ORIGINAL RESEARCH

Vascular Stiffening Mediated by Rho-Associated Coiled-Coil Containing Kinase Isoforms

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BACKGROUND: The pathogenesis of vascular stiffening and hypertension is marked by non-compliance of vessel wall because of deposition of collagen fibers, loss of elastin fibers, and increased vascular thickening. Rho/Rho-associated coiled-coil containing kinases 1 and 2 (ROCK1 and ROCK2) have been shown to regulate cellular contraction and vascular remodeling. However, the role of ROCK isoforms in mediating pathogenesis of vascular stiffening and hypertension is not known.

METHODS AND RESULTS: Hemizygous Rock mice (Rock1+/− and Rock2+/−) were used to determine the role of ROCK1 and ROCK2 in age-related vascular dysfunction. Both ROCK activity and aortic stiffness increased to a greater extent with age in wild-type mice compared with that of Rock1+/− and Rock2+/− mice. As a model for age-related vascular stiffening, we administered angiotensin II (500 ng/kg per minute) combined with nitric oxide synthase inhibitor, L-Nω-nitroarginine methyl ester (0.5 g/L) for 4 weeks to 12-week-old male Rock1+/− and Rock2+/− mice. Similar to advancing age, angiotensin II/L-Nω-nitroarginine methyl ester caused increased blood pressure, aortic stiffening, and vascular remodeling, which were attenuated in Rock2+/−, and to a lesser extent, Rock1+/− mice. The reduction of aortic stiffening in Rock2+/− mice was accompanied by decreased collagen deposition, relatively preserved elastin content, and less aortic wall hypertrophy. Indeed, the upregulation of collagen I by transforming growth factor-β1 or angiotensin II was greatly attenuated in Rock2−/− mouse embryonic fibroblasts.

CONCLUSIONS: These findings indicate that ROCK1 and ROCK2 mediate both age-related and pharmacologically induced aortic stiffening, and suggest that inhibition of ROCK2, and to a lesser extent ROCK1, may have therapeutic benefits in preventing age-related vascular stiffening.

Key Words: aging ■ aortic stiffness ■ eEF1A1 ■ Rho kinase ■ vascular remodeling

Epidemiological studies have shown that vascular stiffening and systolic hypertension are independent predictors of cardiovascular disease.1,2 Although there is a close association between aortic stiffness and the development of systemic hypertension,3,4 the mechanisms leading to vascular stiffening and the temporal relationship between aortic stiffness and the development of hypertension remains unclear. Nevertheless, the pathogenesis of age-related vascular stiffening with or without hypertension is marked by non-compliance of the vessel wall because of deposition of non-compliant collagen fibers, loss or fragmentation of elastin fibers, endothelial dysfunction, and increased vascular matrix remodeling.5,6 Therefore, signaling pathways, which affect the compliance and contractility of the vessel wall, may be important contributors to the pathogenesis of vascular stiffening.

The Rho/Rho-associated coiled-coil containing kinases (ROCK1 and ROCK2) are important mediators of vascular function through their regulation of...
actin cytoskeleton, cellular contraction, and fibrosis. Clinically, we have shown that increased leukocyte ROCK activity is associated with advancing age, systolic hypertension, and increased carotid-femoral pulse wave velocity (PWV). Recently, we identified eukaryotic elongation factor 1-alpha 1 (eEF1A1), a regulator of cellular protrusion and profibrotic gene expression, suggesting ROCK2 is a critical mediator of age-associated vascular remodeling.

CLINICAL PERSPECTIVE

What Is New?
• Aortic Rho/Rho-associated coiled-coil containing kinases (ROCK) activity increases with vascular age.
• ROCK is an important mediator of aortic stiffness in aging mice.
• Loss of Rock2 attenuates hypertension, aortic stiffening, vascular remodeling, and fibrosis in a pharmacologic model of aortic stiffening.

What Are the Clinical Implications?
• Inhibition of ROCK, especially ROCK2, may have therapeutic benefits in preventing age-related aortic stiffening.
• ROCK2 mediates smooth muscle cell proliferation and profibrotic gene expression, suggesting ROCK2 is a critical mediator of age-associated vascular remodeling.

Nonstandard Abbreviations and Acronyms

| Abbreviation | Definition |
|--------------|------------|
| Ang II       | angiotensin II |
| CTGF         | connective tissue growth factor |
| eEF1A1       | eukaryotic elongation factor 1-alpha 1 |
| eNOS         | endothelial nitric oxide synthase |
| L-NAME       | L-Nω-nitroarginine methyl ester |
| MEF          | mouse embryonic fibroblasts |
| MLC          | myosin light chain |
| PWV          | pulse wave velocity |
| ROCK1        | Rho/Rho-associated coiled-coil containing kinase 1 |
| ROCK2        | Rho/Rho-associated coiled-coil containing kinase 2 |
| SMC          | smooth muscle cells |
| TGF-β1       | transforming growth factor β1 |
| VSMC         | vascular smooth muscle cells |
| WT           | wild-type |

neointima formation and regulation of transforming growth factor-β1 (TGF-β1), connective tissue growth factor (CTGF), and procollagen expression. These findings suggest that ROCK could be a critical mediator of the mechanical properties of the vascular wall.

Previous studies with ROCK inhibitors are limited by their relative lack of specificity for ROCK as opposed to other serine/threonine protein kinases such as PKA, protein kinase C, and citron kinase when given in vivo, and are unable to distinguish between the actions of specific ROCK isoforms. Hence, a genetic approach using haploinsufficiency of specific ROCK isoforms is needed to determine the role of ROCK isoforms in vascular diseases. In this study, we used hemizygous Rock1−/+− and Rock2−/+− mice to investigate the role of ROCK isoforms in the pathogenesis of vascular stiffening.

