ROCK Isoform Regulation of Myosin Phosphatase and Contractility in Vascular Smooth Muscle Cells

Yuepeng Wang, Xiaoyu Rayne Zheng, Nadeene Riddick, Meredith Bryden, Wendy Baur, Xin Zhang, Howard K. Surks

Abstract—Abnormal vascular smooth muscle cell (VSMC) contraction plays an important role in vascular diseases. The RhoA/ROCK signaling pathway is now well recognized to mediate vascular smooth muscle contraction in response to vasoconstrictors by inhibiting myosin phosphatase (MLCP) activity and increasing myosin light chain phosphorylation. Two ROCK isoforms, ROCK1 and ROCK2, are expressed in many tissues, yet the isoform-specific roles of ROCK1 and ROCK2 in vascular smooth muscle and the mechanism of ROCK-mediated regulation of MLCP are not well understood. In this study, ROCK2, but not ROCK1, bound directly to the myosin binding subunit of MLCP, yet both ROCK isoforms regulated MLCP and myosin light chain phosphorylation. Despite that both ROCK1 and ROCK2 regulated MLCP, the ROCK isoforms had distinct and opposing effects on VSMC morphology and ROCK2, but not ROCK1, had a predominant role in VSMC contractility. These data support that although the ROCK isoforms both regulate MLCP and myosin light chain phosphorylation through different mechanisms, they have distinct roles in VSMC function. (Circ Res. 2009;104:531-540.)

Key Words: ROCK ▪ myosin phosphatase ▪ myosin light chain ▪ myosin binding subunit

Increased vascular tone plays an important role in the pathophysiology of vascular diseases including hypertension, atherosclerosis, and myocardial infarction. Vascular tone is regulated by the contraction of vascular smooth muscle cells (VSMC) in the blood vessel wall. Vascular smooth muscle contraction is tightly coupled to the phosphorylation of the regulatory myosin light chain (MLC) kinase (MLCK) and myosin light chain phosphatase (MLCP) (reviewed elsewhere). MLCP dephosphorylates MLC, leading to vascular smooth muscle relaxation. Vasoconstrictors, conversely, inhibit MLCP, leading to MLC phosphorylation and smooth muscle contraction (reviewed elsewhere). Vasoconstrictor-mediated MLCP inhibition occurs by either phosphorylation of the MLCP inhibitory protein CPI-17 or by phosphorylation of the myosin binding subunit (MBS) of MLCP at inhibitory sites T696 and T850.

The RhoA/ROCK pathway is the most extensively studied mechanism of MLCP inhibition. Vasoconstrictor G protein-coupled receptor agonists lead to activation of RhoA guanine nucleotide exchange factors and GTP loading of the monomeric GTPase RhoA. GTP-bound RhoA then binds and activates its downstream effector ROCK, which in turn phosphorylates MBS at the 2 phosphorylation sites, leading to inhibition of MLCP activity. Phosphorylation at T850 also has been shown to cause dissociation of MBS from myosin. More recently, T850 has been implicated as the major ROCK phosphorylation site, whereas T696 is thought to be phosphorylated by other kinases. Many studies support that RhoA/ROCK signaling plays a role in the regulation of vascular tone and in the pathogenesis of vascular diseases, yet the precise mechanisms by which ROCK is targeted to and interacts with MLCP are not well understood.

We and others have recently characterized a new member of the MLCP complex, myosin phosphatase–Rho interacting protein (MP-RIP, also M-RIP, p116GSP). MP-RIP is a cytoskeletal scaffold that binds directly to both RhoA and MBS and targets MLCP to the contractile apparatus to dephosphorylate MLC. MP-RIP is also required to colocalize RhoA and MBS to regulate MLCP, however, does not bind ROCK, leaving the issue of how ROCK is targeted to MLCP unresolved.

There are 2 isoforms of ROCK, ROCK1 and -2, that share overall 65% homology at the amino acid level. The tissue distribution of ROCK1 and -2 is similar, and relatively few
Figure 1. The MBS of MLCP interacts with ROCK2 in VSMCs. A, ROCK1 and ROCK2 were immunoprecipitated from A7r5 cells using 1.4 µg of either goat anti-ROCK1 (goat ROCK1), goat anti-ROCK2 (goat ROCK2), or goat nonimmune (NI IP) antibodies. Immunoblots were performed with mouse anti-ROCK1 (m ROCK1) or mouse anti-ROCK2 (m ROCK2) antibodies. These conditions were used for all subsequent immunoprecipitations of ROCK1 and -2. B, ROCK1 and -2 were immunoprecipitated from A7r5 cells, followed by immunoblot to detect the MBS and MP-RIP proteins. A sample of the input protein for ROCK1 and -2 (25, 12.5, and 6.25 µg) is shown by immunoblot below. C, Nonimmune ROCK1 and ROCK2 immunoprecipitations were performed from primary rat aortic smooth muscle cells. The immunopellets were probed by immunoblot with MBS, ROCK1, and ROCK2 antibodies. D, HEK293 cells were transfected with empty plasmid (Mock), Myc-tagged MBS, ROCK1, or ROCK2. Immunoprecipitations were performed with anti-Myc antibodies, followed by immunoblotting for ROCK1 and ROCK2.

