

# Myosin Phosphatase Target Subunit 1 (MYPT1) Regulates the Contraction and Relaxation of Vascular Smooth Muscle and Maintains Blood Pressure\*

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**Background:** MYPT1 is a regulatory subunit of myosin phosphatase.

**Results:** Deleting MYPT1 in vascular smooth muscle enhances myosin phosphorylation, contractility, and blood pressure.

**Conclusion:** Genetic evidence shows MYPT1 plays a role in modulating vascular smooth muscle contractility.

**Significance:** Although MYPT1 is not essential for vascular smooth muscle contractility, it contributes to blood pressure maintenance *in vivo* through signaling to myosin phosphorylation.

Myosin light chain phosphatase with its regulatory subunit, myosin phosphatase target subunit 1 (MYPT1) modulates Ca<sup>2+</sup>-dependent phosphorylation of myosin light chain by myosin light chain kinase, which is essential for smooth muscle contraction. The role of MYPT1 in vascular smooth muscle was investigated in adult MYPT1 smooth muscle specific knock-out mice. MYPT1 deletion enhanced phosphorylation of myosin regulatory light chain and contractile force in isolated mesenteric arteries treated with KCl and various vascular agonists. The contractile responses of arteries from knock-out mice to norepinephrine were inhibited by Rho-associated kinase (ROCK) and protein kinase C inhibitors and were associated with inhibition of phosphorylation of the myosin light chain phosphatase inhibitor CPI-17. Additionally, stimulation of the NO/cGMP/protein kinase G (PKG) signaling pathway still resulted in relaxation of MYPT1-deficient mesenteric arteries, indicating phosphorylation of MYPT1 by PKG is not a major contributor to the relaxation response. Thus, MYPT1 enhances myosin light chain phosphatase activity sufficient for blood pressure maintenance. Rho-associated kinase phosphorylation of CPI-17 plays a significant role in enhancing vascular contractile responses, whereas phosphorylation of MYPT1 in the NO/cGMP/PKG signaling module is not necessary for relaxation.

Blood pressure is influenced by multiple factors, including the pumping action of the heart, vascular resistance, and blood volume. Vascular resistance depends on a balance between contraction and relaxation of vascular smooth muscle cells to establish arterial tone (1–4). Depolarization of the cell membrane by external stimuli activates voltage-gated Ca<sup>2+</sup> channels, resulting in Ca<sup>2+</sup> influx (5, 6). Vascular tone is regulated via G-protein-coupled receptors (GPCRs) acting on two major signaling modules involving the heterotrimeric G proteins G<sub>q</sub>/G<sub>11</sub> and G<sub>12</sub>/G<sub>13</sub>. G<sub>q</sub>/G<sub>11</sub> mediates the activation of phospholipase C to generate inositol 1,4,5-trisphosphate, which releases Ca<sup>2+</sup> from the sarcoplasmic reticulum, leading to Ca<sup>2+</sup>/calmodulin-dependent activation of myosin light chain kinase (7–9). The G<sub>12</sub>/G<sub>13</sub> proteins couple to Rho guanine nucleotide exchange factor (RhoGEF) proteins to activate RhoA and thereby enhance the Ca<sup>2+</sup>-dependent contraction via RhoA kinase-dependent inhibition of myosin light chain phosphatase (MLCP)<sup>3</sup> (Ca<sup>2+</sup>-sensitization). G<sub>q</sub>/G<sub>11</sub>-mediated signaling is required for basal vascular tone induced by vasoactive mediators, whereas G<sub>q</sub>/G<sub>11</sub> and G<sub>12</sub>/G<sub>13</sub> are needed for pathological increases in vascular tone in hypertension (9).

Ca<sup>2+</sup>/calmodulin-dependent myosin light chain kinase (MLCK) phosphorylates myosin regulatory light chain (RLC) leading to displacement of myosin cross-bridges from the thick filament to cycle on actin filaments for force development and cell shortening (10–13). RLC is dephosphorylated by myosin

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<sup>3</sup> The abbreviations used are: MLCP, myosin light chain phosphatase; MYPT1, myosin phosphatase target subunit 1; MLCK, myosin light chain kinase; PP1c $\delta$ , myosin phosphatase catalytic subunit; RLC, myosin regulatory light chain; ROCK, Rho-associated kinase; NE, norepinephrine; Ang II, angiotensin II; Vas, Arg-vasopressin; SNP, sodium nitroprusside; CTR, control group; MYPT1<sup>SMKO</sup>, MYPT1 smooth muscle-specific knockout group; CPI-17, myosin phosphatase inhibitory protein of 17 kDa; p-CPI-17, phospho-CPI-17 (Thr-38); PKG, protein kinase G; CLA, calyculin A; MBS85, myosin phosphatase binding subunit of 85 kDa; mN, millinewtons.

light chain phosphatase (MLCP), thereby resulting in relaxation. Thus, the extent of RLC phosphorylation depends on the relative activities of MLCK and MLCP.

MLCP is a heterotrimer that consists of a catalytic type 1 phosphatase subunit (PP1c $\delta$ ), a regulatory subunit, MYPT1, and a 20-KDa subunit (M20) with unknown function (14, 15). MYPT1 enhances the catalytic activity and specificity of PP1c $\delta$  toward phosphorylated RLC in its binding to myosin (16). MYPT1 and PP1c $\delta$  also bind other proteins, which allow MLCP activity to be regulated through multiple signaling pathways (17–20). Moreover, multiple phosphorylation sites within MYPT1 that regulate PP1c $\delta$  activity toward RLC serve as an important platform for relaying signals for contraction or relaxation (21). Phosphorylation of MYPT1 Thr-696 or Thr-853 inhibits PP1c $\delta$  activity by an intramolecular mechanism (22). Phosphorylation of both sites is catalyzed by a RhoA-associated kinase, ROCK, whereas other Ca<sup>2+</sup>-independent kinases phosphorylate Thr-696 constitutively in vascular smooth muscle (23). Phosphorylation at these Thr sites prevents phosphorylation at the adjacent Ser residues (Ser-695 and Ser-852) by cyclic nucleotide-dependent kinase and hence exerts its inhibitory effect on MLCP activity (24), thereby causing Ca<sup>2+</sup>-sensitization. If Ser-695 and Ser-852 are phosphorylated, phosphorylation of Thr-696 and Thr-853 are inhibited. In addition, MYPT1 changes the conformation of PP1c $\delta$  to increase its sensitivity for binding CPI-17 phosphorylated by PKC, thereby further inhibiting MLCP activity (22). Finally, MYPT1 acts as a scaffold for other proteins including Par-4, HSP27, and M-RIP (25). Thus, MYPT1 appears to play a central role in regulating MLCP activity through direct interactions with PP1c $\delta$ , its phosphorylation by different kinases, and its actions as a scaffold for other proteins that may affect RLC phosphorylation (8, 11, 14, 25).

Although an important role for modulation of RLC phosphorylation by MYPT1 is implicated by numerous biochemical investigations and studies in intact and permeable smooth muscle fibers, insights into its role *in vivo* are lacking. A conventional total body knock-out of MYPT1 is embryonic-lethal in mice (26). Using conditional knock-out mice with a specific deletion of MYPT1 in smooth muscle cells, we previously found that the loss of MYPT1 in smooth muscle was not lethal but did alter the contractile phenotypes of gut phasic smooth muscle (27). We have now investigated the role of MYPT1 in tonic vascular smooth muscle contraction and blood pressure maintenance *in vivo*. The changes in vascular contractile response resulting from MYPT1 deficiency appear to be sufficient to elevate blood pressure *in vivo*.

## EXPERIMENTAL PROCEDURES

**Establishment of MYPT1<sup>SMKO</sup> Animals**—The establishment of MYPT1 conditional knock-out mice is described in our previous report (27). Briefly, two *loxP* sites were inserted at each end of the first exon of the *Mypt1* gene, and the resultant *Mypt1*<sup>fl $\delta$ /fl $\delta$</sup>  mice were crossed with transgenic mice with *Cre* expression driven by a smooth muscle  $\alpha$ -actin promoter (*Mypt1*<sup>fl $\delta$ /fl $\delta$</sup> :SMA-Cre mice, MYPT1<sup>SMKO</sup>) (28). The littermates of the *Mypt1*<sup>fl $\delta$ /fl $\delta$</sup> ;<sup>+</sup>:SMA-Cre mice were used as a control (both male and female mice of 4–60 weeks old). All mice were specific pathogen-free and maintained in compliance with

the Guidelines of the Care and Use of Laboratory Animals in the Model Animal Research Center of Nanjing University.

