

Published in final edited form as:

Circulation. 2005 November 8; 112(19): 2959–2965. doi:10.1161/CIRCULATIONAHA.105.584623.

Decreased Perivascular Fibrosis but Not Cardiac Hypertrophy in ROCK1^{+/-} Haploinsufficient Mice

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Abstract

Background—Rho GTPase and its downstream target, Rho-associated kinase (ROCK), have been implicated in diverse cardiovascular diseases such as cardiac hypertrophy. However, pharmacological inhibitors of ROCK are not entirely specific, nor can they discriminate between the ROCK isoforms ROCK1 and ROCK2. To determine the specific role of ROCK1 in the development of cardiac hypertrophy, we generated ROCK1^{+/-} haploinsufficient mice and determined whether cardiac hypertrophy and remodeling are decreased in these mice.

Methods and Results—Litters of ROCK1^{-/-} mice on C57Bl/6 background were markedly underrepresented, suggesting lethality in utero or postnatally. ROCK1^{+/-} mice, however, are viable and fertile with no obvious phenotypic abnormalities. Basal blood pressure, heart rate, and cardiac dimension and function in ROCK1^{+/-} mice were similar to those in wild-type (WT) littermates. Infusion of angiotensin II (400 ng · kg⁻¹ · min⁻¹ for 28 days) or treatment with N^G-nitro-L-arginine methyl ester (1 mg/mL in drinking water for 28 days) caused similar increases in systolic blood pressure, left ventricular wall thickness, left ventricular mass, ratio of heart weight to tibial length, and cardiomyocyte size in ROCK1^{+/-} mice and WT littermates. In contrast, perivascular fibrosis in hearts was increased to a lesser extent in ROCK1^{+/-} mice compared with WT littermates. This was associated with decreased expression of transforming growth factor-β, connective tissue growth factor, and type III collagen. In addition, perivascular fibrosis induced by transaortic constriction or myocardial infarction was decreased in ROCK1^{+/-} mice compared with WT littermates.

Conclusions—These findings indicate ROCK1 is critical for the development of cardiac fibrosis, but not hypertrophy, in response to various pathological conditions and suggest that signaling pathways leading to the hypertrophic and profibrotic response of the heart are distinct.

Keywords

blood pressure; hypertension; hypertrophy; remodeling; angiotensin

Cardiac remodeling, which occurs in response to changes in mechanical and neurohormonal stimulation, is a complicated process that includes cardiomyocyte hypertrophy, inflammation, fibrosis, and remodeling. Cardiac hypertrophy, an adaptive response to hemodynamic

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Guest Editor for this article was Donald D. Heistad, MD.

Disclosure

Dr Liao has served as a consultant to Asahi-Kasei Pharmaceuticals.

overload, is important physiologically for maintaining normal cardiac function.¹ In some cases, however, the prolonged hypertrophic response becomes pathological and increases the risk of developing cardiac arrhythmias, diastolic dysfunction, congestive heart failure, and death.²⁻⁴ Numerous studies have implicated the involvement of diverse signaling molecules such as G proteins, protein kinase C, mitogen-activated protein kinases, phosphatidylinositol 3-kinase, protein kinase Akt, and calcineurin in the hypertrophic response.⁵ These pathways contribute to cardiomyocyte hypertrophy and the proliferation and survival of other cell types such as fibroblasts and smooth muscle cells.

Angiotensin II (Ang II) plays an important role in blood pressure regulation and hypertrophic response. Ang II induces cardiac hypertrophy not only through the indirect mechanism of blood pressure elevation but also through its direct effect on cardiomyocytes. These direct myocardial effects of Ang II involve activation of the Rho family of small GTPases, which includes RhoA and Rac1.^{6,7} RhoA regulates myofibrin organization, whereas Rac1 is important for generating oxidative stress through the activation of myocardial NAD(P)H oxidase.

Rho-associated kinase (ROCK) is a serine/threonine kinase that mediates some of the downstream signaling of RhoA.⁸ Currently, there are 2 isoforms of ROCK, ROCK1 and ROCK2. Pharmacological inhibition of ROCK suggests that ROCK plays an important role in the pathogenesis of diverse cardiovascular diseases such as cerebral and coronary vasospasm, hypertension, vascular inflammation, and ischemiareperfusion injury.⁹ For example, recent studies with the ROCK inhibitors fasudil and Y27632 suggest the involvement of ROCK in the development of cardiac hypertrophy in rats^{10,11} and mice.¹² In Ang II-infused rat model, oral administration of fasudil suppressed left ventricular (LV) hypertrophy and decreased perivascular fibrosis and endothelial superoxide anion production probably through the inhibition of NAD(P)H oxidase expression.¹⁰ Similarly, in Dahl salt-sensitive rats fed a high-salt diet, Y27632 inhibited the development of LV hypertrophy and improved cardiac contractile dysfunction.¹¹

Although these pharmacological studies have demonstrated the involvement of RhoA/ROCK signaling in cardiac hypertrophy, transgenic mice overexpressing RhoA in the heart do not develop cardiac hypertrophy. Furthermore, ROCK inhibitors are somewhat nonselective and may potentially inhibit other protein kinases such as protein kinase A and C, which also are critical to the development of cardiac hypertrophy. It is also impossible to determine the isoform-specific role of ROCK in cardiac hypertrophy with the use of these pharmacological inhibitors of ROCK because they are unable to discriminate between ROCK1 and ROCK2. Thus, a genetic approach with gene targeting of specific ROCK allele offers the best strategy for dissecting the isoform-specific role of ROCK. Accordingly, we generated haploinsufficient ROCK1-knockout (ROCK1^{+/-}) mice and tested the hypothesis that ROCK1 mediates cardiac hypertrophy and remodeling in response to Ang II.