METHODS

The data, analytic methods, and study materials are available upon request.

Animals

The hemizygous Rock mice, Rock1−/+− and Rock2−/+− mice, were generated as described. Wild-type (WT) littermates were used as the controls for Rock1−/+− or Rock2−/+− mice. All mice are congenic strains on a C57Bl/6J background. Mice were housed on a 12-hours light/dark cycle in a specific pathogen-free facility maintained by the University of Chicago Animal Resources Center. Standard laboratory diet and drinking water were supplied ad libitum. For all surgical procedures, animals were anesthetized with 2% isoflurane and rectal temperature was monitored and maintained at 37 °C ±0.5 °C. The level of anesthesia was monitored with pedal reflex. The analgesic buprenorphine (0.1–0.2 mg/kg) was administered subcutaneously at the beginning of surgery and once every 12 hours for 4 days or until animals are observed to be free of signs of pain. All animals were euthanized by carbon dioxide (CO2) asphyxiation. A gradual fill method was used with a displacement rate of 10% to 30% of the chamber volume/minute (3 L/min). CO2 flow was continued for at least 1 minute after respiratory arrest. Cervical dislocation, a secondary euthanasia method was used for all the animal procedures. All animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Chicago and conform to National Institutes of Health guidelines.

Western Blotting

Protein was extracted from tissues or cells as previously described. The same amount of extracted protein was loaded on SDS-PAGE gel and transferred to polyvinylidene difluoride membranes (Immobilon-P;
and at 4 weeks after treatment. Measurements of aortic stiffness were performed at baseline and blood pressure (BP) were performed at baseline. The duration of treatment was 4 weeks. Measurements of aortic stiffness were performed at baseline and blood pressure (BP) were performed at baseline. A 1.0-cm vertical mid-scapular skin incision was made, followed by a creation of a 3.5-cm deep subcutaneous pocket. The osmotic mini-pump was inserted into the pocket. Skin closure was performed using 4-0 silk sutures, and mice were allowed to recover on a heating plate at 37 °C. The L-Nω-nitroarginine methyl ester (L-NAME, 0.5 g/L, N5751; Sigma-Aldrich) was supplied in the drinking water. The duration of treatment was 4 weeks. Measurements of aortic stiffness and blood pressure (BP) were performed at baseline and at 4 weeks after treatment.

**RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction**

Total RNA was extracted from tissues or cells, using the PureLink RNA Mini Kit (Invitrogen), according to the manufacturer’s instructions. Total RNA was converted to cDNA using the iScript Reverse Transcription Supermix Kit (Bio-Rad). The PowerUp SYBR Green Master Mix (Applied Biosystems) was used to perform amplifications on the StepOnePlus Real-Time PCR System (Applied Biosystems). The Ct value calculated by the StepOne Software version 2.3 (Applied Biosystems) The Ct value calculated by the StepOne Software version 2.3 (Applied Biosystems) for all samples was normalized to the housekeeping gene, Hprt. The relative fold change was computed by the ΔΔCt method. The primers used in this study are listed in Table S2.

**Ang II/L-NAME Administration**

Twelve-week-old male WT, Rock1+/−, and Rock2+/− mice were implanted with Alzet osmotic mini-pump (Model 1004) containing saline or angiotensin II (Ang II) (500 ng/kg per minute, A9525; Sigma-Aldrich). Mice were anesthetized with 2% isoflurane and analgesic buprenorphine (0.1–0.2 mg/kg) was administered subcutaneously before implantation of the osmotic mini-pump. A 1.0-cm vertical mid-scapular skin incision was made, followed by a creation of a 3.5-cm deep subcutaneous pocket. The osmotic mini-pump was inserted into the pocket. Skin closure was performed using 4-0 silk sutures, and mice were allowed to recover on a heating plate at 37 °C. The L-Nω-nitroarginine methyl ester (L-NAME, 0.5 g/L, N5751; Sigma-Aldrich) was supplied in the drinking water. The duration of treatment was 4 weeks. Measurements of aortic stiffness and blood pressure (BP) were performed at baseline and at 4 weeks after treatment.

**Measurements of PWV, Blood Pressure, and Hemodynamic Parameters in Mice**

Mice were anesthetized with 2% isoflurane on a heating (37 °C) board with integrated ECG electrodes. The pulse waveforms in the aortic root and abdominal aorta were obtained by pulse wave Doppler ultrasonography (Vevo 2100 imaging system, VisualSonics) and synchronized with ECG monitoring. The pulse wave, aortic dimensions in systole and diastole were measured by 38-MHz transducer. The PWV was calculated by dividing the distance by the difference of transit time between aortic root and abdominal aorta. Invasive blood pressure was measured using a Millar pressure catheter (1.4F, model SPR-671 recorded on MPVS-300 System) inserted into the aortic root of mice under 2% isoflurane anesthesia. In Ang II/L-NAME treated mice, a second pressure transducer was inserted via the right femoral artery and advanced into the thoracic aorta. The pullback procedure was performed to simultaneously measure PWV, determine the transit distance, and correct for differences in frequency response and amplifier delays between the 2 pressure transducers. The pressure waveforms in the aorta were recorded and analyzed to obtain systolic, diastolic, and mean aortic blood pressure (systolic BP, diastolic BP, and mean BP, respectively). These parameters were used to calculate pulse pressure, the change in pressure from augmentation point to peak systolic pressure (ΔP), and the augmentation index (ΔP/pulse pressure)x100. The aortic systolic and diastolic internal and outer diameter were determined by ultrasonography, and the corresponding lumen area and wall thickness were calculated. Using pulse wave Doppler ultrasonography, the flow velocity waveform and diameter at the left ventricular outflow tract (LVOT) and aortic root were measured. The LVOT area was calculated by measuring the average diameter, assuming a circular orifice. The LVOT flow velocity waveform was multiplied by LVOT area to derive the flow volume in the LVOT, which is equivalent to flow volume in the aorta.