Figure 2. Cell stimulation augments the ROCK2-MBS interaction. A, ROCK1 and -2 were immunoprecipitated from A7r5 cells in serum-free (arrested) conditions or following cell stimulation with either 1 µmol/L LPA or 10% serum. B, A time course of serum stimulation of A7r5 cells followed by ROCK2 immunoprecipitation is shown (top). Pooled results from 4 experiments are shown (bottom). C, A time course of serum stimulation of A7r5 cells followed by measurement of MBS phosphorylation at Thr850 by immunoblot (top). Pooled results from 4 experiments (bottom).
studies have delineated the isoform specific roles of ROCK. Smooth muscle cells have been traditionally thought to express ROCK2 because it was purified from gizzard smooth muscle, although the expression of ROCK1 was not excluded. In this study, the specific roles of the ROCK isoforms in MLCP regulation and VSMC contractility were explored.

**Materials and Methods**

**Cell Culture**

A7r5 cells were purchased from ATCC. Primary rat aortic smooth muscle cells were derived from rat aortas by the explant method and were identified by the expression of smooth muscle α-actin.

**Smooth Muscle Cell Contractility Assay**

Primary rat aortic smooth muscle cells were plated on a polymer substrate consisting of microfabricated posts made by replica molding of polydimethylsiloxane (PDMS). After incubation in serum-free media for 48 hours, the cells were stimulated with contractile agonist and imaged every minute for 30 minutes using a Cool SNAP EZ CCD camera (Photometrics) with NIS Elements software. Cell length change following agonist stimulation was measured using Image Pro Plus 6.0 software.

An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

**Results**

**The MBS of MLCP Interacts With ROCK2 in VSMCs**

To investigate the mechanism whereby ROCK interacts with MLCP, ROCK1 and -2 were individually immunoprecipitated from A7r5 VSMCs without contamination by the other isoform (Figure 1A). When the ROCK1 and -2 immunopellets were probed for MBS binding, ROCK2, but not ROCK1, was found to coimmunoprecipitate MBS (Figure 1B). In primary rat aortic smooth muscle cells, where the immunoreactivity of ROCK1 and -2 was similar, MBS was also found in a selectivity in the ROCK2 immunopellet (Figure 1C). In a
separate approach, ROCK2, but not ROCK1, was found in a myc-MBS immunopellet from transfected HEK293 cells (Figure 1D). Together, these experiments support that the MBS of MLCP interacts specifically with ROCK2.

Cell Stimulation Augments the ROCK2-MBS Interaction

The ROCK2-MBS coimmunoprecipitation in resting VSMCs was augmented by exposure to the contractile agonist lysophosphatidic acid (LPA) or to serum (Figure 2A). These stimuli would be expected to increase ROCK activity, and the data therefore suggest that ROCK activity may modulate its interaction with MBS. When the time course of ROCK2-MBS binding in response to serum was examined, the interaction was significantly increased within 5 minutes of stimulation \( (P=0.008; n=4) \), was diminished by 40 minutes, and reached a second peak at 1 hour \( (P=0.04; n=4) \) (Figure 2B). When phosphorylation of MBS by ROCK was studied, a similar time course was observed \( (P=0.04 \text{ at } 5 \text{ and } 60 \text{ minutes}; n=4) \), suggesting that the ROCK2-MBS interaction correlates with phospho-regulation of MLCP by ROCK (Figure 2C).

Mechanism of ROCK-MBS Binding

To localize the ROCK2 binding domains within MBS, glutathione S-transferase (GST) fusion proteins were created that together encompassed the entire MBS molecule. These were individually tested for binding to ROCK from VSMC lysates. MBS peptides corresponding to amino acids 540 to 858 (with and without the central insert splice variant) and 683 to 866 both bound ROCK2 (Figure 3A). The minimal MBS domain tested that bound ROCK included amino acids 683 to 866, which contain the 2 major inhibitory phosphorylation sites, T696 and T850 (Figure 3C), and a predicted coiled coil structure (amino acids 704 to 764). Interestingly, MBS 683 to 866 was able to bind both ROCK2 and ROCK1 in vitro. When binding of mycROCK1 and mycROCK2 to MBS 683 to 866 was compared, 2-fold more ROCK2 bound MBS than ROCK1 \( (P=0.01, n=3) \); Figure 3B). MP-RIP targets RhoA to regulate MLCP \( 26,28,30 \); however, Koga and Ikebe recently found that MP-RIP does not interact with ROCK.\(^28\) To confirm this observation in our binding studies, the same MBS domains were probed for MP-RIP binding. MP-RIP bound MBS 850 to 1030, confirming our previous findings \( 26,28,30,31 \) and demonstrating that MP-RIP and ROCK bind to distinct domains of MBS (Figure 3A). Furthermore, when GST-MP-RIP domains, together encompassing the entire molecule, were compared with GST-MBS 683 to 866 for ROCK binding, only GST-MBS bound ROCK (Figure I in the online data supplement).

To localize the MBS binding domain of ROCK, and to test direct binding between MBS and ROCK, 4 domains each of ROCK1 and -2, that together encompassed both molecules in their entirety, were separately tested for binding to GST-MBS 683 to 866. Of the ROCK2 domains, a coiled coil domain of ROCK2, 354 to 775, and to a much lesser extent the kinase domain of ROCK2, 1 to 360, bound MBS (Figure 3D). No domains of ROCK1 specifically bound MBS (Figure 3D and data not shown). The homology between ROCK2 354 to 775 and ROCK1 338 to 750 is only 58% (supplemental Figure II), compared to 86% homology between the catalytic domains of the 2 isoforms (data not shown). These studies suggest that ROCK2 amino acids 354 to 775 can directly bind MBS amino acids 683 to 866 (Figure 4).