Methods

Generation of ROCK1^{+/-} Mice

The conditional targeting vector was constructed to delete a genomic fragment containing exon 1b of the ROCK1 gene by homologous recombination (Figure 1A). Two loxP sites flanking exon 1b were introduced. The neomycin resistance gene (Neo) fused to the phosphoglycerokinase (pGK) promoter (pGK-Neo) was inserted between exon 1b and the 3' loxP site. The 5' homology arm and the 3' homology arm were inserted upstream of the 5' loxP site and downstream of 3' loxP site, respectively. The linearized targeting vector was injected into embryonic stem cells derived from C57Bl/6 mice. Neomycin-resistant clones were screened for homologous recombination by polymerase chain reaction (PCR) after identification by genomic Southern blot. The loxP-flanked exon 1b and pGK-Neo gene in

correctly targeted clones were deleted by transfection with Cre. The deletion of exon 1b introduced a frame shift and stop codon in exon 1c, preventing further translation of the functional protein. The correctly targeted embryonic stem cell clones were injected into C57Bl/6 blastocysts. Male heterozygous ROCK1^{+/-} mice were bred to C57Bl/6 females to obtain heterozygous pups. Male ROCK1^{+/-} mice and their counterparts 8 to 12 weeks old were used in this study. The genotypes of offspring were analyzed by Southern blot (Figure 1B). The mice were maintained in the Harvard Medical School animal facilities. The Standing Committee on Animals at Harvard Medical School approved all protocols pertaining to experimentation with animals.

Ang II Infusion

Ang II (400 ng · kg⁻¹ · min⁻¹) dissolved in saline or saline was infused with the use of mini-osmotic pumps (ALZET model 2004) as described previously.⁷ The pumps were subcutaneously implanted into male ROCK1^{+/-} mice or their wild-type (WT) littermates (8 weeks old).

N^G-Nitro-L-Arginine Methyl Ester Treatment

N^G-nitro-L-arginine methyl ester (L-NAME) was administered in drinking water (1 mg/mL) for 28 days to male ROCK1^{+/-} mice or their WT littermates.

Mouse Models of Pressure Overload and Myocardial Infarction

Pressure overload and myocardial infarction (MI) were studied in 8-to 12-week-old male mice. Transaortic constriction (TAC) was produced from suprasternal approach with minimal modification. TAC was created with a 6-0 suture tied around the aorta and a 27-gauge needle. After the needle was retracted, 60% to 80% constriction with an outer aortic diameter of ≈0.3 mm was created. MI was created by ligation of the left anterior descending coronary artery. Sham operation without aortic constriction or coronary ligation was also performed.

Noninvasive Analyses of Blood Pressure and Cardiac Performance

Before and 1, 2, 3, and 4 weeks after implantation of the pumps or L-NAME treatment, systolic blood pressure (SBP) and heart rate were noninvasively measured by the tail-cuff method as described previously.⁷ Four weeks after pump implantation, echocardiography was performed with 2D guided M-mode echocardiography performed with 15-MHz pulsed-wave Doppler echocardiography (SONOS 4500, Agilent Technologies). LV end-diastolic diameter (LVDd), LV end-systolic diameter (LVDs), diastolic interventricular septum thickness (IVS), and diastolic posterior wall thickness (PW) were measured. The percentage of LV fractional shortening (%FS) was calculated as follows: [(LVDd-LVDs)/LVDd]×100 (%). LV ejection fraction (EF) and LV mass were calculated as follows: (LVEDV-LVESV)/LVEDV×100 (%) and 1.055×[(LVDd+PW+IVS)³-(LVDd)³] (mg), respectively, where LVEDV is LV end-diastolic volume and LVESV is LV end-systolic volume.

Northern Blotting

Four weeks after pump implantation, hearts were isolated from all mice. Total mRNA was extracted from the left ventricles with TRIzol reagents (Invitrogen) according to the manufacturer's protocol. Equal amounts of total RNA (15 µg) were separated by 1.0% formaldehyde-agarose gel electrophoresis, and hybridization and washing were performed as described.⁷ The oligonucleotide probe was obtained as a PCR product using specific primers for murine atrial natriuretic factor (ANF) (forward, 5'-ATG GGC TCC TTC TCC ATC AC-3'; reverse, 5'-AAG CTG TTG CAG CCT AGT CC-3') and murine connective tissue growth factor (CTGF) (forward, 5'-GAG TGG GTG TGT GAC GAG CCC AAG G-3'; reverse, 5'-ATG TCT CCG TAC ATC TTC CTG TAG T-3'). Each probe was labeled with a random primer DNA

labeling kit (Takara) and [α - 32 P]dCTP (New England Nuclear Life Science Products). Band intensities were quantified densitometrically with the NIH Image Program. Expression levels of ANF and CTGF mRNAs were standardized by rehybridization with a GAPDH probe.