**Histological Analysis**

Mice were anesthetized with 2% isoflurane and euthanized by CO2 inhalation. At the time of euthanizing, the left ventricle of euthanized mouse was cannulated and perfused with PBS containing heparin. The aorta was then perfused and fixed with 4% paraformaldehyde in PBS under physiological pressure. Following removal of the aorta and further incubation in 4% paraformaldehyde in PBS for 6 hours, the aortic segments are embedded in optimal cutting temperature compound and frozen. Ten sequential cross-sections of 5-μm thickness at 3- to 4-mm intervals distal to the aortic valve were obtained for immunohistochemical and morphometric analysis. Fresh frozen sections were cut on a freezing microtome (Microm; Walldorf). Slides were stained with hematoxylin and eosin, elastin, picrosirus red, and cross-sectional images were analyzed using an image analysis system (Multiscan; Fullerton). The identity of each sample was...
coded to allow data to be analyzed in a "blinded" manner. Measurements of vessel thickness and medial area were determined on cross-sections of aortas on the same slide used for morphometric analysis. Wall thickness was determined in pressure-perfusion fixed aortas (at mean BP of 100 mm Hg). Medial area was determined by elastin stain and defined as the area between the internal and external elastic lamina. The medial areas were measured using National Institutes of Health Image software and the average medial area was calculated. To correct for vessel deformation and off-transverse sectioning, the areas were determined by measuring the circumference of the vessel lumen and calculating the area as a generalized circle based on the measured circumference. For morphometric analysis, sections were stained with picosirius red (24901; Polysciences Inc.) for collagen, and elastin stain for elastin (HT25; Sigma-Aldrich). The areas of collagen and elastin staining were then standardized to medial area or wall thickness.

### Measurement of Hydroxyproline and Desmososine Content

The entire aorta was harvested and snap freezing in liquid nitrogen. The aortic tissues were hydrolyzed in 6 N HCl at 100 °C for 24 hours as described. The amount of cross-linked collagen was quantitated by measuring the amount of hydroxyproline in aortic lysates as described. The amount of desmosine and isodesmosine, which forms cross-links with elastin, was determined by high performance liquid chromatography/mass spectrometry as described. The amount of collagen and elastin were standardized to vessel length (mm). The ratio of hydroxyproline to desmosine and isodesmosine was taken as an index of vascular remodeling.

### Isolation of Mouse Primary Aortic Smooth Muscle Cells

Primary aortic smooth muscle cells (SMC) were isolated from 8- to 10-week-old mice. The thoracic aortas were dissected from the arch to just above the diaphragm. The adventitia and the surrounding adipose tissue were carefully removed. Aorta was cut longitudinally and the endothelial layer was gently removed by sterile cotton swab. The luminal side of aorta was attached to gelatin-coated 35-mm culture dish with growth medium. The dishes were placed in 37 °C and 5% CO₂ incubator and left undisturbed for 5 days, allowing the aortic SMC to migrate and proliferate from the aorta. The growth media containing DMEM/F12 (11320033), 20% fetal bovine serum (FBS), Smooth Muscle Growth Supplement (S00725), 1% GlutaMAX (35050061) and 1% Antibiotic-Antimycotic (15240062) were used for the SMC culture. All the cell culture reagents listed above were purchased from Thermo Fisher Scientific and were used according to the manufacturer’s instructions. Primary SMC between passages 3 to 6 were used for all experiments. The mRNA levels of SMC differentiation markers (Myh11 and Smtn) and stem cell markers (S100b and Sca1) were significantly lower in SMC compared with aortas, suggesting SMC exhibit proliferative phenotype (data not shown). For in vitro studies, SMC were serum (1%)-starved overnight and then treated with or without 20 μmol/L ROCK inhibitor Y-27632 (688001; Millipore) for 1 hour before stimulation with 1 μmol/L Ang II (A9525; Sigma-Aldrich) for 24 hours.

### Transfection of Small Interfering RNA

Control small interfering RNA (siRNA) (sc-37007), Rock1 siRNA (sc-36432), and Rock2 siRNA (sc-36432) were purchased from Santa Cruz Biotechnology. The siRNAs were diluted to 20 nmol/L in Opti-MEM (31985070; Thermo Fisher) and mixed with Lipofectamine RNAiMax (13778075; Thermo Fisher) at room temperature for 20 minutes. Mouse primary aortic SMC were seeded at subconfluent densities in collagen type I-coated 6-well plates or 60-mm culture dishes in growth media without antibiotics. The siRNA-Lipofectamine mixtures were added to SMC and incubated for 48 hours.