ROCK Isoform Regulation of MLCP and MLC Phosphorylation

When overexpressed in VSMCs, ROCK1 and -2 were localized diffusely in the cytoplasm (Figure 5A, right images). Immunofluorescent labeling with phospho-MLC antibodies showed that overexpression of either ROCK1 or ROCK2 dramatically increased MLCP phosphorylation (Figure 5A, left images), whereas GFP did not affect MLC phosphorylation, and a dominant negative ROCK2 peptide reduced MLC phosphorylation in this assay (supplemental Figure III).

When ROCK1 and -2 were individually silenced using RNA interference, upregulation of the other isoform was noted (Figure 5B). Combined ROCK isoform silencing was not as effective as individual silencing. MBS phosphorylation at the 2 inhibitory phosphorylation sites, T696 and T850, was probed in the setting of ROCK silencing. Silencing of either ROCK isoform caused a significant reduction in phosphorylation at T850 \( (P<0.05 \text{ for ROCK1 and ROCK2a; } n=4) \) (Figure 5C). Silencing of both ROCK isoforms combined caused a significantly greater reduction in MBS T850 phosphorylation than individual silencing of either ROCK isoform \( (P=0.04 \text{ versus ROCK1 alone; } n=4) \). ROCK silencing did not affect phosphorylation of MBS at T696 (supplemental Figure IV).

Phosphorylation of the MLCP downstream substrate MLC, the biochemical determinant of smooth muscle contraction, was also examined in the setting of ROCK isoform silencing. ROCK1 and -2 silencing both reduced MLC phosphorylation, although only ROCK2 silencing reached statistical significance \( (P<0.002; n=4) \) (Figure 5D). Combined ROCK silencing caused greater inhibition of MLC phosphorylation than silencing of individual ROCK isoforms \( (P<0.05 \text{ for R1, R2a, or R2b versus R1+R2; } n=4) \) (Figure 5D). When examined by immunofluorescence microscopy, silencing of
either ROCK isoform reduced phosphorylation of MLC, consistent with the biochemical data above (Figure 5E). These studies support that the ROCK isoforms both regulate MLCP activity and MLC phosphorylation and have an additive effect when silenced in combination.

**Figure 5.** ROCK isoform regulation of MLCP and MLC phosphorylation. A, A7r5 cells transfected with Myc-ROCK1 or Myc-ROCK2 and immunostained with both anti–phospho-MLC-Cy3 (left images) and anti-Myc-FITC (right images). The arrowheads indicated transfected cells. The scale bar is 30 μm. B, Immunoblot showing specific silencing of ROCK1 (R1) expression, ROCK2 (R2a and R2b) (2 separate oligonucleotides) expression, or both ROCK1 and ROCK2 (R1+R2) expression in A7r5 cells using dsRNA oligonucleotides. Scr indicates scrambled negative control dsRNA (100 nmol/L). 2xScr indicates scrambled control dsRNA concentration adjusted to match R1+R2 (200 nmol/L). GAPDH is shown as a loading control. C, phosphorylation of MBS at the known inhibitory site Thr850 in A7r5 cells, following the silencing conditions described in B (top). Total MBS is shown as a loading control. Pooled data from 4 experiments of MBS phosphorylation at Thr850 following ROCK isoform silencing (bottom). The values are represented as Thr850 phosphorylation normalized to MBS expression for each sample. D, Phosphorylation of MLC following silencing of ROCK isoforms in A7r5 cells as described in B (top). Total MLC is shown as a loading control. Pooled data from 4 separate experiments of MLC phosphorylation following ROCK isoform silencing (bottom). The values are represented as MLC phosphorylation normalized to total MLC expression for each sample. E, Immunofluorescence microscopy of primary rat aortic smooth muscle cells following control (Scr) or ROCK isoform silencing. The cells were labeled with phalloidin to identify actin fibers (left column) and phospho-MLC (same antibody used for immunoblotting above) (right column). The scale bar is 30 μm.

**ROCK Isoform Regulation of VSMC Morphology**

The ROCK isoforms were found to have distinct morphological effects on primary rat aortic smooth muscle cells (Figure 6A and supplemental Figures V and VI). ROCK1-silenced cells had a smaller cell area (47% reduction, \( P < 0.001 \) versus...
scrambled, 1-way ANOVA on ranks; Figure 6A and 6B), fewer stress fibers (39% reduction, \( P < 0.001 \) versus scrambled, 1-way ANOVA on ranks; Figure 6A and 6C), and increased numbers of focal adhesions (40% increase, \( P < 0.05 \), ANOVA Holm–Sidak test; Figure 6A and 6D) than scrambled control transfected cells. ROCK2-silenced cells had a larger cell area (65% increase, \( P < 0.001 \) versus scrambled, 1-way ANOVA on ranks; Figure 6A and 6B) and greater number of stress fibers (49% increase, \( P < 0.001 \) versus scrambled, 1-way ANOVA on ranks; Figure 6A and 6C) and fewer focal adhesions (63% reduction, \( P < 0.001 \), ANOVA Holm–Sidak test; Figure 6A and 6D) compared to control VSMCs. These experiments support that whereas both ROCK isoforms regulate MBS and MLC phosphorylation, they have opposing effects on cell morphology. Treatment with the contractile agonist LPA modestly increased the number of stress fibers and reduced the number of focal adhesions under all silencing conditions (supplemental Figure V). Treatment with the ROCK inhibitor Y27632 dramatically reduced the numbers of stress fibers and focal adhesions under all silencing conditions, indicating that the contraction required actin–myosin interactions (supplemental Figure VIII). ROCK1-silenced VSMCs also underwent a vigorous contraction (Figure 7A and supplemental Video 2). ROCK2-silenced cells contracted significantly less than scrambled control and ROCK1-silenced cells (Figure 7A and 7B and supplemental Video 3; \( P < 0.001 \) versus scrambled siRNA cells, 1-way ANOVA).