Real-Time PCR

Total RNA was extracted from the PBS-perfused mouse heart with TRIzol. The Quantitect SYBR Green RT-PCR kit (Qiagen) was used to perform amplifications with the 1-step protocol as described by the manufacturer. The following primers were used to amplify collagen III, transforming growth factor- β (TGF β), and GAPDH partial cDNA: collagen III: forward, 5'-TGA ATG GTG GTT TTC AGT TCA G-3', and reverse, 5'-GGT CAC TTG CAC TGG TTG ATA A-3'; TGF β : forward, 5'-CTC CCA CTC CCG TGG CTT CTA G-3', and reverse, 5'-GTT CCA CAT GTT GCT CCA CAC TTG-3'; and GAPDH: forward, 5'-GCA GTG GCA AAG TGG AGA TT-3', and reverse, 5'-CAC ATT GGG GGT AGG AAC AC-3'. The samples were then placed in a LightCycler (Roche), and fluorescence curves were analyzed with included software. GAPDH was used as an endogenous control reference. Fold change is shown as relative to that of WT control mice.

Histology

Paraffin sections (6 μ m) were obtained from heart specimens and stained with hematoxylin-eosin and Masson's trichrome. Photomicrographs were quantified with NIH Image software to measure the cross-sectional area of cardiomyocytes and to assess the area of perivascular fibrosis, which was calculated as the ratio of the fibrosis area surrounding the vessel to the total vessel area. Ten sections or \approx 40 vessels were examined in each heart; results obtained from 8 hearts in each group were averaged.

Statistical Analysis

Results are expressed as mean \pm SEM. All data were analyzed by means of Student's *t* tests or 1-way ANOVA, followed by Fisher's exact test for post hoc analyses. A value of $P < 0.05$ was considered statistically significant.

Results

Analysis of ROCK1 $^{+/-}$ Mice

We analyzed 61 offspring obtained by intercrossing ROCK1 $^{+/-}$ mice on pure C57Bl/6 background. Analysis of genotype distribution in offspring from heterozygous crosses revealed that the homozygous ROCK1 $^{+/-}$ mice (1 of 61, 1.6%) were markedly underrepresented among littermates at 3 weeks of age (WT, $n=20$; ROCK1 $^{+/-}$, $n=40$; ROCK1 $^{-/-}$, $n=1$, respectively). We then analyzed 61 offspring that were 3 weeks old obtained by crossing male ROCK1 $^{-/-}$ and female ROCK1 $^{+/-}$ mice and observed that the ROCK1 $^{-/-}$ mice (10 of 61, 16.4%) were also underrepresented compared with the value expected by mendelian ratio (ROCK1 $^{+/-}$, $n=51$; ROCK1 $^{-/-}$, $n=10$, respectively). Some but not all ROCK1 $^{-/-}$ mice exhibit defects in eyelid closure and omphalocele at birth, similar to results reported by Shimizu et al.¹³ Thus, ROCK1 $^{-/-}$ mice are abnormal, and most die either in utero or postnatally.

The partial and complete deletion of ROCK1 expression was confirmed by Western blot with specific ROCK1 monoclonal antibody (Figure 1C). As expected, the expression level of ROCK1 in ROCK1 $^{+/-}$ heart was approximately half of that in WT heart. There was no compensatory upregulation of ROCK2 expression for the loss of ROCK1. In this study, we used ROCK1 $^{+/-}$ mice, which are viable and fertile and show no developmental or gross anatomic abnormalities.

Effect of Haploinsufficiency of ROCK1 on Ang II–Induced Hypertension

We examined the effect of Ang II on hemodynamic parameters such as blood pressure and heart rate in WT and ROCK1^{+/-} mice. At baseline, there was no difference in body weight (27.8±0.4 versus 27.4±0.7 g; *P*>0.05), blood pressure (117±2 versus 120±2 mm Hg; *P*>0.05), and heart rate (502±2 versus 504±22 bpm; *P*>0.05) between WT and ROCK1^{+/-} mice (*n*=16 in each group). Mice were randomly divided into 2 groups: a saline-infused group (control) and an Ang II–infused group. Infusion of Ang II but not saline led to sustained increases in SBP in WT and ROCK1^{+/-} mice (Table 1). There was no significant difference in Ang II–induced SBP between WT and ROCK1^{+/-} mice. Body weight was increased in all groups except Ang II–infused ROCK1^{+/-} mice at 4 weeks after infusion compared with that before treatment. Heart rate was unchanged by Ang II infusion except in saline-infused WT mice. These results indicate that haploinsufficient ROCK1 does not affect Ang II–induced increase in SBP.

Effect of Haploinsufficiency of ROCK1 on Ang II–Induced Cardiac Hypertrophy

Echocardiography showed that there were no differences in cardiac dimensions such as LVDD, LVDs, EF, and %FS between the 4 groups, indicating that ROCK1 haploinsufficiency did not affect cardiac structure or contractile performance (Table 1). Wall thickness and LV mass were increased to a similar extent in both Ang II–infused WT and ROCK1^{+/-} mice compared with saline-infused mice. There was no difference in the ratio of heart weight to tibial length between saline-infused WT and ROCK1^{+/-} mice. The ratio of heart weight to tibial length was increased to a similar extent by infusion of Ang II in both WT and ROCK1^{+/-} mice. These results are consistent with the findings of echocardiographic measurements. Similarly, cardiomyocyte size as determined by the cross-sectional area of cardiomyocytes was increased by Ang II infusion in both WT and ROCK1^{+/-} mice (Figure 2A). However, there was no difference between WT and ROCK1^{+/-} mice in response to Ang II (*P*>0.05). The expression of cardiac fetal genes such as ANF, an established marker for cardiac hypertrophy, was upregulated in hearts by infusion of Ang II in both WT and ROCK1^{+/-} mice. However, there was no difference in ANF expression between WT and ROCK1^{+/-} hearts (Figure 2B). Taken together, these results indicate that ROCK1 is not involved in Ang II–induced cardiac hypertrophy.