**Blood Pressure Measurements**—The systolic, diastolic, and mean blood pressures of conscious adult mice were recorded using a tail-cuff system (ALC-Non-invasive Blood Pressure System, Shanghai Alcott Biotech, China) as previously reported (13). Mice were trained by repeated blood measurement for 7 days before measurements were recorded. Mice were kept in a prewarmed box at 32 °C and pressure-measured for 20 min at the same time every day. Data were calculated as the mean value of three sequential days.

**Western Blot Analysis**—Proteins were extracted from isolated arteries fixed in trichloroacetic acid. Briefly, the arteries were frozen quickly by immersion in 10% trichloroacetic acid and 10 mM dithiothreitol in acetone cooled in liquid nitrogen to –78 °C. After homogenizing in 10% trichloroacetic acid and 10 mM dithiothreitol in H<sub>2</sub>O, the protein pellets were collected by centrifugation and then washed 3 times with ether. The resultant dried protein was dissolved in sample buffer, boiled for 4 min, and then resolved by SDS-polyacrylamide gel electrophoresis. The blotted membrane was probed with specific primary antibodies (anti-MYPT1 (1:1,000), anti-MLCK (1:10,000), anti-CPI-17 (1:5,000), anti-ROCKII (1:1,000), or anti-PP1c $\delta$  (1:500)), and the appropriate horseradish peroxidase-conjugated secondary antibodies and then visualized with enhanced chemiluminescence (ECL, Pierce).

**RNA Extraction and RT-PCR**—Total RNA was extracted from vessels and reverse-transcribed with PrimeScript RT reagent kit (Takara). The following primers were used for RT-PCR: MBS85 forward (ATCTGGACGAGGCTCGCCTGAT) and reverse (TCCCCGTCAGTGTGACAGCAG), PCR product size 322 bp; MYPT2 forward (AGACGGTGCAGTCTTTCTAGCT) and reverse (CCTCTGCAAGGTCAGAAGGAAC), PCR product size 326 bp; MYPT3 forward (AGCAAGAAGGCCATGGGGA) and reverse (AATCCAGCGTCTGTGCGTCC), PCR product size 418 bp;  $\beta$ -actin forward (5-CACCCTGTGCTGCTCACC-3) and reverse (5-GCACGATTTCCCTC-TCAG-3), PCR product size 328 bp.

**Histological Analysis and Immunofluorescent Staining**—Tissues were fixed with 4% paraformaldehyde overnight and then embedded in paraffin for histological analyses. Transverse sections (5  $\mu$ m) were stained with hematoxylin/eosin and examined under a microscope. For the immunofluorescence analysis, fresh arteries were embedded with OCT (Leica), and sections were blocked with phosphate-buffered saline containing 0.1% Triton X-100, 0.1% Tween 20, 1% bovine serum albumin, and 5% non-immune goat serum for 1 h at room temperature. Primary antibodies (anti-MYPT1 (1:200), Upstate Biotechnology; anti-smooth muscle  $\alpha$ -actin (1:200), Thermo Scientific) and fluorescent secondary antibodies (Invitrogen) were used. The fluorescent staining was examined with a confocal microscope (Olympus).

**Echocardiography**—Echocardiographic parameters were collected using a Micro-Echocardiography system (Vevo 770TM, Visual Sonics, Toronto, ON, Canada). Mice were anesthetized with avertin (250 mg/kg, intraperitoneal injection) and maintained on a platform at 37 °C. Transthoracic M-mode

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echocardiography was performed with a 30-MHz RMV-707B scanning head.

**Measurement of Plasma Renin, Angiotensin II, and Aldosterone**—Plasma concentrations of renin, angiotensin II, and aldosterone were assayed with an ELISA kit (angiotensin II and aldosterone ELISA kits were from Enzo Life Sciences; mouse renin ELISA kit were from Xinqidi Biological Technology) and performed according to the protocols provided by the manufacturers. Plasma samples were collected, and EDTA was used as an anticoagulant. After centrifugation at  $1000 \times g$  for 15 min at  $4^\circ\text{C}$ , the supernatant was aliquoted and stored at  $-80^\circ\text{C}$  before the measurements were performed.

**Myography Assay for Vessel Contractility**—The secondary branch of the mesenteric artery was isolated from adventitial tissues and mounted for isometric tension measurements (610-M, Danish Myo Technology) as previously reported (13). After equilibrating in pre-oxygenated HEPES-Tyrode buffer (137 mM NaCl, 2.7 mM KCl, 1.8 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ , 5.6 mM glucose, and 10 mM HEPES, pH 7.4) at  $37^\circ\text{C}$  for 30 min, the resting tension was adjusted to a value comparable to 100 mm Hg pressure *in vivo*. The artery segments were then equilibrated for 20 min before testing contractile capacity by exposure to 124 mM KCl-containing buffer (15.7 mM NaCl, 124 mM KCl, 1.8 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$ , 5.6 mM glucose, and 10 mM HEPES, pH 7.4). The contractile ability of the vessels was then determined by exposure to KCl and norepinephrine.

**Measurement of Myosin Light Chain Phosphorylation**—Arterial strips were frozen by immersion in 10% trichloroacetic acid in acetone chilled to a slurry in liquid nitrogen at the indicated time points. After thawing in trichloroacetic acid/acetone to room temperature, the samples were transferred to 10% trichloroacetic acid in  $\text{H}_2\text{O}$  and homogenized. The extracted proteins were resolved with urea-glycerol PAGE followed by membrane transfer. Western blotting analysis was performed with a monoclonal antibody against myosin light chain (29). The percentage of phosphorylated myosin light chain relative to the total amount of myosin light chain was calculated as described previously (13).

**Chemicals and Antibodies**—Norepinephrine, angiotensin II, U46619, Arg-vasopressin, sodium nitroprusside (SNP), HA1077, acetylcholine, and G66976 were purchased from Sigma; endothelin-1, Y27632, and H1152 were purchased from Calbiochem. Calyculin A and GF109203X were purchased from Tocris. ECL was purchased from Thermo Scientific. Antibodies against MYPT1 and PP1c $\delta$  were obtained from Millipore, p-CPI-17 (phosphorylated CPI-17) was from Bio-world, MLCK (K36),  $\beta$ -actin was from Sigma, ROCKII was from Santa Cruz Biotechnology, PKGI $\alpha$  was from Cayman Chemical, and PKGI $\beta$  was from Stressgen Bioreagents. Antibody against CPI-17 was previously described (30).

**Statistics**—Data are presented as the mean  $\pm$  S.E. Statistics was analyzed with GraphPad Prism 5.0. Student's *t* test was used between two groups data analysis. For multiple groups, significance was analyzed with two-way analysis of variance analysis followed by the Bonferroni post test. Significant differences between groups were considered when  $p < 0.05$ .