### SMC Proliferation and Protein Synthesis Assay

Mouse aortic SMC in logarithmic growth phase were harvested and plated at 1.5×10⁵ cells/cm² and cultured overnight in DMEM with 10% FBS. SMC were transfected the following day with control vector, WT eEF1A1 cDNA vector or the mutant eEF1A1 (ΔT432A) cDNA vector, which cannot be phosphorylated by ROCK2. After transfection for 48 hours, SMC were synchronized to G0 with serum-starvation for 16 hours. SMC proliferation was assessed by cell count and [³H]-thymidine uptake and protein synthesis by [³H]-leucine (New England Nuclear-Life Science Products) uptake in response to 10% FBS. SMC were treated with the indicated conditions in the presence of diluents, [³H]-thymidine or [³H]-leucine (1 μCi/mL) for 24 hours. After incubating at room temperature for 45 minutes, cellular proteins were precipitated with 5% trichloroacetic acid, resuspended in 0.4 N NaOH, and the radioactivity was counted in a scintillation counter (Beckman LS 6000IC). Transfection efficiency was 30% to 35% and standardized to transfection with β-gal cDNA. The results were expressed as counts per minute (cpm) per microgram protein.

### Isolation and Immortalization of Mouse Embryonic Fibroblasts

Primary mouse embryonic fibroblasts (MEF) were cultured from 13-day-old embryos derived from WT,
Rock1−/−, and Rock2−/− mice. The embryos were separated from their yolk sac and placed in the complete growth medium, DMEM with 10% FBS, 1% penicillin and streptomycin, and 1% GlutaMAX (Invitrogen). After the embryonic head and innards were removed, the trunk was homogenized and trypsinized at 37 °C for 45 minutes. After neutralizing the trypsin with the complete growth medium, the MEF were isolated from the remaining embryonic body by repeated pipetting and cultured until confluent. After the third passage, the MEF from each strain were immortalized by infection with a retrovirus vector expressing SV40 large T antigen, using neomycin selection for 2 weeks. MEF were cultured in gelatin-coated dishes. After serum starvation overnight, MEF were cotreated with or without ROCK inhibitor Y-27632 (20 μmol/L; 688001, Millipore) and stimulated for 24 hours with TGF-β1 (10 ng/mL; 100-21C, PeproTech).

Statistical Analysis
All results are expressed as the mean±SEM. Comparisons of parameters were performed with 1-way ANOVA followed by post-hoc Tukey Honest Significant Difference test for multiple comparisons. Statistical significance was evaluated with GraphPad Prism 7 (GraphPad Software) and JMP 7 (SAS Institute Inc.). A P value of <0.05 was considered to be statistically significant.

RESULTS

Effects of Age on ROCK Activity
Age is associated with increased aortic stiffening and systolic hypertension.22 To determine whether increased ROCK expression and activity are associated with aging in mice, we measured ROCK activity and expression in the aortas of 4-, 11-, and 20-month-old male WT C57BL/6J mice. Compared with 4-month-old mice, 11- and 20-month-old mice exhibited increased aortic ROCK activity (P<0.001 for both, n=6 in each group) as determined by phosphorylation of myosin-binding subunit of myosin light chain (MLC) phosphatase (Figure 1A and 1B). Interestingly, there was no increase in ROCK1 or ROCK2 expression in aortas of 11- and 20-month-old mice (Figure 1C). These findings suggest that aging may lead to conditions, which increases ROCK activity but not ROCK expression in the aorta.

Effects of ROCK Isoforms on Age-Associated Changes in Endothelial Nitric Oxide Synthase and Extracellular Matrix
We next determine the effects of ROCK isoforms on age-associated changes in endothelial nitric oxide synthase (eNOS) expression and extracellular matrix in aortas of 4 and 12-month-old male WT littermate, and hemizygous Rock1+/− and Rock2+/− mice. The expression of eNOS was similar between 4-month-old WT, Rock1+/− and Rock2+/− mice (Figure 1F). However, compared with 4-month-old mice, eNOS expression was decreased by 64% and 60% in 12-month-old WT and Rock1+/− mice (P<0.001 for both, n=5 in each group), respectively, whereas there were no changes in eNOS expression between 4 and 12-month-old Rock2+/− mice (P>0.05, n=5 in each group). These findings suggest that Rock2 mediates the downregulation of eNOS expression during vascular aging.

In 4-month-old male mice, the hydroxyproline and desmosine content in the thoracic aortas were similar between WT, Rock1+/− and Rock2+/− mice (Figures 1G and 1H). However, in 12-month-old mice, the hydroxyproline...
content was increased in aortas of WT and Rock1+/− mice, but not in the aortas of Rock2+/− mice (Figure 1G). In contrast, the reduced aortic desmosine or elastin content in 12-month-old WT mice was not observed in aortas of corresponding age Rock1+/− and Rock2+/− mice (Figure 1H). Consequently, the increased ratio of collagen to elastin in 12-month-old WT and Rock1+/− aortas was substantially attenuated in 12-month-old Rock2+/− aortas (Figure 1I). These findings indicate that Rock2 mediates age-associated aortic fibrosis, which could account for the increased pulse pressure and stiffness with age.

**ROCK Isoforms Mediate Pharmacologically-Induced Vascular Stiffening**

To determine the hemodynamic and histological effects of Rock1 and Rock2 on age-associated aortic stiffening, we used a pharmacologically-induced model of vascular stiffening to induce age-like vascular changes in mice. Accordingly, 12-week-old male WT, Rock1+/− and Rock2+/− mice were treated with Ang II (500 ng/kg per minute) and L-NAME (0.5 g/L) for 4 weeks. With saline and Ang II/L-NAME treatment, body weights...
were not different between the groups of mice (Table). However, treatment with Ang II/L-NAME increased the heart weights as well as the heart weight to body weight ratios of WT and Rock1+/− mice to a greater extent compared with that of Rock2+/− mice. These findings indicate that treatment with Ang II/L-NAME increased myocardial hypertrophy in WT and Rock1+/− mice, but to a lesser extent, in Rock2+/− mice. Indeed, systolic BP and mean BP were substantially increased with Ang II/L-NAME treatment in WT and Rock1+/− mice, and to a much lesser extent, in Rock2+/− mice (Table).

