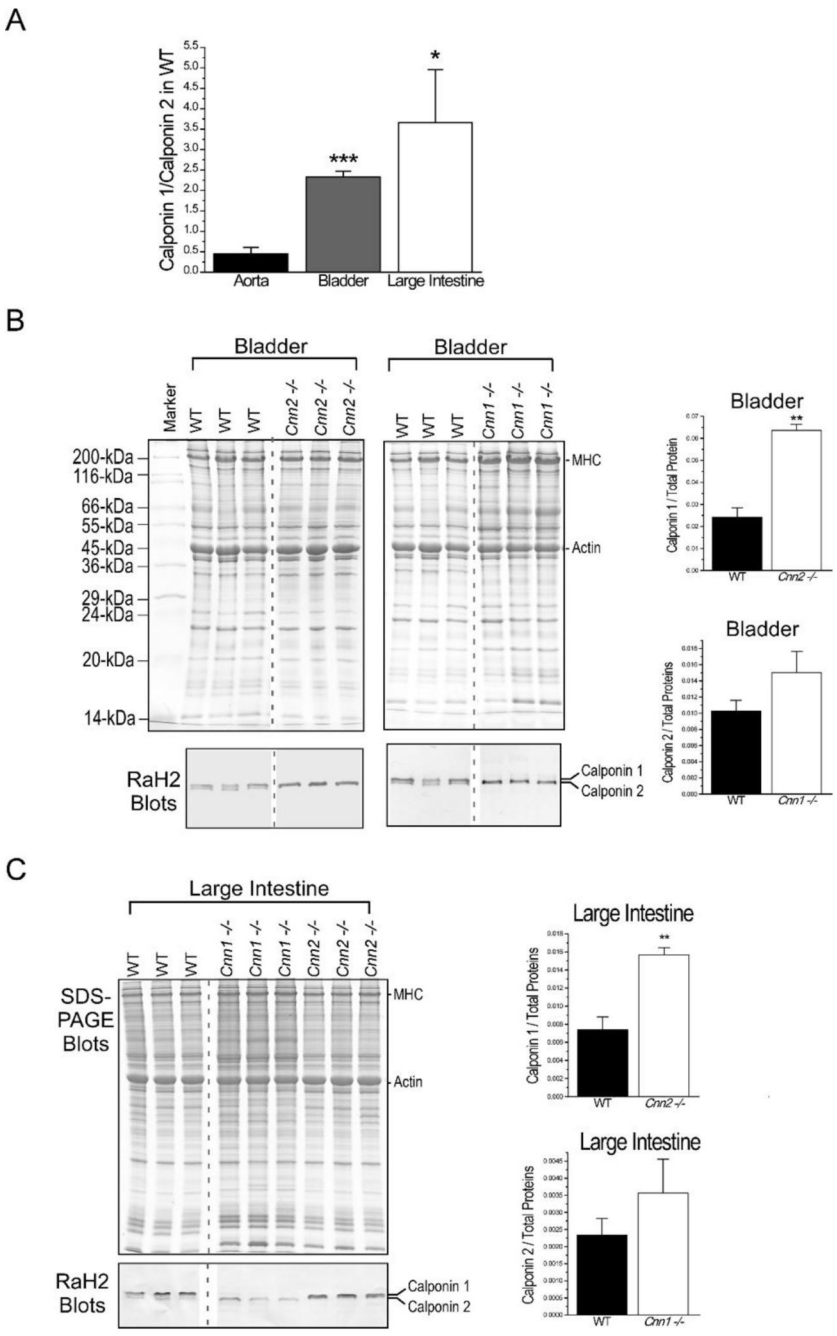

Figure 9. Compensations between calponin 1 and calponin 2 in urinary bladder and large intestine smooth muscles of *Cnn1*$^{-/-}$ and *Cnn1*$^{-/-}$ single knockout mice.

(A) The ratios between calponin 1 and calponin 2 determined by densitometry quantification of SDS-gel and Western blot in aortic, bladder and large intestinal smooth muscles of wild type (WT) showed significant differences. (B) SDS-gel, Western blot and densitometry quantification of bladder smooth muscle showed a significant increase of calponin 1 in *Cnn2*$^{-/-}$ mice as compared to WT control. (C) Large intestinal smooth muscle of *Cnn2*$^{-/-}$ mice also showed a significant upregulation of calponin 1 as compared to that of WT control. A trend of calponin 2 increases was seen in *Cnn1*$^{-/-}$ mouse bladder and large

intestine but statistical significance was not established. Values are presented as Mean ± SEM. N = 3 mice each in WT, *Cnn1*$^{-/-}$ *and Cnn2*$^{-/-}$ groups. **P<0.01; ***P<0.001 vs. WT in Student's *t* test.

Table 1.

Cnn1$^{-/-}$ single, *Cnn2*$^{-/-}$ single, *Cnn1*$^{-/-}$,*Cnn2*$^{-/-}$ double knockout and wild type mice had no significant difference in body weight

Mice	WT	*Cnn1*$^{-/-}$	*Cnn2*$^{-/-}$	*Cnn1*$^{-/-}$, *Cnn2*$^{-/-}$
Body Weight (g)	25.02±1.21	25.55±1.89	22.89±1.60	24.40±1.25

Three to 4.5 months old male and female *Cnn1*$^{-/-}$ single, *Cnn2*$^{-/-}$ single, *Cnn1*$^{-/-}$,*Cnn2*$^{-/-}$ double knockout and wild type mice showed similar body weight, indicating that the deletion of calponin 1 and/or calponin 2 had no destructive effects on viability and overall development. N=13 for WT, 7 for *Cnn1*$^{-/-}$, 5 for *Cnn2*$^{-/-}$ and 5 for *Cnn1*$^{-/-}$,*Cnn2*$^{-/-}$ double knockout groups. Values are Mean ± SEM. Statistical analysis was performed using one-way ANOVA with Bonferroni Test for mean comparison between groups. The slightly less-than-WT value of the *Cnn2*−/− group did not have statistical significance (P=0.77).