Intracellular force generation was calculated from the displacement of microfabricated posts. Force maps of control and ROCK1- and ROCK2-silenced cells (Figure 7A) confirmed that ROCK1 silencing did not significantly affect force generation, whereas ROCK2 silencing significantly reduced force (Figure 7C; scrambled versus ROCK1, \( P = \text{NS} \); scrambled versus ROCK2, \( P < 0.05 \); ANOVA on ranks). Interestingly, the difference in force between ROCK2 and controls cells was more pronounced than the difference in cell contraction in Figure 7B. When examined in more detail, control and ROCK1-silenced cells had a similar relationship between force production and the degree of contraction. However, ROCK2-silenced cells exhibited a steeper slope for this relationship, indicating that force production yielded a greater degree of contraction in these cells (Figure 7D).

The differences in force production and contraction between ROCK1- and ROCK2-silenced cells were explored further using immunofluorescence microscopy to image actin–myosin fibers and phosphorylation of MLC after treatment with LPA or Y27632 (Figure 7E and supplemental Figures IX and X). The stress fibers in control silenced VSMCs were

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**ROCK Isoform Regulation of VSMC Contractility**

The role of the ROCK isoforms in VSMC contractility was explored using an assay in which VSMCs are cultured on microfabricated posts which do not hinder cell contraction and whose movement can be used to measure cellular force production.\(^{34-39}\) Cells transfected with scrambled and ROCK2 small interfering (si)RNA were elongated, whereas cells transfected with ROCK1 siRNA appeared smaller than scrambled control and ROCK2-silenced cells, consistent with the immunofluorescence labeling in Figure 6 (also Figure 7A and supplemental videos). When the contractile agonist LPA was applied, control cells underwent a gradual contraction, primarily along the long axis of the cells, over a period of 15 to 20 minutes (see supplemental Video 1). LPA-mediated contractions were abolished by pretreatment with the ROCK1 and ROCK2 inhibitor Y27632, supporting that the contractility was mediated by ROCK (supplemental Figure VII) and by pretreatment with blebbistatin, indicating that the contraction required actin–myosin interactions (supplemental Figure VIII). ROCK1-silenced VSMCs also underwent a vigorous contraction (Figure 7A and supplemental Video 2). ROCK2-silenced cells contracted significantly less than scrambled control and ROCK1-silenced cells (Figure 7A and 7B and supplemental Video 3; \( P < 0.001 \) versus scrambled siRNA cells, 1-way ANOVA).

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Figure 7. Contraction and force production in ROCK1- and ROCK2-silenced VSMCs. A, Contractility and force production in representative scrambled negative control (Scr) and ROCK1- and ROCK2-silenced primary rat aortic VSMCs. For each silencing condition, the phase-contrast image of the cell is shown before and 30 minutes after administration of 1 μmol/L LPA. Adjacent to the cell is the displacement map of the microfabricated posts. The displacement map was used to generate the force map shown to the far right of each image, as described in Materials and Methods. B, Contraction, as measured by the change in cell length, in scrambled negative control (Scr) and ROCK1- and ROCK2-silenced VSMCs treated with LPA (N=60, 69, 54 cells, respectively). C, Force production in 20 scrambled negative control (Scr) and ROCK1- and ROCK2-silenced cells treated with LPA. D, Plot of force versus contraction for scrambled negative control (Scr, black squares), ROCK1-silenced (red triangles), and ROCK2-silenced (blue circles) VSMCs. E, Detail of scrambled negative control (Scr) and ROCK1- and ROCK2-silenced unstimulated VSMCs labeled with phalloidin for actin filaments and phospho-MLC as in Figure 5E. The scale bar is 30 μm. F, Plot of force direction analyzed in the longitudinal and transverse axes for scrambled negative control (black squares), ROCK1-silenced (red triangles), and ROCK2-silenced (blue circles) LPA-stimulated VSMCs. Force direction was determined by the direction in which the posts were bent and the average longitudinal and transverse components were plotted.
aligned with and extended across the long axis of the cell and were diffusely localized throughout the cell body. The stress fibers displayed abundant MLC phosphorylation despite lack of agonist stimulation (Figure 7E). ROCK1-silenced cells had fewer stress fibers than control cells, yet these stress fibers extended across the long axis of the cell and were frequently localized at the cell periphery. MLC phosphorylation colocalized with the peripheral actin–myosin stress fibers (Figure 7E). The ROCK2-silenced cells displayed abundant stress fibers, but in many cells the stress fibers were short and haphazardly arranged. MLC phosphorylation also colocalized with stress fibers in the ROCK2-silenced cells (Figure 7E). For all silencing conditions, stimulation with LPA increased both stress fiber number and MLC phosphorylation slightly (supplemental Figure IX), whereas Y27632 dramatically reduced both stress fiber number and MLC phosphorylation (supplemental Figure X). The differences in stress fiber distribution and orientation between ROCK1- and ROCK2-silenced VSMCs raised the possibility that the axis of force generation may be altered when ROCK isoform expression is silenced. The direction of movement of the microfabricated posts was used to determine the average longitudinal and transverse components of force, which were plotted for each silencing condition (Figure 7F). For both scrambled and ROCK1-silenced cells, the longitudinal and transverse forces were similar and the force vectors pointed toward the center of the cell. However, in ROCK2-silenced cells, the force vectors were random and small, possibly reflecting the haphazard distribution of stress fibers as seen in Figures 5E, 6A, and 7E.