The pressure waveforms of invasive blood pressure monitoring showed that the increases in pulse pressure, ΔP, and augmentation index following Ang II/L-NAME treatment in WT mice were attenuated in Rock1+/− mice, and more substantially reduced in Rock2+/− mice (Figure 2A through 2C). This correlated with greater distensibility as measured by changes in the difference between systolic and diastolic internal diameters in the aortic root of Rock2+/− mice compared with that of WT and Rock1+/− mice (Figure 2D through 2F). Compared with control (saline), aortic wall thickness in WT and Rock1+/− mice was also increased with treatment of Ang II/L-NAME, but this thickening of the aortic wall was absent in Rock2+/− mice (Figure 2G). These findings suggest greater distensibility or elasticity of aortas from Rock2+/− mice compared with that of WT and Rock1+/− mice.

Using ultrasonography and pulse wave Doppler to measure the diameter of left ventricular outflow tract (LVOT) and the peak and mean velocities through LVOT, we calculated the peak and mean flow in LVOT. The diameter of LVOT for all groups were similar and did not change with Ang II/L-NAME treatment (Table). However, peak LVOT velocities were higher in Rock2+/− mice, both with saline and Ang II/L-NAME treatment, suggesting higher flow across LVOT. Indeed, treatment with Ang II/L-NAME decreased peak and mean flow in WT mice, but had little or no effect on these parameters in Rock1+/− and Rock2+/− mice. This corresponded to a smaller and no increase in PWV in Ang II/L-NAME-treated Rock1+/− and Rock2+/− mice, respectively, compared with that of WT mice (PWV: WT, 4.93±0.06 m/s; Rock1+/−, 4.16±0.10 m/s; Rock2+/−, 3.25±0.03 m/s, P<0.001 compared with WT mice, Figure 2H). These results suggest that Rock1, and to a greater extent Rock2, plays an important role in regulating aortic distensibility and flow.

### Effects of ROCK on Aortic Hypertrophy and Vascular Remodeling

Next, we examined the morphology and extracellular matrix of aortas from WT and Rock mutant mice. Using hematoxylin and eosin staining, we found that treatment with Ang II/L-NAME increased the medial area of the aortic wall, as determined by the area between the internal and external elastic lamina. This increase in medial area was smaller in Rock2+/− mice compared with that of WT mice (Figure 3A and 3B). Indeed, treatment with Ang II/L-NAME increased aortic wall thickness by ultrasonography in WT mice, which was attenuated...
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in Rock1+/− mice, and to a greater extent, in Rock2+/− mice (Table). This attenuation in aortic wall and medial thickness was associated with less collagen fiber deposition (Figure 3C) and greater elastic fiber preservation in Rock2+/− mice compared with that of WT mice (Figure 3D). This correlated with decreased amount of hydroxyproline, an indicator of collagen fiber content, in the aortas of Rock2+/− mice compared with that of WT mice (Figure 3E). Furthermore, the level of desmosine and isodesmosine, which cross-links with elastin, was higher in aortas from Rock2+/− mice compared with that of WT mice after Ang II/L-NAME treatment (Figure 3F). Using the ratio of collagen to elastin as an index of extracellular matrix remodeling of the aorta, we found that WT mice have higher remodeling compared with that of Rock1+/− and Rock2+/− mice following Ang II/L-NAME treatment (Figure 3G). These results indicate that Rock1, and to a greater extent Rock2, are critical mediators of wall thickening and extracellular matrix remodeling of the aortic wall that could lead to aortic stiffening.

**Figure 2.** Hemodynamic parameters in Angiotensin II/L-Nω-nitroarginine methyl ester-treated hemizygous Rock mice.
Twelve-week-old, male wild-type littermates, Rock1+/−, and Rock2+/− mice were treated with angiotensin II (Ang II; 500 ng/kg per minute) and L-Nω-nitroarginine methyl ester (L-NAME, 0.5 g/L, drinking water) for 4 weeks. A, Pulse pressure, B, ΔP, augmentation point to peak systolic pressure, and C, augmentation index were obtained by pressure waveforms from pressure transducers in the aorta. D, Systolic internal diameter, E, diastolic internal diameter, F, change in the systolic and diastolic internal diameters of the aortic root, and G, aortic wall thickness were determined by ultrasonography. H, Pulse wave velocity was determined by pressure waveforms in aortic root and abdominal aorta at the level of renal artery. Saline group: wild-type (n=4), Rock1+/− (n=3), and Rock2+/− (n=3); Ang II/L-Nω-nitroarginine methyl ester group: wild-type (n=5), Rock1+/− (n=3), and Rock2+/− (n=4). Results are expressed as mean±SEM. P values were calculated using 1-way ANOVA with Tukey Honest Significant Difference test. Ang II indicates angiotensin II; PP, pulse pressure; ΔP, augmentation point to peak systolic pressure; Rock, Rho/Rho-associated coiled-coil containing kinase; and WT, wild-type.