Effect of Haploinsufficiency of ROCK1 on L-NAME–Induced Cardiac Hypertrophy

Treatment with L-NAME increased SBP in WT and ROCK1^{+/-} mice. After 4 weeks, there was no significant difference in the L-NAME–induced increase in SBP between WT and ROCK1^{+/-} mice (Table 2). The ratio of heart weight to tibial length was increased to a similar extent by treatment with L-NAME in both WT and ROCK1^{+/-} mice. Haploinsufficiency of ROCK1 also did not affect cardiac dimension and indexes of cardiac hypertrophy (ie, ratio of heart weight to tibial length), as well as contractile performance as assessed by echocardiography, indicating that ROCK1 is not involved in L-NAME–induced cardiac hypertrophy.

Effect of Haploinsufficiency of ROCK1 on Cardiac Fibrosis

In contrast to no observable differences in cardiac hypertrophy in WT and ROCK1^{+/-} mice, there was a substantial difference in Ang II– and L-NAME–induced increase in cardiac fibrosis between WT and ROCK1^{+/-} mice (Figure 3A). Cardiac fibrosis was observed quite prominently in areas surrounding the coronary arteries in WT but not ROCK1^{+/-} mice. Quantitative immunohistochemical analyses showed that Ang II infusion and L-NAME treatment increased perivascular fibrosis area in the heart to a greater extent in WT than in ROCK1^{+/-} mice (Figure 3B). Similarly, there was a substantial difference in TAC- and ischemia/MI-induced increase in perivascular fibrosis between WT and ROCK1^{+/-} mice (Figure 4A and 4B). In both animal models of cardiac remodeling, perivascular fibrosis

occurring 4 weeks after surgery was substantially less in ROCK1^{+/-} mice compared with WT mice despite comparable changes in myocardial wall thickness and ratio of heart weight to tibial length in WT and ROCK1^{+/-} mice (data not shown).

Effect of Haploinsufficiency of ROCK1 on Profibrotic Gene Expression

To determine whether profibrotic gene expression was altered in ROCK1^{+/-} mice, we analyzed cardiac expression of CTGF, a critical regulator of fibrosis in diverse tissues, including heart.¹⁴ Infusion of Ang II increased CTGF mRNA expression in WT mice (Figure 5A). This increase in Ang II-induced CTGF expression was markedly attenuated in ROCK1^{+/-} mice. In addition, Ang II increased other critical markers of fibrosis such as TGF β and type III collagen in hearts of WT mice (Figure 5B). However, the increase in TGF β and type III collagen expression was also markedly less in ROCK1^{+/-} mice compared with WT mice. These findings indicate that ROCK1 plays a critical role in mediating cardiac fibrosis under various pathological conditions.

Discussion

There is growing evidence that ROCK plays an important role in the pathogenesis and development of a variety of cardiovascular diseases. Most of the evidence, however, was based on studies using pharmacological inhibitors of ROCK such as Y27632 and fasudil.⁹ ROCK consists of 2 isoforms, ROCK1 and ROCK2, the kinase domains of which share 92% homology. Because ROCK inhibitors target the kinase domains of ROCK1 and ROCK2, they have similar potency in inhibiting both ROCK isoforms. Therefore, it is impossible to determine the isoform-specific role of ROCK by treatment with ROCK inhibitors. In addition, ROCK inhibitors such as Y27632 and fasudil may not be entirely selective and could potentially inhibit other protein kinases such as protein kinase C and A at relatively higher concentrations.

To determine the isoform-specific roles of ROCK in cardiovascular diseases, we generated ROCK1^{+/-} mice and investigated the effects of ROCK1 deficiency on Ang II changes in blood pressure and cardiac remodeling. For this purpose, we used ROCK1^{+/-} mice because heterozygotes show no developmental or anatomical abnormalities. In addition, our preliminary data revealed that homozygous ROCK1^{-/-} mice were markedly underrepresented among littermates obtained by intercrossing ROCK1^{+/-} mice (1 of 61, 1.6%) and by crossbreeding male ROCK1^{-/-} with female ROCK1^{+/-} mice. A recent study showed that targeted ROCK1 gene deletion by replacing exons 3 and 4 with an *lacZ* reporter gene resulted in mutant mice with eyes open at birth and omphalocele.¹³ These phenotypes result from disorganization of actomyosin cables in the eyelid epithelium and a defect in the actin cytoskeleton assembly in the umbilical ring, respectively. In agreement with our observation, they also demonstrated that ROCK1^{-/-} mice were significantly underrepresented among littermates obtained by intercrossing ROCK1^{+/-} mice (12 of 316, 3.8%). Interestingly, despite the relatively high expression levels of ROCK1 in hearts, ROCK1^{-/-} mice apparently exhibit no abnormalities in the heart.