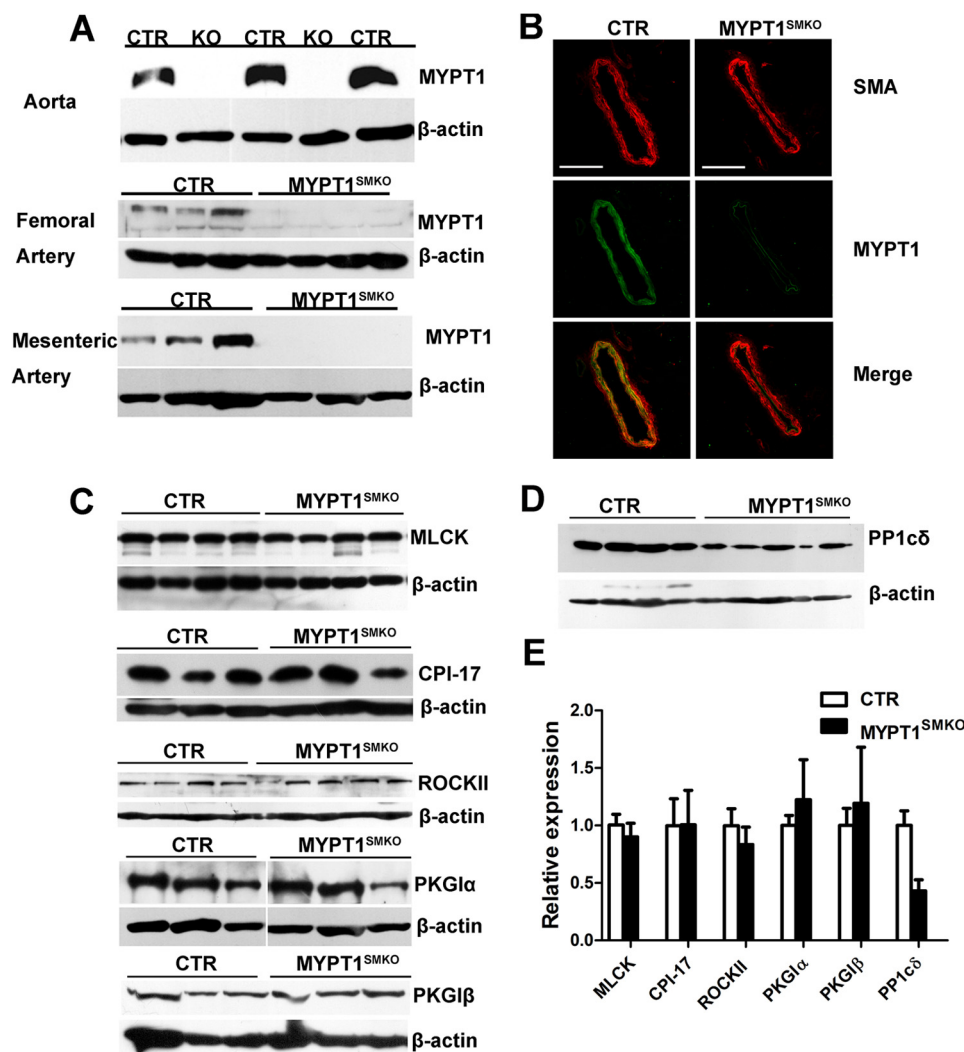
## RESULTS

**Ablated Expression of MYPT1 in Vascular Smooth Muscle**—We disrupted the *Mypt1* gene specifically in smooth muscle cells by crossing *Mypt1*-floxed mice (*Mypt1*<sup>flox/flox</sup>) with SMA-Cre transgenic mice (28). To examine the knock-out efficiency of *Mypt1* in vascular smooth muscle from the resultant mice (*Mypt1*<sup>flox/flox</sup>; SMA-Cre, MYPT1<sup>SMKO</sup>), we estimated the amount of MYPT1 protein via Western blot analysis and immunofluorescence. Western blotting showed either a trace or undetectable amounts of MYPT1 protein in mesenteric, femoral, and aortic smooth muscle tissues from MYPT1<sup>SMKO</sup> mice (Fig. 1A). Immunofluorescence analyses showed no obvious staining for MYPT1 within the smooth muscle layer of tissues from knock-out animals (Fig. 1B). We also analyzed the expression of MLCK, ROCKII, CPI-17, PKGI $\alpha/\beta$ , and PP1c $\delta$  in smooth muscles from MYPT1<sup>SMKO</sup> and control mice. For MLCK, ROCKII, CPI-17, and PKGI $\alpha/\beta$  no significant differences in expressed amounts were observed between the MYPT1<sup>SMKO</sup> and control groups (Fig. 1, C and E). We also measured the expression pattern of structural proteins by SDS-PAGE as we reported previously (27), and no difference among the proteins was observed (data not shown). However, PP1c $\delta$  expression in the MYPT1-deficient arteries was reduced  $\sim 50\%$  compared with the control vessels (Fig. 1, D and E). The reduction in PP1c $\delta$  is likely due to the cellular degradation of unbound PP1c $\delta$  compared with PP1c $\delta$  in complex with MYPT1 (31). We examined the expression of MYPT2, MYPT3, and MBS85 in the knock-out vascular smooth muscle by RT-PCR and found no significant MYPT2 and MYPT3 expression in mutant or control smooth muscles (Fig. 2). A weak expression of MBS85 could be detected but was similar in both mutant and control smooth muscles. Thus, these three MYPT family members are unlikely to compensate for the loss of MYPT1.

**MYPT1 Knock-out Mice Displayed Permanent Hypertension**—We measured the blood pressure of mice at different ages. The systolic arterial pressure of 8–48-week-old MYPT1<sup>SMKO</sup> mice was  $138 \pm 2.18$  mm Hg ( $n = 14$ ), which was significantly greater than that of control mice ( $114 \pm 2.31$  mm Hg,  $n = 14$ ,  $p < 0.001$ , Fig. 3A); mean arterial pressure of the knock-out mice was also significantly greater than that of the control mice ( $115 \pm 2.03$  versus  $95 \pm 1.94$  mm Hg; \*\*\*,  $p < 0.001$ ,  $n = 14$ ). Elevated blood pressures were detected as early as 1 month postnatal and lasted up to 15 months of age (Fig. 3B).

Because the increased blood pressure might be affected by multiple factors, we examined cardiac and renal functions in addition to vascular properties of MYPT1<sup>SMKO</sup> mice. The heart rate of the KO mice ( $528 \pm 11$  beats per min) was comparable with the CTR heart rate ( $558 \pm 21$  beats per min,  $p > 0.05$ ). Cardiac performance measures, including fractional shortening, ejection fraction, and ventricular volume, were also comparable in the MYPT1<sup>SMKO</sup> mice to values obtained with CTR mice (Table 1). The concentrations of renin, angiotensin II, and aldosterone in the peripheral blood were not altered in MYPT1<sup>SMKO</sup> mice (renin: CTR  $19.41 \pm 0.93$  pg/ml,  $n = 6$ ; MYPT1<sup>SMKO</sup>,  $19.02 \pm 1.91$  pg/ml,  $n = 6$ ,  $p > 0.01$ ; angiotensin II (Ang II): CTR  $79.61 \pm 10.21$  pg/ml; MYPT1<sup>SMKO</sup>,  $86.33 \pm 8.71$  pg/ml,  $n = 12$ ,  $p > 0.01$ ; aldosterone: CTR  $145.99 \pm 20.54$





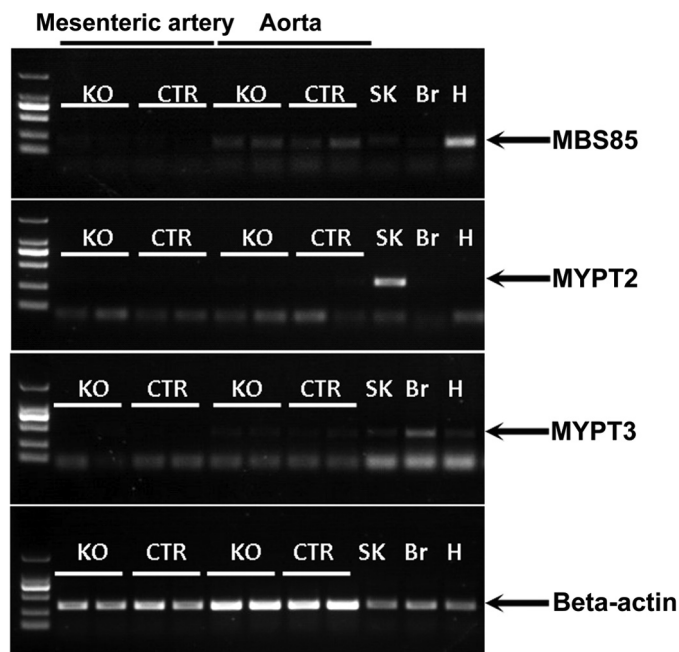
**FIGURE 1. MYPT1 was deleted in vascular smooth muscles from MYPT1<sup>SMKO</sup> mice.** *A*, Western blot of MYPT1 in different vessels from MYPT1<sup>SMKO</sup> and CTR mice (8–10 weeks old), including the aorta, mesenteric artery, and femoral artery with  $\beta$ -actin loading control. *B*, immunofluorescence staining of mesenteric arteries. Smooth muscle  $\alpha$ -actin (SMA, red) was stained as smooth muscle-specific marker. MYPT1 (green) was detected in the CTR smooth muscle layer and endothelium but only in the endothelium in MYPT1<sup>SMKO</sup> vessels. *C*, expression of other contractile proteins in MYPT1<sup>SMKO</sup> vascular smooth muscle. Western blots of MLCK, CPI-17, and ROCKII PKGI $\alpha$  and PKGI $\beta$  from mesenteric arteries showed no significant differences between MYPT1<sup>SMKO</sup> and CTR. *D*, measurement of PP1c $\delta$  expression in MYPT1<sup>SMKO</sup> and CTR mesenteric arteries by Western blotting. *E*, relative amounts of MLCK ( $n = 4$ ), CPI-17 ( $n = 3$ ), ROCKII (CTR  $n = 4$ , MYPT1<sup>SMKO</sup>  $n = 5$ ) PKGI $\alpha$  ( $n = 3$ ), PKGI $\beta$  ( $n = 4$ ), and PP1c $\delta$  (CTR  $n = 4$ , MYPT1<sup>SMKO</sup>  $n = 5$ ) in mesenteric arteries are shown.

pg/ml; MYPT1<sup>SMKO</sup>,  $141.61 \pm 24.14$  pg/ml,  $n = 12$ ,  $p > 0.01$ ). Histological examination of MYPT1-deficient mesenteric vessels showed no gross changes in vessel diameter or thickness or in the organization of the smooth muscle layer (Fig. 3C). To assess arterial compliance in the MYPT1<sup>SMKO</sup> mice, we compared the circumference corresponding to a transmural pressure of 100-mm Hg using a previously described method (13). IC<sub>100</sub> values for mesenteric arteries ( $n = 9$ ) were comparable between the MYPT1<sup>SMKO</sup> and CTR vessels ( $362 \pm 20$   $\mu$ m versus  $361 \pm 14$   $\mu$ m,  $p > 0.05$ ). Collectively, our results suggest that hypertension in the MYPT1<sup>SMKO</sup> mice is unlikely attributable to alterations of cardiac and renal function or vascular structural properties.