**ROCK2 Mediates SMC Proliferation Through eEF1A1 Phosphorylation**
To determine the mechanism by which ROCKs regulate vascular remodeling and stiffness, we hypothesized that phosphorylation of eEF1A1 by ROCK2 could be involved in aortic SMC proliferation and...
hypertrophy. Previously, we have shown that ROCK2 can phosphorylate eEF1A1 at Thr^{432} and eEF1A1 has been shown to play an important role in regulating the actin cytoskeleton. Using an antibody against phospho-Thr^{432} of eEF1A1, we found that treatment with the ROCK inhibitor, Y-27632, decreased eEF1A1 phosphorylation in primary aortic SMC (Figure 4A).

Compared with SMC treated with control or Rock1 siRNA, eEF1A1 Thr^{432} phosphorylation was reduced in SMC treated with Rock2 siRNA (Figure 4B).

To confirm that eEF1A1 Thr^{432} phosphorylation by ROCK2 contributes to ROCK2-mediated SMC proliferation and

Figure 3. Morphology of aortas from Rho/Rho-associated coiled-coil containing kinase (Rock)^{+/−} and Rock^{2+/−} mice with saline or Angiotensin II/L-N^ω-nitroarginine methyl ester treatment.

A. Hematoxylin and eosin staining of aortas after saline or angiotensin II (Ang II)/L-N^ω-nitroarginine methyl ester treatment for 4 weeks. The medial area is indicated by white dash lines. Scale bar represents 100 μm. B. Quantification of medial area from the aortic section of hematoxylin and eosin staining. C. Representative images of picrosirius red-stained aortic sections. Black arrowhead indicates collagen deposition. Scale bar represents 100 μm. D. Morphology of elastic fibers in elastin-stained aortic sections. White arrowhead indicates the distribution of elastic fibers. Scale bar represents 50 μm. Cross-linked (E) collagen and (F) elastin content in aortas were determined by measurement of hydroxyproline content and HPLC/MS detection of total desmosine (desmosine+isodesmosine). G. The ratio of collagen (hydroxyproline) to elastin (total desmosine) content in the aorta. Saline group: wild-type (n=4), Rock^{1+/−} (n=3), and Rock^{2+/−} (n=3). Ang II/ L-N^ω-nitroarginine methyl ester group: wild-type (n=5), Rock^{1+/−} (n=3), and Rock^{2+/−} (n=4). Results are expressed as mean±SEM. P values were calculated using 1-way ANOVA with Tukey Honest Significant Difference test. Ang II indicates angiotensin II; L-NAME, L-N^ω-nitroarginine methyl ester; Rock, Rho/Rho-associated coiled-coil containing kinase; and WT indicates wild-type.
hypertrophy, we made a mutant eEF1A1 (ΔT432A), which cannot be phosphorylated by ROCK2. Compared with control vector, overexpression of WT eEF1A1 in SMC led to increased cell proliferation and protein synthesis, while overexpression of mutant eEF1A1 (ΔT432A) had no effect on SMC proliferation (Figure 4C through 4E). These results suggest that phosphorylation of Thr432 of eEF1A1 by ROCK2 mediates SMC proliferation and hypertrophy, which could contribute to medial SMC proliferation and wall thickening in the aorta.

**ROCK2 Mediates Profibrotic Gene Expression**

To determine the role of ROCK isoforms in Ang II-induced extracellular matrix remodeling, we first examined the expression of profibrotic genes in Ang II-stimulated primary aortic SMC with and without the ROCK inhibitor, Y-27632. Treatment with Ang II increased ROCK activity and upregulated TGF-β1 and collagen I protein and mRNA expression in SMC, which were substantially attenuated by Y-27632 (Figure 5A and 5B). Selective knockdown of Rock1 or Rock2 in SMC led to a comparable reduction in Ang II-induced TGF-β1 and collagen I protein expression (Figure 5C). Similarly, using MEF that were isolated from WT, Rock1−/− and Rock2−/− embryos, we found that the expression of collagen I, and the downstream effector of TGF-β1, CTGF, were both reduced in response to TGF-β1 stimulation (Figure 5D). These findings suggest that ROCK inhibition, and in particular, ROCK2 inhibition attenuates Ang II-induced TGF-β1 signaling and fibrosis, in part, through downregulation of TGF-β1 expression and its downstream profibrotic effectors, CTGF, and collagen I.
DISCUSSION

We have found that aortic ROCK activity increases with age and haploinsufficiency of Rock1, and to a greater extent, Rock2, confers protection against age-associated aortic stiffening. Using a pharmacologically-induced vascular stiffening model with Ang II/L-NAME, we found that hypertension, myocardial hypertrophy, aortic stiffening, and vascular remodeling were also reduced in hemizygous Rock mice, especially in Rock2+/− mice. The attenuation in aortic stiffening in Rock2+/− mice was accompanied by reduced vascular wall thickness, greater distensibility, less collagen deposition, and more preserved elastin content. In vitro, we showed that the phosphorylation of eEF1A1 by ROCK2 contributes to SMC proliferation and hypertrophy, which could account for the medial thickening of the aorta in older mice. Furthermore, the loss of Rock2 led to decreased profibrotic gene expression. These findings indicate that ROCK2 is an important mediator of vascular remodeling and fibrosis, and suggest that ROCK could be a therapeutic target for age-associated aortic wall remodeling and stiffening.