In the present study, we demonstrated that haploinsufficiency of the ROCK1 gene in mice resulted in the inhibition of cardiac fibrosis without affecting Ang II-induced hypertension and cardiac hypertrophy. Similar results were obtained in L-NAME-treated mice. L-NAME-induced perivascular fibrosis was decreased in ROCK1^{+/-} mice despite the equivalent response to L-NAME in terms of hypertension and cardiac hypertrophy between WT and ROCK1^{+/-} mice. Although previous studies have demonstrated that administration of ROCK inhibitors to hypertensive rats decreases blood pressure,^{15,16} given that the Ang II-induced increase in systolic blood pressure was comparable between WT and ROCK1^{+/-} mice, these findings suggest that ROCK1 may not be involved in Ang II-induced increase in blood pressure. Long-

term treatment with the ROCK inhibitor fasudil does not affect Ang II–induced hypertension in animals.^{10,12}

Chronic inhibition of ROCK activity by ROCK inhibitors has been shown to inhibit Ang II–induced cardiac hypertrophy and fibrosis in rats¹⁰ and apolipoprotein E–knockout mice.¹² Other studies have shown that ROCK inhibitors suppress the hypertrophic response and fibrotic change in hearts of Dahl salt-sensitive rats.¹¹ In contrast to the results of these studies, haploinsufficiency of the ROCK1 gene in mice did not prevent Ang II–induced cardiac hypertrophy. Thus, it appears that ROCK1 may not play a prominent role in Ang II–induced cardiac hypertrophy.

The most striking finding of this study is the decrease in cardiac fibrosis in haploinsufficient ROCK1^{+/-} mice under various pathological conditions. We analyzed cardiac fibrosis using 4 models of fibrosis: Ang II infusion, L-NAME treatment, MI, and TAC. In all of these models, ROCK1^{+/-} mice exhibited reduced fibrosis compared with WT controls. The critical role of ROCK in fibrosis has been demonstrated not only in the heart^{10–12} but also in other tissues, including the kidney.¹⁷ Involvement of ROCK in Ang II–induced fibrosis has been shown in vitro.¹⁸ In the present study, we showed that the decrease in cardiac fibrosis was associated with a decrease in CTGF expression. CTGF, which was initially characterized as a downstream mediator of TGFβ, is implicated in fibrosis. CTGF is induced in hearts of animal models and cultured cardiac myocytes in response to diverse stimuli and injury, including Ang II infusion.^{19,20} The expression of TGFβ and collagen type III was decreased in ROCK1^{+/-} mice. These results suggest that ROCK1 may be an important therapeutic target for fibrotic diseases.

Haploinsufficiency of the ROCK1 gene, however, did not prevent the development of cardiac hypertrophy in response to Ang II, L-NAME, or TAC. Therefore, our results suggest that signaling pathways that lead to cardiac fibrosis may be distinct from that of cardiac hypertrophy. For example, transgenic mice with cardiac-specific overexpression of dominant negative forms of p38α and p38β develop cardiac hypertrophy without changes in cardiac fibrosis in response to pressure overload.²¹ In addition, transgenic mice with cardiac-specific overexpression of activated forms of MKK3bE or MKK6bE, the direct upstream activators of p38, resulted in cardiac fibrosis and systolic and diastolic dysfunction but not cardiac hypertrophy.²² Collectively, these studies demonstrate that MKK-p38 signaling pathway may play an important role in the development of cardiac fibrosis but not in cardiac hypertrophy. Although inhibition of ROCK1 in cardiomyocytes appears to play a predominant role in the prevention of cardiac fibrosis, we cannot exclude the possibility that ROCK1 in cardiac fibroblasts could also be mediating these effects. Further studies using cardiomyocyte-specific deletion of ROCK1 are needed to sort out these issues.

Acknowledgment

This work was supported by grants from the National Institute of Health (HL-52233) and the American Heart Association (Bugher Foundation Award). Y. Rikitake is a recipient of an AHA Postdoctoral Fellowship Award–Northeast Affiliate. Y. Rikitake, N. Oyama, and K. Noma are recipients of the Japan Heart Foundation/Bayer-Yakuin Research Grant Abroad.

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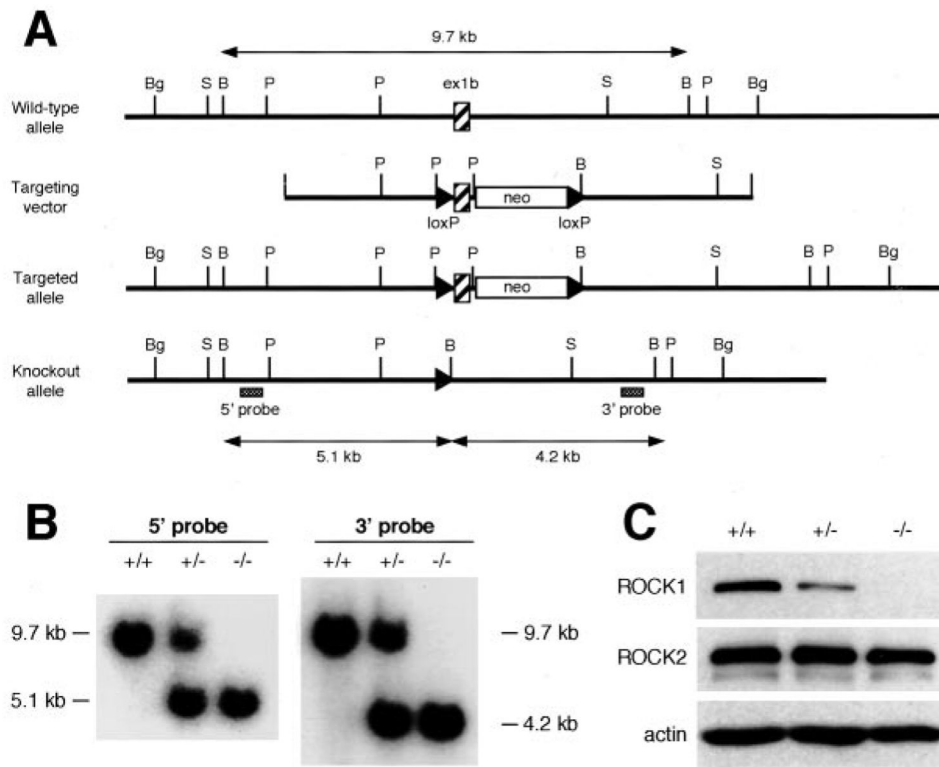