**Deletion of MYPT1 Enhanced Contractile Responses of Vascular Smooth Muscle**—The contractile responses of mesenteric artery segments from MYPT1<sup>SMKO</sup> mice to different stimuli were modified compared with control tissues. Upon stimulation with 124 mM KCl, mesenteric smooth muscle from CTR

mice produced a rapid and robust contraction followed by a lower sustained force (Fig. 4A). With deletion of MYPT1, segments developed a significantly greater initial force ( $4.27 \pm 0.25$  mN (KO) versus  $3.33 \pm 0.21$  mN (CTR);  $p < 0.05$ ) and a greater sustained force ( $3.98 \pm 0.33$  mN (KO) versus  $1.92 \pm 0.21$  mN (CTR);  $p < 0.05$ ; Fig. 4, A' and H), indicating the functional involvement of MYPT1 in both phases of contraction. Similarly, upon stimulation with 10  $\mu$ M norepinephrine, mesenteric arteries from MYPT1<sup>SMKO</sup> mice developed greater maximal force ( $4.47 \pm 0.64$  mN (nitric oxide (NO)) versus  $2.38 \pm 0.26$  mN (CTR),  $p < 0.05$ ; Fig. 4B, B'). We further examined the concentration-response effect of norepinephrine on force development (Fig. 4J). Both the control and mutant arteries initially developed force at 1  $\mu$ M norepinephrine that increased up to 10  $\mu$ M norepinephrine. At 10, 30, and 100  $\mu$ M norepinephrine, the corresponding forces developed by MYPT1-deficient arteries were significantly greater than the control arteries (all  $p < 0.05$ ; Fig. 4J). The norepinephrine concentration required

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**FIGURE 2. Expression of MYPT family members in vascular smooth muscles.** Fresh mesenteric artery and aorta were isolated from MYPT1<sup>SMKO</sup> and CTR mice (8–10 weeks old). The total RNA extracted from these tissues was subjected to RT-PCR assay for 30 reaction cycles.  $\beta$ -Actin mRNA was used for internal control and mRNA from skeletal muscle (SK), brain (Br), heart (H) was used for a positive control. The MBS85 panel showed weak signals, whereas no signal showed in MYPT2 and MYPT3 panels of vascular smooth muscle. Nonspecific bands are present in MYPT2 and MYPT3 measurements, which migrate differently from expected products marked by arrows.

for 50% of the maximal force ( $E_{50}$ ) was  $1.97 \pm 0.65$  and  $3.80 \pm 0.76 \mu\text{M}$  for arteries from MYPT1<sup>SMKO</sup> and CTR mice, respectively. We also measured the contractile responses of mesenteric artery segments to other vasoconstrictors. Upon the addition of endothelin, Ang II, U46619 (a thromboxane analog) and Arg-vasopressin (Vas), all of the treated arteries from MYPT1<sup>SMKO</sup> mice developed significantly greater forces compared with the control arteries (endothelin 1:  $4.55 \pm 0.36$  versus  $2.25 \pm 0.12$  mN,  $p < 0.01$ ; Ang II:  $3.04 \pm 0.38$  versus  $1.95 \pm 0.12$  mN,  $p < 0.05$ ; U46619:  $5.46 \pm 0.62$  versus  $2.52 \pm 0.31$  mN,  $p < 0.01$ ; Vas:  $4.74 \pm 0.37$  versus  $2.75 \pm 0.40$  mN,  $p < 0.01$ ; Fig. 4, C–F and I). Therefore, we conclude that deletion of MYPT1 enhanced the contraction of vascular smooth muscle in response to depolarization and various vasoconstrictors. Signaling through MYPT1 appears to be shared by many vasoconstrictors in G-protein-coupled receptor-mediated contraction. The enhanced contractile response to vasoconstrictors might contribute to the development of hypertension in MYPT1<sup>SMKO</sup> mice.

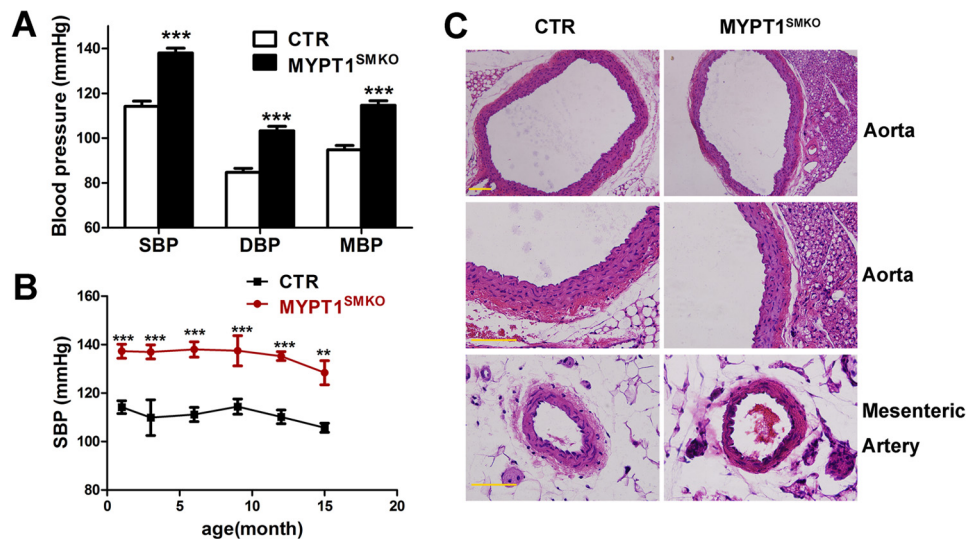
**Deletion of MYPT1 Enhanced Myosin Light Chain Phosphorylation Responses of Vascular Smooth Muscle**—Myosin light chain phosphorylation of vascular smooth muscle in response to depolarization and agonist stimulation was measured in mesenteric arteries (Fig. 5). With KCl stimulation, RLC phosphorylation in MYPT1-deficient arteries reached  $49 \pm 16\%$  at 10 s and remained elevated ( $43 \pm 6\%$  at 60 s and  $31 \pm 6\%$  at 300 s; Fig. 5, A and B). RLC phosphorylation in the control arteries also reached maximal values at 10 s ( $53 \pm 9\%$ ) but rapidly declined subsequently ( $15 \pm 5\%$  at 60 s and  $9 \pm 5\%$  at

300 s; Fig. 5, A and B). These results are consistent with the marked increase in sustained force development in mesenteric arteries from MYPT1<sup>SMKO</sup> mice. With norepinephrine stimulation for 10 s, RLC phosphorylation increased to  $63 \pm 9$  and  $49 \pm 5\%$  in MYPT1-deficient and CTR mesenteric arteries, respectively (Fig. 5, C and D). RLC phosphorylation in MYPT1-deficient vessels was greater than CTR values at 60 s, and both then decreased to  $27 \pm 8\%$  at 300 s (Fig. 5, C and D). Thus, deletion of MYPT1 results in enhanced RLC phosphorylation with depolarization- and agonist-induced contractions.