Aortic stiffness is associated with impaired endothelial function and increased vascular tone. An essential mediator of endothelial function and vasodilation is eNOS. The contribution of endothelial dysfunction in aortic stiffness is supported by higher blood pressure and aortic stiffness in eNOS knockout mice. In our pharmacologically-induced vascular stiffening model, Ang II/L-NAME increased ROCK activity, and increased ROCK activity has been shown to downregulate eNOS, impair endothelial function, and promote vascular remodeling. The accelerated aortic stiffening model required the treatment of L-NAME, further highlighting the involvement of eNOS in the vascular stiffening process. Indeed, ROCK2 downregulates eNOS expression via phosphorylation of eEF1A1, which directly binds to eNOS mRNA and limits its mRNA stability. The impaired endothelium-dependent dilation in aging cerebral arteries was reversed by selective sROCK2 inhibitor SLX-2119. Consistent with these previous studies, we also found that eNOS mRNA level was decreased in the aortas of older WT and Rock1+/− mice but not in older Rock2+/− mice compared with that of younger corresponding mice. These findings suggest that ROCK2-mediated downregulation of eNOS and endothelial dysfunction may be one of the mechanisms by which ROCK2 mediates vascular stiffening and hypertension. Further studies using endothelial-specific ROCK isoform deletion in mice would be helpful in determining the mechanisms of endothelial ROCK2 in vascular stiffening.

Vascular aging is associated with enhanced contractile response to vasoconstrictors and increased myogenic tone. ROCK could increase blood pressure through their direct effects on SMC contraction via changes in actin cytoskeletal dynamics. ROCK phosphorylates myosin-binding subunit of MLC phosphatase and inhibits MLC phosphatase activity, leading to increased MLC phosphorylation, actomyosin interaction, and contraction. The eEF1A mediates binding and cross-linking F-actin into non-contractile bundles. Domain III of eEF1A has been shown to cross-link actin filaments with a unique binding rule that excludes other proteins from cross-linking F-actin into contractile bundles, such as phosphorylated MLC. The binding of eEF1A to F-actin could negatively affect cellular contractility and alter cell shape by preventing actin-myosin interaction. Interestingly, the unphosphorylated form of eEF1A has been shown to bind to and bundle F-actin, whereas phosphorylated eEF1A at Thr432 by ROCK is unable to bind F-actin. Thus, by phosphorylating eEF1A and preventing its interaction with F-actin, ROCK could induce SMC contraction by permitting the available F-actin to cross-link with phosphorylated MLC. Although both ROCK1 and ROCK2 can regulate MLC phosphatase, the ROCK2-eEF1A interaction may be more important for vascular contraction, which is reflected by the finding that knockdown of ROCK2, but not ROCK1, reduces the contractility in rat vascular smooth muscle cells (VSMC). Moreover, the ROCK2-specific inhibitor, KD025, significantly inhibits the myogenic tone in mice at 2, 6 to 8, and 13 to 14 months of age via ROCK2-mediated Ca2+ sensitization. Thus, these findings highlight the distinct effects of ROCK2 in mediating vascular contraction as well as age-related hypertension.

Epidemiological studies have shown that premenopausal women are protected against arterial stiffening compared with age-matched men. Increased contractions to vasoconstrictors in aortas from male mice are attributable to higher in RhoA/ROCK activation in smooth muscles, independent of differences in the expression of RhoA or ROCK isoforms. Basal activity of RhoA but not ROCK is higher in aortas of male mice and there is no difference in levels of ROCK1 and ROCK2 in aortas from male and female mice. Interestingly, 17β-estradiol attenuates vascular contraction through inhibition of RhoA/ROCK activity. The inhibitory effect of estrogen on RhoA/ROCK activity may be attributable to the activation of G protein-coupled estrogen receptors, leading to the downstream Epac/Rap1- and PKA-mediated RhoA/ROCK inhibition. Our findings indicate that the PWV was lower in old female WT mice than in age-matched male mice. The haploinsufficiency of ROCK isoforms seems to exhibit similar protective effects on age-related vascular stiffness in both male and female mice.

Vascular thickening and remodeling are predominant structural changes that occur in vascular aging. Upregulation of ROCK2, but not ROCK1, is found in the media of pulmonary arteries from patients with idiopathic
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Figure 5. Regulation of transforming growth factor-β1 and collagen I expression by Rho/Rho-associated coiled-coil containing kinase (ROCK) isoforms. A. Protein and (B) mRNA levels of transforming growth factor-β1 and collagen I in wild-type primary aortic smooth muscle cells (SMC) treated with Ang II (10 μmol/L) or Y-27632 (20 μmol/L) for 24 hours. n=4 cell isolates in each group. C. Representative immunoblots and densitometric quantification of transforming growth factor-β1 and collagen I in wild-type SMC treated with control (scrambled), Rock1 or Rock2 small interfering RNA. After transfection of small interfering RNA for 48 hours, SMC were stimulated with or without Ang II (10 μmol/L) for 24 hours, n=4 cell isolates in each group. D. Representative immunoblots and densitometric quantification of connective tissue growth factor and collagen I in wild-type, Rock1−/− or Rock2−/− mouse embryonic fibroblasts treated with transforming growth factor-β1 (10 ng/mL) for 24 hours; n=4 cell isolates in each group. Results are expressed as mean±SEM. P values were calculated using 1-way ANOVA with Tukey Honest Significant Difference test. Ang II indicates angiotensin II; CTGF, connective tissue growth factor; p-MBS indicates phosphorylated myosin-binding subunit; ROCK/Rock, Rho/Rho-associated coiled-coil containing kinase; siRNA, small interfering RNA; TGF, transforming growth factor; t-MBS, total myosin-binding subunit; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Hprt, hypoxanthine phosphoribosyltransferase; Col1α1, collagen type I alpha 1 chain; and WT, wild-type.

pulmonary arterial hypertension.37 The hypoxia-induced VSMC proliferation and pulmonary arterial thickening are reduced in VSMC-specific Rock2−/− mice, whereas the overexpression of ROCK2 in VSMC enhances both features of pulmonary arteries.37 The arterial SMC from patients with pulmonary hypertension further confirms that ROCK2 is required for ERK1/2-mediated cell proliferation and vascular remodeling in pulmonary arteries.37 These results support the notion that ROCK2 in VSMC regulates vascular remodeling through ERK-mediated proliferative signal, which may be a similar mechanism to ROCK2-mediated eEF1A1 phosphorylation-induced SMC proliferation in the present study.