Figure 1. Generation of ROCK1-knockout allele. **A**, Schematic of structures of the WT ROCK1 allele, targeting vector, targeting allele, and ROCK1-knockout allele. The targeting vector containing loxP sites flanking exon 1b of ROCK1 was injected into embryonic stem cells. The loxP-flanked exon 1b and pGK-Neo gene in correctly targeted clones were deleted by transfection with Cre. Shown are positions of neomycin resistance (neo), loxP sites (loxP); restriction enzyme sites for *Bgl*II (Bg), *Bam*HI (B), *Stu*I (S), and *Pes*tI (P); and 5' and 3' genomic probes. **B**, Southern blots showing the presence of WT (+) and deleted (-) ROCK1 allele. Genomic DNA was isolated from tails of WT (+/+), ROCK1^{+/-} (+/-), and ROCK1^{-/-} (-/-) mice, digested with *Bam*HI, and subjected to Southern blots. **C**, Western blots showing the protein levels of ROCK1 and ROCK2. Proteins were extracted from hearts of WT (+/+), ROCK1^{+/-} (+/-), and ROCK1^{-/-} (-/-) mice and analyzed by Western blots using anti-ROCK1, anti-ROCK2, and anti-actin antibodies.

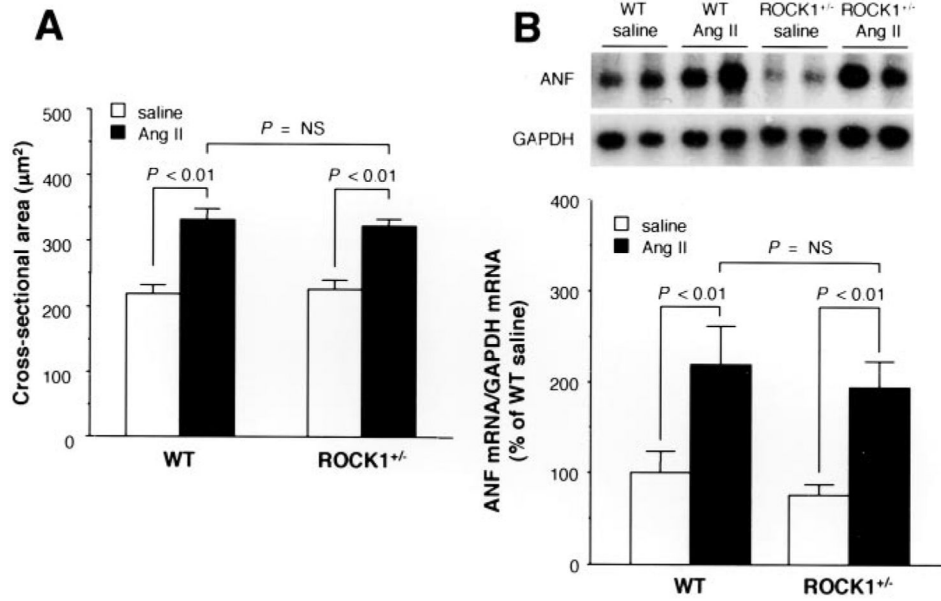


Figure 2. Effect of Ang II on cardiac hypertrophy in WT and ROCK1^{+/-} mice. Hearts were isolated from mice receiving saline or Ang II for 4 weeks. A, Quantified data of cross-sectional area of cardiomyocytes are shown. B, Northern blots showing the expression of ANF mRNA expression. Expression levels of ANF mRNA were standardized by expression levels of GAPDH mRNA. n=8 in each group (A and B).