**Signaling to MLCP in the Vascular Smooth Muscle Lacking MYPT1**—Inactivation of  $G_{12}$ - $G_{13}$ /Rho/ROCK signaling by ROCK inhibitors causes disinhibition of MLCP activity, resulting in reduced RLC phosphorylation and force development (23). Y27632, an inhibitor of ROCK, inhibits either the KCl- or norepinephrine-induced contraction of control arteries (Fig. 6). Upon pretreatment with Y27632, MYPT1-deficient mesenteric arteries developed a normal initial response induced by KCl, but the sustained force was greatly inhibited in a manner similar to that which affected the control muscle (Fig. 6, A–C). Upon treatment with Y27632 during the sustained contraction, the control arteries almost completely relaxed when contracted in response to KCl (Fig. 6D) or norepinephrine (Fig. 6G) treatment. Adding Y27632 to MYPT1-deficient arteries also inhibited sustained contractile responses induced by KCl (Fig. 6E) or norepinephrine (Fig. 6H). The norepinephrine-induced force response of MYPT1-deficient arteries was as sensitive to Y27632 as the responses in control muscles ( $IC_{50}$  of  $2.51 \pm 0.91 \mu\text{M}$  (KO) versus  $1.81 \pm 0.62 \mu\text{M}$  (CTR),  $p > 0.05$ ; Fig. 6I). The KCl-induced force responses for the MYPT1-deficient muscles also appeared similarly sensitive to Y27632 compared with the control at the higher concentration of Y27632 (Fig. 6F). To further analyze the effect of ROCK on MYPT1-deficient mesenteric arteries, we examined the effects of other ROCK-specific inhibitors, HA1077 and H1152 (11). No differences in sensitivity to these inhibitors were observed between arteries from CTR and MYPT1<sup>SMKO</sup> mice (data not shown). These results collectively showed that the loss of MYPT1 was not sufficient to prevent inhibition of vascular smooth muscle contraction by inhibition of ROCK activity.

Inactivation of PKC signaling by PKC inhibitors also results in disinhibition of MLCP activity by preventing CPI-17 phosphorylation (11). To assess the effect of PKC inhibitors on vascular smooth muscle force, we added Gö6976 and GF109203X to KCl- and NE-stimulated segments, respectively. The norepinephrine/KCl-induced contraction of MYPT1-deficient arteries was as sensitive as to Gö6976 and GF109203X as control muscle (Fig. 6, J–M).

To assess the role of MYPT1 in endothelium-dependent relaxation, a critical process in regulating vessel tone, we examined the effects of acetylcholine and nitric oxide on relaxing MYPT1-deficient arteries. In the control arteries, acetylcholine relaxed KCl-induced force with a similar reduction in force in the knock-out arteries (Fig. 7, A and B), showing a similar sensitivity to acetylcholine (ACh; Fig. 7C). Thus, MYPT1-deficient mesenteric segments were as sensitive to relaxation to acetylcholine as control segments. Because NO is a downstream product of acetylcholine signaling, we also examined the effect



**FIGURE 3. Blood pressure of MYPT1<sup>SMKO</sup> mice was significantly increased without changes in vascular structure.** *A*, blood pressure recordings of conscious MYPT1<sup>SMKO</sup> and control adult mice (8–48 weeks old). Systolic, diastolic, and mean blood pressures (SBP, DBP, MBP) were significantly elevated in MYPT1<sup>SMKO</sup> mice (\*\*\*,  $p < 0.001$ ,  $n = 14$ ). *B*, systolic blood pressure in MYPT1<sup>SMKO</sup> and CTR mice at different ages. The systolic blood pressure of MYPT1<sup>SMKO</sup> mice was increased in 1-month-old mice ( $137 \pm 2.9$  mm Hg (MYPT1<sup>SMKO</sup>) versus  $109.9 \pm 7.3$  mm Hg (CTR), \*\*\*,  $p < 0.001$ ,  $n = 3$ ) and maintained up to 15 months of age ( $128.4$  mm Hg (MYPT1<sup>SMKO</sup>) versus  $105.7$  (CTR), \*\*,  $p < 0.01$ ,  $n = 3$ ). *C*, histology of aortic and mesenteric arteries. Tissue slices of the arteries were stained with hematoxylin and eosin. The results showed apparently normal vascular morphology of the MYPT1<sup>SMKO</sup> blood vessels compared with CTR vessels. Scale bar, 100  $\mu$ m.

**TABLE 1**

**Echocardiography parameters of MYPT1<sup>SMKO</sup> mice**

Electrocardiography was performed as described under "Experimental Procedures." Data are presented as the mean  $\pm$  S.E. LV Vol;d, diastolic left ventricular volume; LV Vol;s, systolic left ventricular volume; % EF, percent ejection fraction; % FS, percent fractional shortening; V mass, left ventricular mass. NS, no significant difference,  $p > 0.05$ ;  $n$ , at least 11 values for each measurement.

Cardiac parameters	KO	CTR	Summary
LV Vol;d ( $\mu$ l)	45.3 $\pm$ 3.9	45.7 $\pm$ 2.5	NS
LV Vol;s ( $\mu$ l)	16.4 $\pm$ 2.5	14.8 $\pm$ 1.3	NS
% EF (% ejection fraction)	65.3 $\pm$ 2.9	67.8 $\pm$ 2.2	NS
% FS (% fractional shortening)	35.4 $\pm$ 2.1	37.3 $\pm$ 1.8	NS
LV mass(mg)	83.6 $\pm$ 4.8	73.4 $\pm$ 3.7	NS

of the NO donor, SNP, on the relaxation of KCl-induced contraction, and a similar conclusion was obtained (Fig. 7, *G–I*). We next examined the relaxation of NE-induced contraction in mesenteric arteries after treatment with ACh and SNP. The norepinephrine-induced forces in arteries from MYPT1<sup>SMKO</sup> and control mice were relaxed by acetylcholine with similar concentration dependence (Fig. 7, *D–F*). Surprisingly, the deletion of MYPT1 did affect SNP-mediated relaxation of norepinephrine-induced force.

**MLCP Activity Is Regulated in Mesenteric Segments from MYPT1<sup>SMKO</sup> Mice**—The minimal effect of MYPT1 deletion on inhibition of norepinephrine contractile responses to ROCK inhibitors and NO generators led us to consider the existence of redundant regulation of MLCP activity. Because vascular smooth muscles predominantly express CPI-17, which inhibits PP1c $\delta$  activity after phosphorylation by PKC or ROCK, we hypothesized that CPI-17 phosphorylation might inhibit PP1c $\delta$  activity and, hence, relax vessels in the absence of MYPT1. To test this hypothesis, we pretreated intact mesenteric segments with the PP1c inhibitor calyculin A and measured the relaxation effect of H1152. In control segments, pretreatment with calyculin A attenuated H1152-mediated inhibition of the contractile response (Fig. 8*A*). In MYPT1-deficient segments pre-

treated with calyculin A, the attenuation of the H1152-mediated relaxation effect was greater compared with CTR (Fig. 8, *B* and *C*). Upon stimulation with norepinephrine, CPI-17 phosphorylation in the MYPT1-deficient smooth muscle was reduced compared with the control vessels, whereas the total amount of CPI-17 was unchanged (Fig. 8, *D–F*). Y27632 inhibited CPI-17 phosphorylation in response to norepinephrine treatment in both MYPT1-deficient and CTR mesenteric arteries. Collectively, the results show that ROCK acting on CPI-17 may regulate MLCP activity in vascular smooth muscle.

**DISCUSSION**

Fundamental to blood pressure maintenance are cardiac output, circulating volume, and vascular tone. The latter is actively regulated by contraction and relaxation of vascular smooth muscle cells in response to mechanical stresses and vasoactive signals from sympathetic nerves, endocrine organs, and parenchymal cells. As a focal point for smooth muscle contractile signaling, RLC phosphorylation is regulated by multiple pathways involving MLCK activity regulated by Ca<sup>2+</sup>/calmodulin and phosphorylation (10). MLCP activity is regulated by MYPT1 and CPI-17 phosphorylation (11, 14, 15, 25). Interacting temporal and spatial signaling responses basically control the ratio of MLCK to MLCP activities, thereby physiologically fine-tuning the extent of RLC phosphorylation and arterial tone.

Smooth muscle-specific knock-out of MLCK is lethal and results in general smooth muscle failure with markedly impaired contractile responses in ileal (32), airway, vascular (13, 33), and bladder (34) (30) smooth muscle tissues. In contrast smooth muscle-specific knock-out of MYPT1 is not lethal (27) but does lead to measurable changes in contractile properties of smooth muscle tissues with *in vivo* consequences as with hypertension described herein. These results support the conclusion that whereas MYPT1 is not an essential regulatory effector for myosin dephosphorylation, it serves a modulatory role.