Discussion

There is little known about the ROCK isoform specificity of MLCP regulation or the mechanism whereby ROCK interacts with MLCP. We have found that ROCK2 specifically is found in a complex with MBS in the cell. Although MP-RIP targets RhoA to MLCP,30 MP-RIP does not bind ROCK.28 Our data confirm that ROCK and MP-RIP bind separate domains of MBS. The data presented here support a model where RhoA bound to MP-RIP and ROCK bound to MBS are brought into proximity by MP-RIP/MBS binding.

In the first demonstration of ROCK2-MLCP binding, we found that amino acids 683 to 866 bind directly to amino acids 354 to 775 of ROCK2. The ROCK2 binding domain on MBS is predicted to contain a coiled coil structure between amino acids 704 and 764. The MBS binding domain of ROCK2, amino acids 354 to 775, is also predicted to be a coiled coil structure, suggesting that coiled-coil binding mediates their interaction. Future studies will further characterize the specific residues involved in their interaction. Although ROCK1 338 to 750 is also predicted to form a coiled coil, the amino acid homology between ROCK1 and -2 in this domain is only 58%, suggesting that the specific residues that mediate the ROCK2-MBS interaction differ between the 2 ROCK isoforms. In fact, this domain of ROCK1, but not ROCK2, has been found to bind to RhoE and PDK1, further supporting that this region of ROCK1 and ROCK2 mediates isoform-specific interactions.40,41 Interestingly, in GST fusion protein interaction assays, both ROCK1 and -2 from cell lysates could interact with MBS 683 to 866. However, twice as much ROCK2 bound MBS. These data suggest that there may be an intermediary protein(s) involved in ROCK1-MBS binding and that this interaction may become apparent when relatively high concentrations of GST-MBS peptide are used for binding studies. This potential indirect interaction between ROCK1 and MBS will require further investigation.

When the roles of the ROCK isoforms in MBS and MLC phosphorylation were tested, overexpression of both isoforms increased MLC phosphorylation. A previous study has shown that the ROCK isoforms lose their specificity when overexpressed,33 and therefore silencing studies were also undertaken. Interestingly, silencing of each ROCK isoform led to upregulation of the other isoform, suggesting that the expression level of the ROCK isoforms is tightly controlled and inter-related in VSMCs. Silencing of either ROCK isoform lead to reduced MBS and MLC phosphorylation. Furthermore, combined ROCK isoform silencing, despite that down-regulation of the ROCK isoforms was less efficient, lead to a significantly greater reduction in MBS and MLC phosphorylation than either isoform alone. These data support that both ROCK isoforms regulate MBS and MLC phosphorylation in VSMCs and, further, taken together with the binding data showing different mechanisms of interaction with MBS, suggest that their respective mechanisms are potentially distinct.

ROCK is known to regulate cell morphology, including the formation of actin–myosin stress fibers and focal adhesion complexes. Recently, 2 studies examined the roles of ROCK1 and -2 on fibroblast morphology with different results,33,42 and thus the effects of the ROCK isoforms on actin cytoskeletal architecture remains unresolved. In VSMCs, ROCK1 and ROCK2 had opposing effects, suggesting a cooperative role for the 2 ROCK isoforms where both are required to regulate cell morphology.

The measurement of contractility in single VSMCs is complicated by the strong focal adhesion contacts between the VSMC and the underlying substrate, limiting movement of the cell. This limitation has been addressed previously by placing VSMCs on flexible polymer substrates that can be pulled by the contracting cell. Force can be estimated by counting wrinkles in the polymer substrate or by measuring the movement of beads embedded in the polymer. In this study, a recently developed technology to precisely calculate intracellular forces from single cells was adapted to measure contractility in VSMCs in which ROCK1 or ROCK2 expression was silenced.

The present study is the first to demonstrate that ROCK2 is the isoform that regulates VSMC force production and contractility. Furthermore, the specificity of ROCK2 for direct MLCP binding suggests that this interaction may be critical for contractile regulation and is thus a potential target for the treatment of cardiovascular diseases. The regulation of actin cytoskeletal organization may also contribute to the differential roles of the ROCK isoforms in contractility. The plot of force direction versus contraction axis suggests that force remains aligned with the major axes of the cell in ROCK1 but not in ROCK2-silenced cells. This is corrobor-
rated by immunofluorescence microscopy showing preserved phospho-MLC along the long axis of the cell in ROCK1-silenced cells, but in fragmented multidirectional fibers in ROCK2-silenced cells. These data suggest that the abnormal distribution of actin–myosin fibers may cause a disorganized orientation of force production and thus contribute to the reduced contraction in ROCK2-silenced VSMCs. These data further suggest that both force production and the orientation of force producing fibers are important in VSMC contractility. Interestingly, the reduction in force production in ROCK2-silenced cells was more pronounced than the reduction in contractility. This discrepancy may be related to the fewer focal adhesions in ROCK2-silenced cells, causing reduced tethering of the cell to the microfabricated posts and allowing more length change per unit of force.