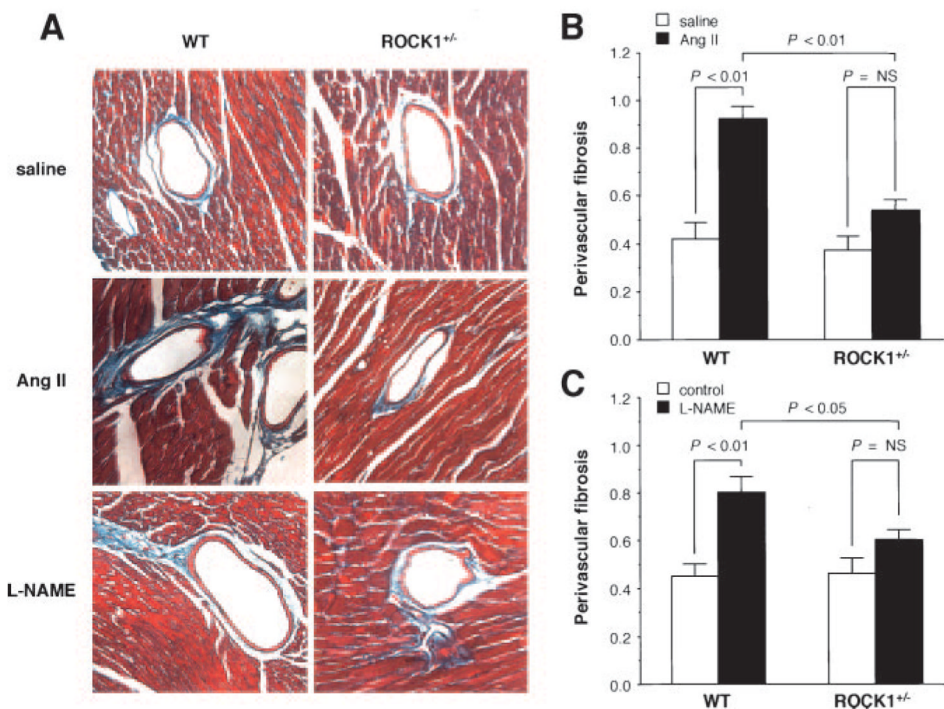


Figure 3. Effects of Ang II and L-NAME on cardiac fibrosis in WT and ROCK1^{+/-} mice. Hearts were isolated from WT and ROCK1^{+/-} mice receiving either saline or Ang II for 4 weeks or treated with or without L-NAME for 4 weeks. A, Perivascular fibrosis was analyzed by Masson's trichrome staining. Representative photo images are shown. B, C, Quantified data are shown. n=8 (B) and n=4 (C) in each group.

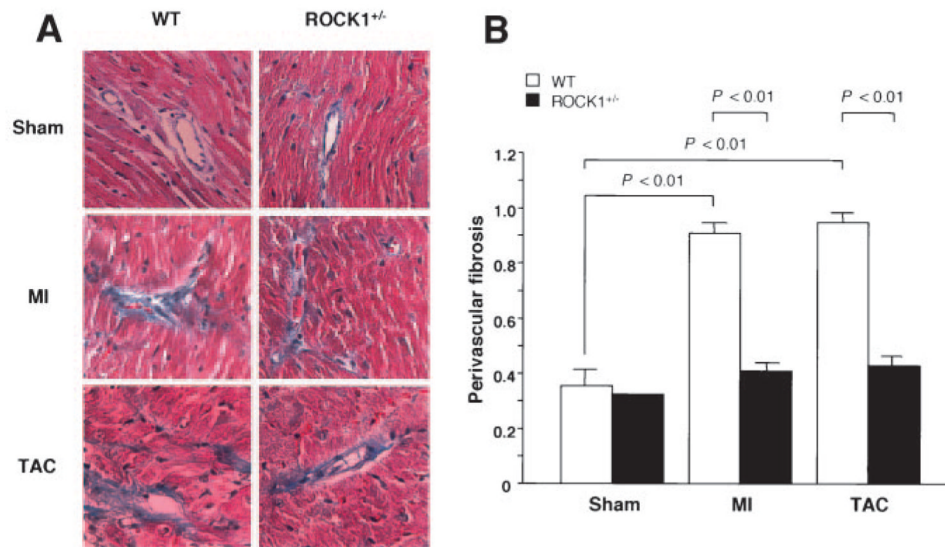


Figure 4. Cardiac fibrosis in WT and ROCK1^{+/-} mice after ischemia/MI or TAC. Hearts were isolated from WT and ROCK1^{+/-} mice subjected to sham, MI, or TAC operation. Hearts were isolated from mice 4 weeks after operation. A, Perivascular fibrosis was analyzed by Masson's trichrome staining. Representative photo images are shown. B, Quantified data are shown. n=3 to 4 in each group.