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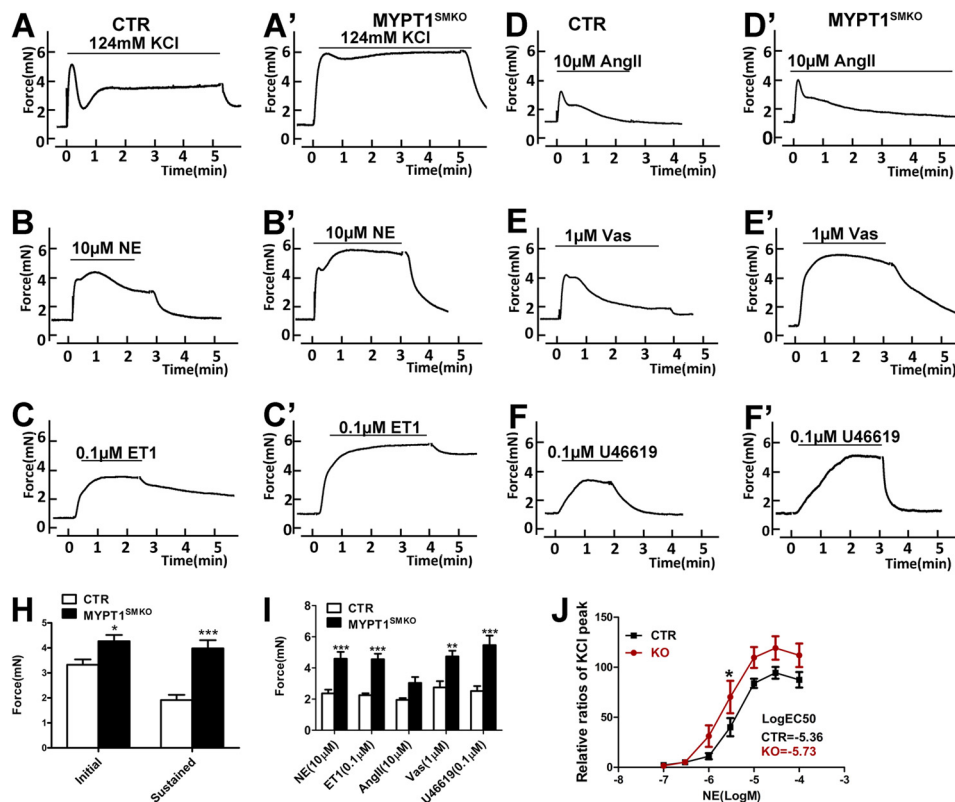


FIGURE 4. Vasoconstrictor responses of MYPT1<sup>SMKO</sup> mesenteric arteries. Representative force responses of CTR and MYPT1<sup>SMKO</sup> mesenteric arteries (8–10 weeks old) to 124 mM KCl (A and A'), 10 μM NE (B and B'), 0.1 μM endothelin 1 (ET1; C and C'), 10 μM Ang II (D and D'), 1 μM Vas (E and E'), and 0.1 μM U46619 are shown. G, quantified initial and sustained force responses to KCl stimulation of vessels from CTR and MYPT1<sup>SMKO</sup> mice (\*,  $p < 0.05$ ; \*\*\*,  $p < 0.001$ ,  $n = 12$ ). H, quantified peak force development responses for different vasoconstrictors showing significant differences between vessels from CTR and MYPT1<sup>SMKO</sup> mice (NE:  $n = 12$ ; \*\*\*,  $p < 0.001$ ; endothelin 1:  $n = 7$ ; \*\*,  $p < 0.01$ ; Ang II:  $n = 7$ ; Vas,  $n = 9$ ; \*\*,  $p < 0.01$ ; U46619:  $n = 8$ , \*\*\*,  $p < 0.001$ ). I, cumulative dose responses to NE relative to responses to 124 mM KCl for mesenteric arteries from CTR and MYPT1<sup>SMKO</sup> mice (\*,  $p < 0.05$ ,  $n = 7$ ).

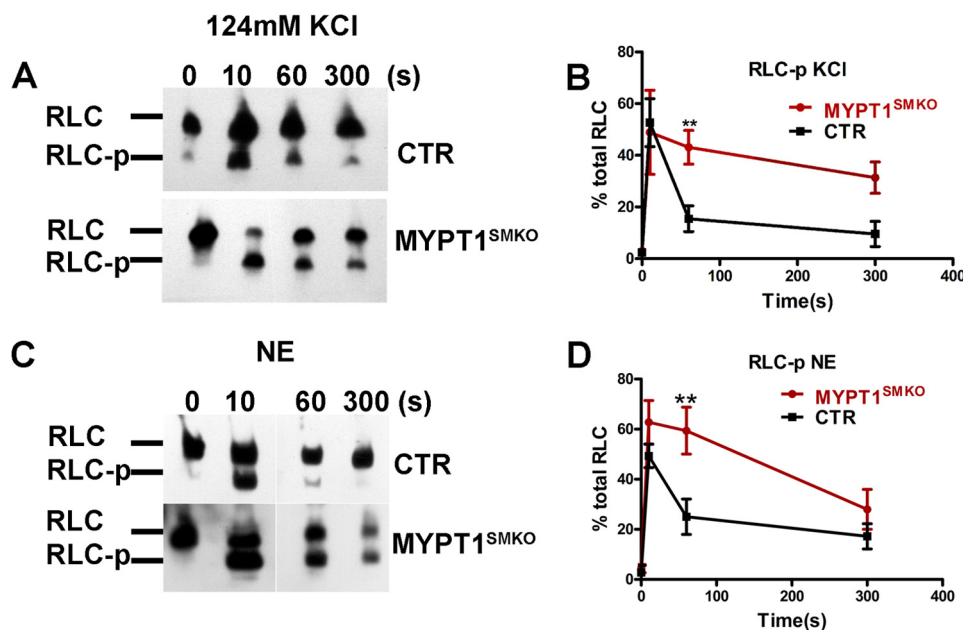


FIGURE 5. RLC phosphorylation is enhanced in mesenteric arteries from MYPT1<sup>SMKO</sup> mice. A and C, representative Western blots after urea/glycerol PAGE for vessels from MYPT1<sup>SMKO</sup> and CTR mice (8–10 weeks old) treated for 300 s with 124 mM KCl (A) or 10 μM NE (C). Nonphosphorylated (RLC) and phosphorylated (RLC-p) RLCs are identified. B and D, temporal responses for RLC phosphorylation to KCl (B) and NE (D) treatments. \*\*,  $p < 0.01$  compared with CTR tissue responses (KCl,  $n = 4$ ; NE,  $n = 6$ ).

Biochemically, MYPT1 is a primary PP1c $\delta$ -binding protein in smooth muscle where it regulates the activity of MLCP holoenzyme by localizing the catalytic subunit to myosin fila-

ments (35). In MYPT1-deficient smooth muscles from mesenteric artery or intestine, 50 or 100% PP1c $\delta$  protein remained, respectively, and RLC was dephosphorylated after treatments