The role of the RhoA/ROCK signaling pathway in the regulation of vascular smooth muscle contractility and vascular tone is well established, although the mechanisms are incompletely understood. ROCK inhibitors have been shown to ameliorate vascular disease in animal models and are promising future therapies for human cardiovascular disease (reviewed elsewhere). Although relatively specific for ROCK, the inhibitors may be active against other kinases, and do not exhibit specificity for the individual ROCK isoforms. A careful dissection of the specific roles of the 2 ROCK isoforms in vascular function may provide the opportunity to create therapies that target specific ROCK-mediated functions, while leaving other essential ROCK functions intact. This study indicates that whereas a balance of ROCK1 and ROCK2 activities is required to regulate VSMC actin cytoskeletal structure, ROCK2 is the predominant isoform that regulates VSMC contractility.

Acknowledgments

The authors wish to thank Dr Shuh Narumiya for the ROCK-Rho–binding domain cDNA, and Dr Kozo Kaibuchi for the ROCK-Rho–binding domain cDNA. We also thank Drs Richard Karas and Michael E. Mendelsohn for helpful discussions.

Sources of Funding

Supported by NIH grants HL074069 and HL077378 to (H.K.S.). X.Z. has grant support from the National Science Foundation.

Disclosures

None.

References


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_Circ Res._ 2009;104:531-540; originally published online January 8, 2009;
doi: 10.1161/CIRCRESAHA.108.188524

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://circres.ahajournals.org/content/104/4/531

Data Supplement (unedited) at:
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Supplement Material

Materials and Methods:

Reagents: Antibodies used were as follows; goat anti-ROCK1, goat anti-ROCK2, rabbit anti-myosin light chain and anti-6xHis from Santa Cruz Biotechnology (Santa Cruz, CA), mouse anti-ROCK1, mouse anti-ROCK2, mouse anti-MYPT1 (MBS), anti-phosphothreonine 18, serine19 myosin light chain and anti-p116RIP from BD Biosciences (Franklin Lakes, NJ), MYPT1 anti-phosphothreonine 696 and 850 from Millipore (Billerica, MA), anti-GAPDH was from Sigma-Aldrich (St. Louis, MO). Mouse anti-vinculin was from Abcam (Cambridge, MA). 1-Oleoyl-sn-glycerol 3-phosphate (lysophosphatidic acid) was purchased from Sigma-Aldrich. Y27632 and blebbistatin were purchased from Calbiochem Biochemicals. 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI), Alexa Fluor 488 phalloidin, donkey anti-mouse Cy3 and Slowfade reagent were from Molecular Probes.

Co-immunoprecipitation Assays: A7r5 or primary rat aortic smooth muscle cells were maintained in DME with 10% serum, or were serum-deprived for 24 hours and treated with 10% serum or 1µM lysophosphatidic acid for 5 minutes. The cells were rinsed with cold PBS, then lysed in buffer A (50mM Tris pH 7.5, 150mM NaCl, 5% glycerol, 1% Triton X, 10mM MgCl₂, 1mM EGTA, 1mM dithiothreitol, 25mM NaF, 20mM β-glycerophosphate, 1mM Na₃VO₄, 0.01μg/ml each of aprotinin, leupeptin and pepstatin A, 2mM phenylmethylsulfonyl fluoride). Immunoprecipitations were performed as described¹. The cell lysates were microcentrifuged at 4°C for 5 minutes, then incubated with protein G beads for 15 minutes at 4°C and centrifuged. The supernatants were then incubated with pre-coupled ROCK1 or ROCK2 antibodies bound to protein G
beads for 30 minutes at 4°C, rocking. The beads were centrifuged and washed three times in buffer A, then boiled in protein SDS sample buffer. Samples were resolved on 7.5% SDS PAGE gels and bound proteins determined by immunoblotting with the indicated antibodies.

GST-fusion protein Interaction Assays: The indicated sequences of human MBS (Full-length MYPT1, kind gift of Dr. Masumi Eto) were amplified by PCR and ligated into pGEX vectors for expression as GST-fusion proteins in bacteria. The PCR primers for each MBS domain are as follows (5’ to 3’): 1-850-5’ (amino acids 1-283):

TAGGATCCAAAGATGGCGGACGCGAAGCAGAAG, 1-850-3’:

GCGAATTGCAGAACTCTTTCTAAATATCCTAAAAA, 800-1650-5’ (amino acids 266-550):

TAGGATCCCCAACAGCCTTTTGTATGTAGCAGAT, 800-1650-3’:

GCGAATTCTGATACGTTGATCCTTCATTAAC, 1620-2575-5’ (amino acids 540-858):

TAGGATCCAAATAGCTCAGTTAATGAAGGATCA, 1620-2575-3’:

GCGAATTCTCCAAATAGAACTCCTGTGATC, 2050-2600-5’ (amino acids 683-866):

TAGGATCCAAAGCAAGATCTAGACAAGCAAGA, 2050-2600-3’:

GCGAATTCTGTTATTTTCATCAGTATCTTGT. MBS 2550-3090 (amino acids 850-1030) was from 2. All PCR products were sequenced in both directions. GST-fusion proteins were expressed in bacteria and purified on glutathione-agarose beads as described 3. Untransfected A7r5 cells and HEK293 cells transfected with mycROCK1 or mycROCK2 (plasmids kindly provided by Prof. Shuh Narumiya) were lysed in buffer A as above, microfuged and the supernatant incubated with GST-fusion proteins for 1 hour at 4°C, rocking. The beads were then washed three times with buffer A, then
boiled in SDS sample buffer. The bound proteins were resolved by 7.5% SDS-PAGE and immunoblotted with the indicated antibodies.

Direct Protein Domain Interaction Assays: The indicated sequences of human ROCK1 and 2 were amplified from the full length cDNA by PCR and ligated into pQE vectors for expression of 6xHis-tagged proteins in bacteria. The PCR primers for the ROCK domains are listed as follows (5' to 3'): ROCK1 1-1020 5' (amino acids 1-340): CCGATCCTCGACTGGGGGACAGTTTTGAGACTCGA, ROCK1 1-1020-3': GCAAGCTTATTTTGAAGAAGAGATGTCGTTTGAT, ROCK2 1-1080-5' (amino acids 1-360): CCGATCCAGCCGGCCCGCCCGACGGGGAAAATG, ROCK2 1-1080-3': GCAAGCTTATTCTTACATTTACATTTAAAGAAAGG, ROCK1 1014-2250-5' (amino acids 338-750): CCGATCCTTTAAAAATGACCAGTGGGCTTGGGAA, ROCK1 1014-2250-3': GCAAGCTTTGGCTTCCAGATCAACGTCTAGCATGGA, ROCK2 1062-2325-5' (amino acids 354-775): CCGATCCAAGAATGATCAATGGAATTGGGATAAC, ROCK2 1062-2325-3': GCAAGCTTTATTTAGCTTCTGCTGAGACTGTTTGAG, ROCK1 2100-3354-5' (amino acids 1118-1354): CCGATCCTCAAGAATTGAAGGTTGGCTTTCAGTA, ROCK1 2100-3354-3': GCAAGCTTTCTCTGGAGGTACCATCAGTTCATC, ROCK2 2175-3450-5' (amino acids 725-1150): CCGATCCGAAGAAGCTTAAATCAGAAGCCATGAAA, ROCK2 2175-3450-3': GCAAGCTTTTCTGGAAATCCATCATCAGGCTCAGC, ROCK1 3354-4062-5' (amino acids 1150-1388): CCGATCCTCAAGAGATGATTGGTGTTGTCATTG,
ROCK2 3450-4164-3': GCAAGCTTGCTTGGCTTGTTTGGAGCAAGCTGTCG. All ROCK constructs were fully sequenced in both directions. ROCK1 and 2 domains were expressed in bacteria, affinity purified and the 6xHis-fusion proteins eluted from the 6xHis beads with 250mM imidazole. The soluble 6xHis-fusion proteins were mixed with GST or GST-MBS683-866 for 1 hour at 4°C in buffer B (50mM Tris pH 7.6, 7mM MgCl$_2$, 2mM EDTA, 2mg/ml n-dodecyl-B-maltoside, 0.4mg/ml cholesteryl hemisuccinate, 1% Triton X, 0.6M NaCl, 10mM Na molybdate, 0.01µg/ml each of aprotinin, leupeptin and pepstatin A, 2mM phenylmethylsulfonyl fluoride). The beads were washed three times in buffer B, and boiled in SDS sample buffer. Bound 6xHis-fusion proteins were detected by SDS-PAGE on 10% gels and immunoblotting with anti-6xHis antibodies.

Transfection: A7r5 cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. HEK293 cells were transfected with Polyfect (Qiagen) according to the manufacturer’s instructions.

MBS and MLC Phosphorylation Assays: A7r5 cells were maintained in DME with 10% serum following siRNA transfection. Some cells were serum-deprived for 24 hours before the assay, and others were serum-deprived then treated with 10% serum for the indicated times for the phosphorylation time course experiments. The cells were then rinsed once with cold PBS. To the cells was added 2mM EDTA, 10mM dithiothreitol and 10% trichloroacetic acid in DME. The cells were scraped on ice, microcentrifuged for 20 minutes at 4°C, then washed three times in cold acetone with 2mM dithiothreitol. The
washed cell pellets were air dried, then solubilized by sonication in SDS sample buffer. The protein was resolved by SDS-PAGE followed by immunoblotting with phospho-specific antibodies against MBS Thr696 and Thr850 and MLC Thr18/Ser19.