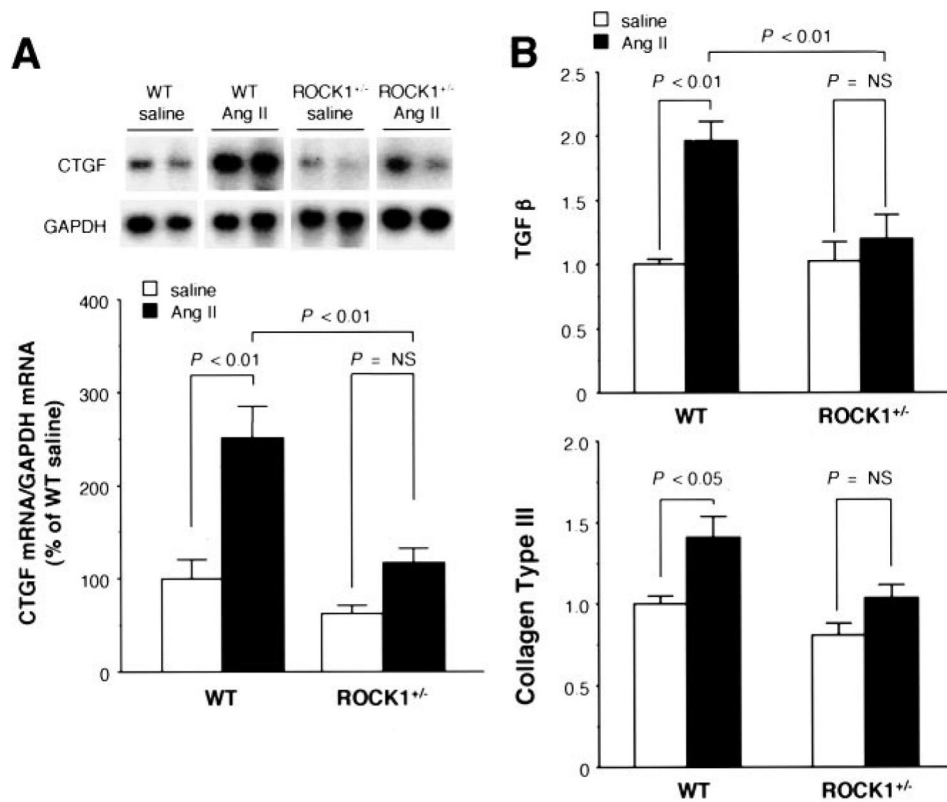


Figure 5. Expression of profibrotic genes in WT and ROCK1^{+/-} mice. Hearts were isolated from mice receiving saline or Ang II for 4 weeks. A, CTGF mRNA expression was analyzed by Northern blotting. Expression levels of CTGF mRNA were standardized by expression levels of GAPDH mRNA. n=8 in each group. B, Expression of TGFβ and collagen type III mRNAs was analyzed by real-time PCR. Values were standardized by expression levels of GAPDH mRNA. n=4 in each group.

TABLE 1
Hemodynamic and Echocardiographic Assessment: Ang II Model

	WT		ROCK1 ^{+/-}	
	Saline (n=8)	Ang II (n=8)	Saline (n=8)	Ang II (n=8)
BW, g				
Before	27.9±0.6	27.8±0.6	27.6±0.6	27.4±0.7
At 4 wk	30.8±0.6 [†]	29.6±0.6 [‡]	30.2±0.9 [‡]	27.6±0.8
SBP, mm Hg				
Before	118±3	117±3	121±2	120±2
At 4 wk	117±3	146±4 ^{*‡}	114±5	138±4 ^{*‡}
HR, bpm				
Before	503±28	501±24	535±27	474±32 [*]
At 4 wk	606±19 [†]	549±38	533±31	523±26
LVDd, mm	1.87±0.08	3.03±0.09	3.21±0.12	3.11±0.16
LVDs, mm	1.87±0.08	1.77±0.05	1.91±0.08	1.83±0.08
FS, %	41.2±2.0	41.6±1.0	40.4±0.9	42.0±1.1
EF, %	77.3±1.6	78.7±1.0	77.5±1.0	77.0±2.6
IVS, mm	0.71±0.02	0.91±0.02 [*]	0.72±0.01	0.89±0.03 [*]
PW, mm	0.73±0.02	0.94±0.02 [*]	0.74±0.02	0.90±0.02 [*]
LV mass, mg	70±4	94±5 [*]	74±5	94±9 [*]
HW/TL, mg/mm	7.1±0.1	7.8±0.2 [*]	7.1±0.1	7.8±0.2 [*]

BW indicates body weight; HR, heart rate; PW, posterior wall; HW, heart weight; TL, tibial length.

* $P < 0.05$ vs saline;

[†] $P < 0.05$,

[‡] $P < 0.01$ vs before.

TABLE 2
Hemodynamic and Echocardiographic Assessment: L-NAME Model

	WT		ROCK1 ^{+/-}	
	Control (n=4)	L-NAME (n=4)	Control (n=4)	L-NAME (n=4)
BW, g				
Before	28.1±1.7	27.9±1.1	27.8±2.4	27.6±1.5
At 4 wk	31.3±2.7	30.1±1.4	29.8±2.9	29.1±2.1
SBP, mm Hg				
Before	112±2	115±3	109±5	112±4
At 4 wk	110±3	147±4 ^{†‡}	106±5	145±2 ^{†‡}
HR, bpm				
Before	539±17	518±18	552±24	539±26
At 4 wk	542±22	541±31	548±29	563±26
LVDd, mm	3.12±0.08	3.19±0.05	3.26±0.09	3.17±0.08
LVDs, mm	1.86±0.06	1.93±0.03	1.97±0.07	1.95±0.07
FS, %	40.2±0.9	39.4±0.4	39.6±0.4	38.6±0.8
EF, %	76.8±0.9	76.0±0.5	76.2±0.6	75.1±1.0
IVS, mm	0.72±0.08	0.90±0.07 [*]	0.74±0.18	0.88±0.12 [*]
PW, mm	0.73±0.08	0.87±0.09 [*]	0.73±0.07	0.86±0.15 [*]
LV mass, mg	69±3	95±3 [*]	76±3	91±4 [*]
HW/TL, mg/mm	7.1±0.3	7.7±0.3 [*]	7.0±0.3	7.7±0.2 [*]

Abbreviations as in Table 1.

* $P < 0.05$ vs control;

[†] $P < 0.01$ vs control;

[‡] $P < 0.01$ vs before.