We found a relatively intact aortic wall structure and relative maintenance of elastic fibers in Rock2−/− aortas after Ang II/L-NAME treatment. These observations suggest that Ang II/L-NAME-induced elastolysis may be attenuated by ROCK2 deficiency, suggesting ROCK2 could increase the activity of elastin-degrading enzymes in the vasculature. Indeed, pharmacological inhibition of ROCK decreases aortic elastin fragmentation, matrix metalloproteinase-2 activity, and vascular inflammation in Ang II-induced aneurysm animal models.38,39 Our previous study also reported that leukocyte/macrophage ROCK1 is a mediator of vascular inflammation, neointima formation, and atherosclerosis.14,40 However, the mechanisms by which ROCK regulates the production of elastolytic enzymes in vascular or inflammatory cells remains to be determined.
Although previous studies show that ROCK1 deletion mediates cardiovascular remodeling and fibrosis through transcriptional regulation of TGF-β1 and its downstream profibrotic genes,14,15 the specific role of ROCK2 in vascular fibrosis is not entirely known. ROCK2, but not ROCK1, have been shown to mediate kidney fibrosis through TGF-β1-induced expression of CTGF and profibrotic genes via the proinflammatory transcription factor, nuclear factor-kB, in mesangial cells.41 This observation agrees with our previous findings about the profibrotic effect of ROCK2 in cardiac fibroblasts.16 The expression of ROCK2, but not ROCK1, is increased in activated cardiac fibroblasts after Ang II treatment and ROCK2 deletion in cardiac fibroblasts leads to a reduction in Ang II-induced cardiac fibrosis, as well as decreases in collagen and CTGF expression. Similarly, in this study, we showed that ROCK2 affects vascular stiffening and remodeling, in part, through TGF-β1 signaling pathways that control the expression of CTGF and collagen. Moreover, since the levels of Ang II12 and TGF-β143 are elevated in circulation of aging animals and humans, these 2 factors could act as the inducers of vascular stiffness, or even the ROCK2 activators, which further highlight the importance of ROCK2 inhibition in alleviation of aging-related vascular stiffness.

In summary, using Rock1 and Rock2 haploinsufficient mice, we have shown that Rock2 is the predominant mediator of vascular remodeling and stiffening that occurs with aging. Our findings suggest that ROCK2 may be a potential therapeutic target for the prevention of age-associated vascular disease.

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Dr Liao serves as a consultant for Esperion. The remaining authors have no disclosures to report.

**Supplementary Material**

Tables S1–S2

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SUPPLEMENTAL MATERIAL
### Table S1. List of primary antibodies

| Target antigen                     | Vendor or Source     | Catalog # | Working concentration       |
|------------------------------------|---------------------|-----------|-----------------------------|
| ROCK1                              | BD Biosciences      | 611136    | 1:1000 (250 ng/ml)          |
| ROCK2                              | BD Biosciences      | 610623    | 1:1000 (250 ng/ml)          |
| Phos-MYPT1 (p-MBS, Thr850)         | Millipore           | 36-003    | 1:1000 (1000 ng/ml)         |
| Myosin Phosphatase (t-MBS)         | Biolegend           | 925101    | 1:1000 (N/A)                |
| beta-actin                         | Proteintech         | 66009     | 1:5000 (86 ng/ml)           |
| Phospho-eEF1A1                     | Dr. James K. Liao   | N/A       | 1:1000 (N/A)                |
| EF-1 α/2                           | Santa Cruz          | 377439    | 1:1000 (200 ng/ml)          |
| GAPDH                              | Cell Signaling Technology | 2118 | 1:5000 (N/A) |
| TGFb1                              | Santa Cruz          | sc-146    | 1:1000 (100 ng/ml)          |
| COL1A1                             | Santa Cruz          | sc-8784   | 1:1000 (100 ng/ml)          |
| CTGF                               | Santa Cruz          | 365970    | 1:1000 (200 ng/ml)          |

### Table S2. List of primers used in quantitative real-time PCR

| Gene name | Forward Primer (5’-3’) | Reverse Primer (5’-3’) |
|-----------|------------------------|------------------------|
| Tgfb1     | GAAGCGGACTACTATGCTAAA  | CCCGAATGTCTGACGTATTG   |
| Col1a1    | CAATGGTGCTCCTGGTATTG   | CACCAGTGTCCTCTTTGTGG   |
| Rock1     | GGAGATGTGTACAGAGCAGAAA | GAAAGTGTTAGAGGTTAGGAATG |
| Rock2     | ACAGATGAAAGCGGAAGACTATG | TGAACATCCAGGGACTGTTC |
| Nos3      | GACAGACTACACGACATTGAG  | ATGGTCCAGTTGGGAGCATC   |
| Hprt      | AGTGTGGGATACAGGCCAGAC  | CGTGATTCAATCCCTGAAGT   |