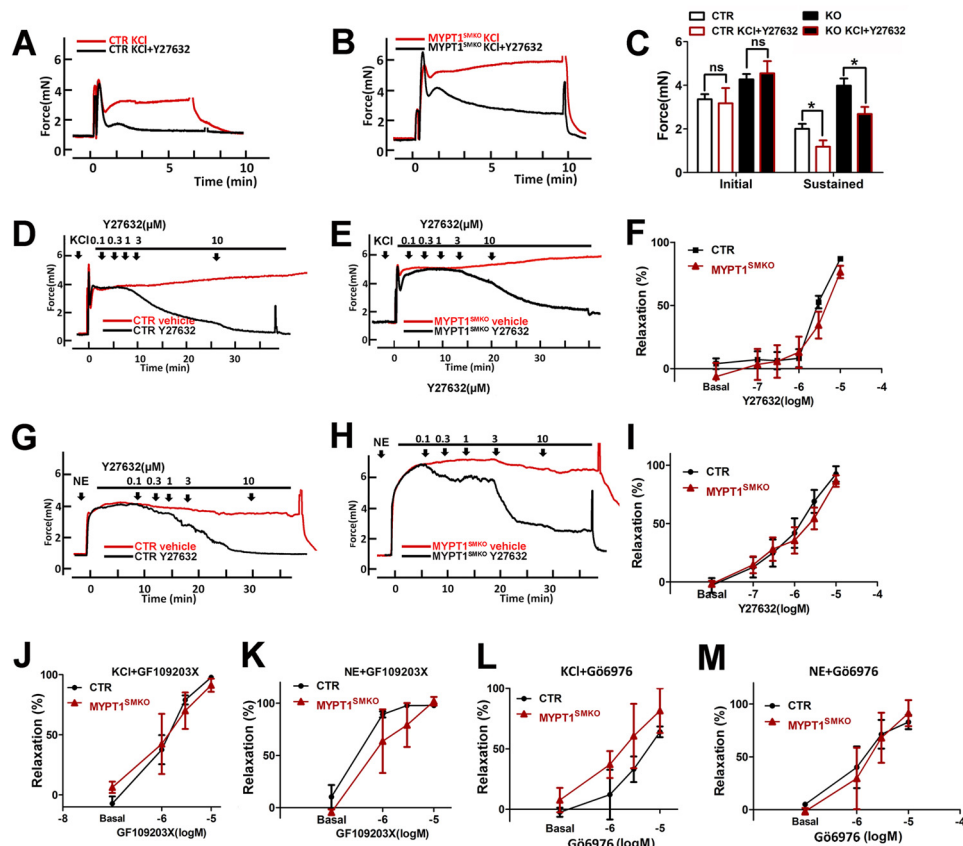


FIGURE 6. **ROCK and PKC inhibitors decrease force responses of MYPT1<sup>SMKO</sup> mesenteric arteries.** *A* and *B*, mesenteric arteries from the mice (8–10 weeks old) were pretreated with the ROCK inhibitor Y27632 and then stimulated with 124 mM KCl. *C*, quantified results of *A* and *B* ( $n = 4$ ; ns:  $p > 0.05$ ; \*,  $p < 0.05$ ). *D* and *E*, mesenteric arteries were precontracted with 124 mM KCl followed by cumulative increases in the concentration of Y27632 (0.1–10 μM) or vehicle ( $n = 7$ ). *F*, quantified force responses for KCl contracted tissues treated with Y27632. Relative ratios of the relaxed force in steady state are expressed as the percent of the force development to KCl at the same time point of vehicle (relaxation % =  $(F_{\text{vehicle}} - F_{\text{inhibitor}})/F_{\text{vehicle}}$ , where  $F$  = force) ( $n = 7$ ). *G* and *H*, mesenteric arteries from the mice (8–10 weeks old) were precontracted with 10 μM NE followed by cumulative increases in the concentration of ROCK inhibitor Y27632 (0.1–10 μM) or vehicle ( $n = 7$ ). *I*, quantified force responses for NE-contracted tissues treated with Y27632. Relative ratio of sustained force is expressed as the percent of the peak force to NE ( $n = 7$ ). *J* and *M*, mesenteric arteries were precontracted with 124 mM KCl or 10 μM NE followed by cumulative increases in the concentration of the PKC inhibitors GF109203X ( $n = 3$ ) and G66976 ( $n = 3$ ).

that increase its phosphorylation (27). Myosin targeting subunits normally expressed in other non-smooth muscle cells and tissues were not abundant and did not compensate by increased expression in MYPT1-deficient smooth muscles. Thus, MYPT1 is not essential for phosphatase activity directed toward phosphorylated RLC. We, therefore, suggest that the remaining PP1cδ is sufficient to dephosphorylate phosphorylated RLC, although the activity is less than in the holoenzyme (36).

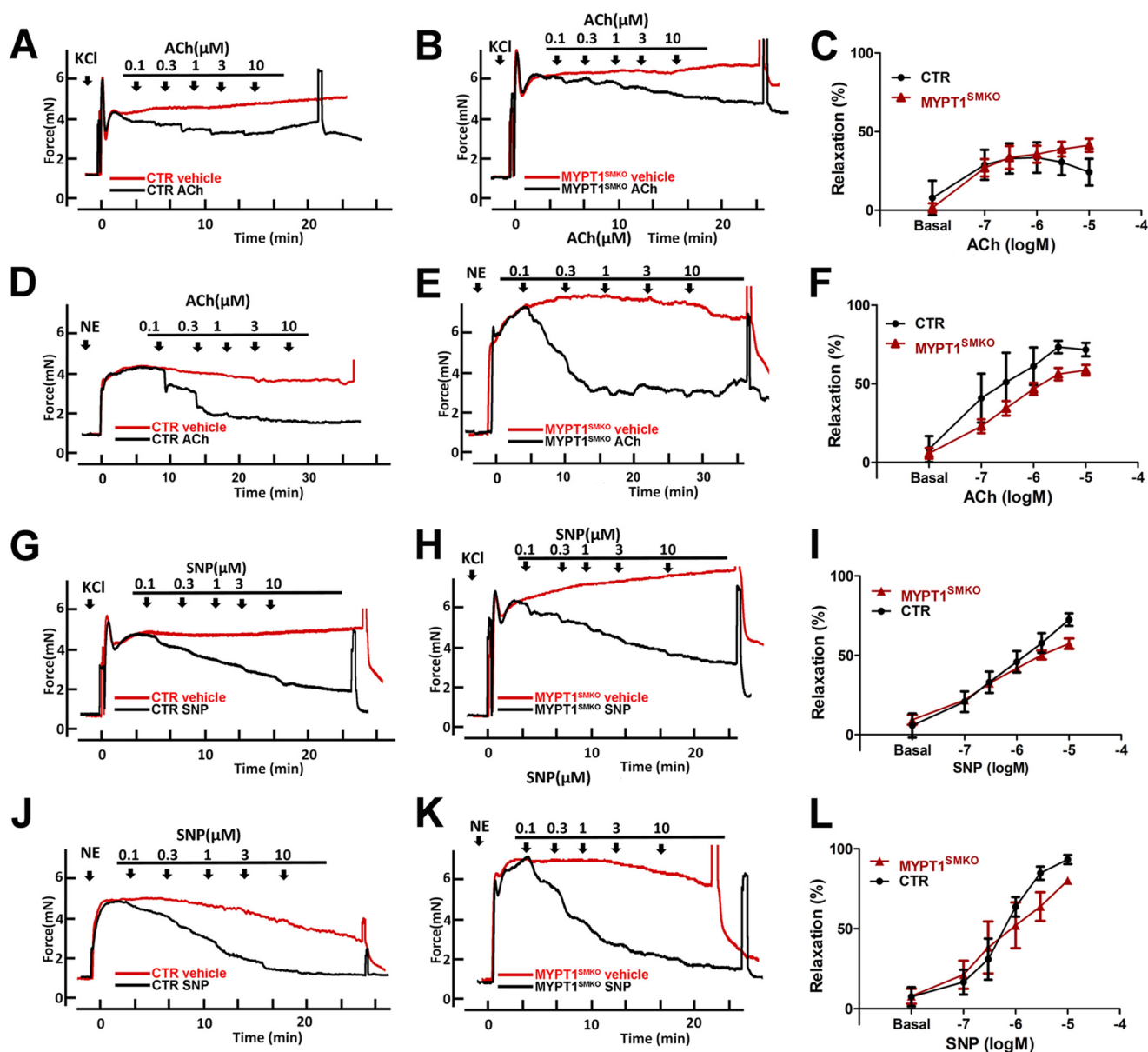
Using a smooth muscle-specific knock-out of MYPT1 in mice, we found that its deletion enhanced RLC phosphorylation and contractility of isolated vascular smooth muscle in response to various vasoactive stimuli and resulted in hypertension. These results are consistent with a decrease in MLCP activity due to the loss of MYPT1 and the partial decline in PP1cδ protein (15, 25, 37). The smooth muscle knock-out of MYPT1 in mice led to no apparent changes in cardiac performance or blood volume regulating hormones so the increased blood pressure may result from the enhanced arterial contractile responses.

In response to KCl depolarization and norepinephrine, vascular smooth muscle produces an initial rapid and then a sustained contraction (38–40). The signaling mechanisms for this

contraction involve a rapid activation of MLCK by  $\text{Ca}^{2+}$ /calmodulin for rapid RLC phosphorylation followed by recruitment of  $\text{Ca}^{2+}$ -sensitization mechanisms for RLC phosphorylation. In general,  $\text{Ca}^{2+}$ -sensitization mechanisms may be mediated by  $G_{12}$ - $G_{13}$ /RhoA/ROCK inhibition of MLCP activity by MYPT1 and CPI-17 phosphorylation (9, 22, 23). The relative physiological significance of these two distinct regulatory pathways in smooth muscle is not completely clear, although published reports indicate that phosphorylation of these proteins fine-tune contractile responses in different smooth muscle tissues in which MYPT1 and CPI-17 are differentially expressed (23, 41). The inhibition of MLCP activity by ROCK-mediated MYPT1 phosphorylation is thought to play a key role in  $\text{Ca}^{2+}$ -sensitized contractions in different kinds of smooth muscles, including different vascular smooth muscles. In MYPT1-deficient mesenteric arteries, force enhancement in the initial and sustained contraction phases was associated with enhanced RLC phosphorylation. Considering the proposed central role MYPT1 plays in regulating MLCP activity, it was surprising that MYPT1 deletion in vascular smooth muscle was not more consequential. The apparent decrease in MLCP activity in tissues was associated with diminished amounts of PP1cδ. Because MYPT1 is absent in MYPT1<sup>SMKO</sup> mice, the enhanced



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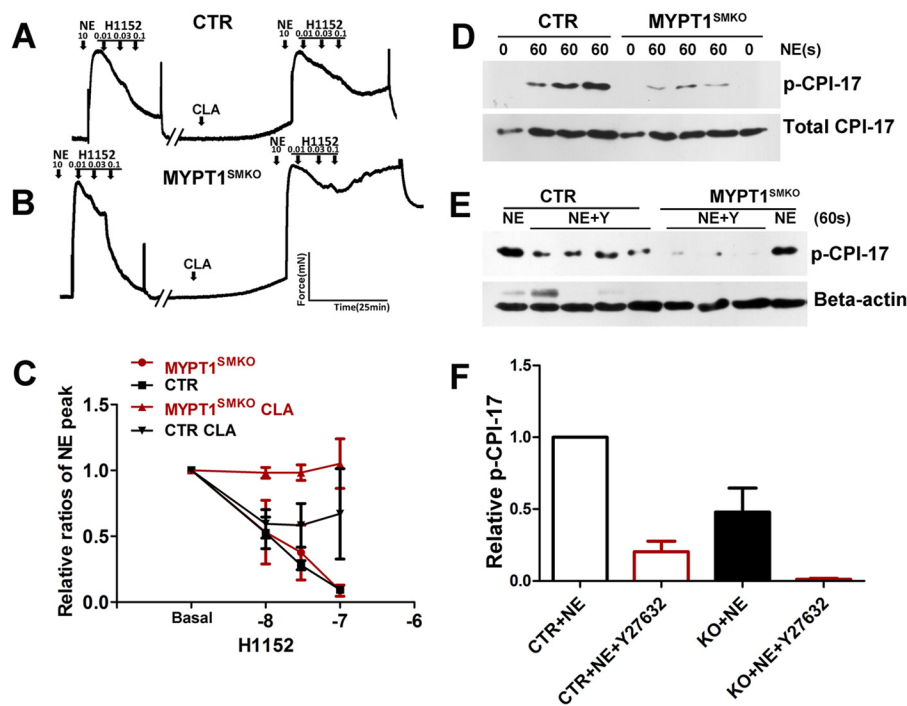


**FIGURE 7. Nitric oxide signaling relaxes mesenteric arteries from MYPT1<sup>SMKO</sup> mice.** *A* and *B*, relaxation responses to the cumulative addition of ACh or vehicle for mesenteric arteries from 8–10 week-old-mice with intact endothelium contracted with 124 mM KCl ( $n = 4$ ) (*A* and *B*). *C*, concentration dependence of relaxation responses to ACh with KCl-induced contraction is shown ( $n = 4$ ). (Relaxation % =  $(F_{\text{vehicle}} - F_{\text{inhibitor}})/F_{\text{vehicle}}$ , where  $F$  = force). *D* and *E*, relaxation responses to the cumulative addition of ACh or vehicle for mesenteric arteries with intact endothelium contracted with 10  $\mu\text{M}$  NE ( $n = 6$ ). *F*, concentration dependence of relaxation responses to ACh with NE-induced contractions are shown ( $n = 6$ ). *G* and *H*, representative concentration-dependent relaxation responses to the cumulative addition of SNP or vehicle for mesenteric arteries contracted with 124 mM KCl ( $n = 8$ ). *I*, concentration dependence of relaxation responses to SNP with KCl-induced contraction is shown. *J* and *K*, relaxation responses to the cumulative addition of SNP or vehicle for mesenteric arteries contracted with 10  $\mu\text{M}$  NE. *L*, concentration dependence of relaxation responses to SNP with NE-induced contraction are shown ( $n = 4$ ).

response to norepinephrine may result from CPI-17 phosphorylation with inhibition of the reduced amount of PP1c $\delta$ . Vascular smooth muscles have an abundance of CPI-17 protein relative to other kinds of smooth muscles (42). Both ROCK and PKC inhibitors inhibited agonist-induced sustained contractions in MYPT1-deficient vascular smooth muscle similar to control smooth muscles, suggesting a contribution of CPI-17 phosphorylation by ROCK in regulating PP1c $\delta$  activity. Contractile responses were not inhibited by a ROCK inhibitor in MYPT1-deficient intestinal smooth muscle, which has much less CPI-17 compared with vascular smooth muscle (27). Rock inhibition also resulted in inhibi-

tion of CPI-17 phosphorylation in MYPT1-deficient mesenteric arteries. Additionally, our results are consistent with a recent report suggesting  $\alpha$ -adrenergic receptor activation acted through CPI-17, not MYPT1 phosphorylation, in rat mesenteric arteries to affect Ca<sup>2+</sup> sensitization (43). Thus, PKC and ROCK pathways may both serve as important Ca<sup>2+</sup>-sensitization mechanisms involving CPI-17 phosphorylation in mesenteric arteries.

In contracting smooth muscles, relaxation may result from a decrease in [Ca<sup>2+</sup>]<sub>i</sub> and/or reduction in Ca<sup>2+</sup>-sensitization of RLC phosphorylation and force development. NO initiates vascular smooth muscle relaxation by stimulating cGMP forma-



**FIGURE 8. Regulation of CPI-17 phosphorylation in mesenteric arteries from MYPT1<sup>SMKO</sup> mice.** *A* and *B*, relaxation effects of the ROCK inhibitor H1152 before and after inhibition of PP1 $\delta$  by calyculin A treatment. After force development with 10  $\mu$ M NE, mesenteric arteries from CTR (*A*) and MYPT1<sup>SMKO</sup> (*B*) mice from 8–10-week-old mice were treated with cumulative concentrations of H1152 (0.01–0.10  $\mu$ M) in the absence of calyculin A (CLA). The vessels were washed and then incubated with 10 nM CLA for 30 min. The tissues were treated again with 10  $\mu$ M NE + 10 nM CLA to induce force development followed by treatment with increasing concentrations of H1152 (*right*). The inhibitory effect of H1152 was attenuated after CLA treatment. *C*, quantified results for responses shown in *A* and *B* (MYPT1<sup>SMKO</sup>,  $n = 3$ ; CTR,  $n = 4$ ; MYPT1<sup>SMKO</sup> CLA,  $n = 3$ ; CTR CLA,  $n = 5$ ). *D*, measurement of p-CPI-17 before and after treating mesenteric arteries with 10  $\mu$ M NE for 60 s in MYPT1<sup>SMKO</sup> and CTR mesenteric arteries. *E*, inhibition of CPI-17 phosphorylation in mesenteric arteries by 10  $\mu$ M NE for 60 s. Note that this panel shows enhanced signal of p-CPI-17 by longer exposure so as to make a weak band clear. *F*, quantified results for responses shown in *C* and *D*. p-CPI-17 was expressed as fraction of p-CPI-17 level in CTR mesenteric arteries treated with NE for 60 s. CPI-17 phosphorylation of NE-treated MYPT1<sup>SMKO</sup> arteries (KO+NE) was  $48 \pm 17\%$  of that of CTR arteries (CTR+NE) ( $n = 8$ ). Y27632 decreased p-CPI-17 in both KO and CTR arteries (KO+NE+Y27632,  $3 \pm 1\%$  ( $n = 3$ ); CTR+NE+Y27632,  $27 \pm 4\%$ ,  $n = 3$ ).

tion and activating PKG (44–46). PKG activation results in a decrease in  $[Ca^{2+}]_i$  by inhibiting PLC and inositol 1,4,5-trisphosphate formation (47, 48), a desensitization of RLC phosphorylation to  $Ca^{2+}$  by phosphorylating MYPT1, resulting in MLCP activation (49), an activation of the large conductance  $Ca^{2+}$ -activated  $K^+$  channel (BK) (50), and RhoA phosphorylation that inhibits activation and membrane translocation (51). The latter inhibits activation of ROCK by RhoA (52). Mice containing selective mutations in the amino-terminal leucine zipper domain of PKG show both decreased MYPT1 phosphorylation and increased activation of RhoA in vascular smooth muscles (4). This disruption of PKG regulation contributes to abnormal relaxation responses and increased systemic blood pressure. The NO/cGMP/PKG signaling module is an important physiological system modulating vascular tone that has multiple cell targets affecting contractility. We found that activation of the NO/cGMP/PKG signaling pathway relaxed evoked force in MYPT1-deficient vascular smooth muscle similar to results obtained with control tissues. This finding suggests that NO/cGMP/PKG signaling mechanisms effecting reduction in  $[Ca^{2+}]_i$  are sufficient to relax contraction of mesenteric vessels. In summary, deletion of MYPT1 in vascular smooth muscle enhanced RLC phosphorylation and contractile activity, which may contribute to the development of hypertension *in vivo*.

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