Immunofluorescence microscopy: Cells grown on glass coverslips coated with fibronectin were rinsed in PBS, then fixed in 3.7% paraformaldehyde. The cells were then permeabilized with 0.3% Triton X in 10% donkey serum, and blocked an additional 1 hr in 10% donkey serum. The cells were labeled with Alexa Fluor 488 phalloidin, anti-vinculin or anti-phospho-MLC for 1 hr. Vinculin and phospho-MLC labeled cells were then rinsed in PBS and labeled with donkey anti-mouse Cy3 for 1 hr, and all coverslips were incubated with DAPI stain for 5 mins. The coverslips were mounted on slides with SlowFade reagent. Cells were imaged on a Nikon Optiphot-2 microscope with Nikon Plan 20/0.50 and Plan 40/0.70 objectives and coupled to a SPOT CCD camera. Cell area was measured using NIS Elements software. Stress fibers and focal adhesions were counted manually.

siRNA: A7r5 and primary rat aortic smooth muscle cells were transfected at 20% confluency using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Oligonucleotides for rat ROCK1 and ROCK2 were as described \(^1\) and were synthesized by Dharmacon (Lafayette, CO) (ROCK1 UCG GCA GAG GUG CAU UUG G, ROCK2 CGU GGA, AAG, CCU, GCU, GGA, U). A second ROCK2 oligonucleotide (R2b), was purchased from Dharmacon HP GenomeWide siRNA (GUC UAU UAA UAC UCG UCU A).
Smooth muscle cell contractility assay: Measurement of contractility of single VSMCs was performed by plating VSMCs on a polymer substrate consisting of microfabricated posts. The cells form adhesions on the tops of the flexible posts, and are thus free to contract, unhindered by their attachments, causing movement of the posts which can subsequently be measured allowing calculation of the mechanical forces exerted upon them. This method has been previously published for contractile force measurement in cardiomyocytes\textsuperscript{4, 5} and traction force measurement\textsuperscript{6-9}. The posts are made by replica molding from the polymer polydimethylsiloxane (PDMS), which is nontoxic and supports cell growth (see\textsuperscript{4} for details). The tops of the posts were coated with fibronectin, to facilitate VSMC attachment. The diameter of the posts was 2-4μm and the height was 6-10μm. Primary rat aortic smooth muscle cells were seeded onto the microfabricated post structures in perfusion chambers in DME with 10% bovine growth serum overnight, then changed to serum-free media for 48 hrs. For imaging of contractions, the samples were moved to the microscope stage (Nikon TE2000-U microscope with Nikon S Fluor 20X/0.75 objective) and enclosed in a LiveCell environment chamber (Neue biosciences, Inc) where they were maintained at 37°C and at 5% CO\textsubscript{2}. After the addition of agonist, the cells were imaged every minute for 30 minutes using a Cool SNAP EZ CCD camera (Photometrics) with NIS Elements software. Cell length change following agonist stimulation was measured using Image Pro Plus 6.0 software.

Cellular Force Measurement: Background noise from captured images was filtered (MATLAB) and the displacement of microfabricated posts derived from comparison to a
reference array. A displacement map was generated and force calculated based on the relationship $F=Kx$ where $x$ is the lateral deformation and $K$ is the spring constant of the post (determined from height, diameter and Young’s Modulus of the cantilever)\(^4\). Force was measured in 20 cells from each silencing condition.

Statistics: One way ANOVA was used in Figures 2, 5, 6 and 7. A p value of < 0.05 was considered statistically significant.

Amino acid alignment was performed using the BLAST 2 sequences program from the National Center for Biotechnology Information (NCBI):


**Online Figure Legends:**

**Online Figure I:** GST and GST-fusion proteins encompassing the entire M-RIP molecule and the MBS domain that binds ROCK in Figure 3A were tested for ROCK2 binding from A7r5 cell lysates. Minimal ROCK binding to M-RIP was detected. ROCK2 bound MBS 683-866 as in Figure 3A.

**Online Figure II:** Amino acid alignment of human ROCK1 338-750 with the ROCK2 MBS binding domain, 354-775. ROCK2 and ROCK1 sequences are labeled on the left. There is 58% homology between the two sequences. The alignment was performed using BLAST 2 sequences from the NCBI:

Online Figure III: A7r5 cells transfected with GFP (top row) or Myc-ROCK-Rho-binding domain (Myc-ROCK-RBD, bottom row). The GFP transfected cells were immunostained with anti-phospho-MLC-Cy3 (top, left panel) and imaged for green fluorescence (top, right panel). The Myc-ROCK-RBD transfected cells were immunostained with both anti-phospho-MLC-Cy3 (bottom, left panel) and anti-Myc-FITC (bottom, right panel). The arrowheads indicated transfected cells. The scale bar is 30μm.

Online Figure IV: Phosphorylation of MBS at the known inhibitory site Thr696 in A7r5 cells, following the silencing conditions described in 5B (Top). Total MBS is shown as a loading control. Pooled data from 4 experiments of MBS phosphorylation at Thr696 following ROCK isoform silencing (Bottom). The values are represented as Thr696 phosphorylation normalized to MBS expression for each sample.

Online Figure V: Immunofluorescence microscopy of representative primary rat aortic VSMCs following scrambled negative control (Scr, top row), ROCK1 (middle row) and ROCK2 (bottom row) silencing. The cells were plated on fibronectin coated coverslips, serum-deprived for 48 hours and treated with LPA for 30 minutes. Immunofluorescence labeling was performed with phalloidin (left column) to image actin fibers and vinculin (right column) to image focal adhesions. The scale bar is 30μm.
**Online Figure VI:**  Primary rat aortic VSMCs after silencing of ROCK isoform expression as in Supplementary Figure 5 above, and treated with Y27632 for 30 minutes before fixation. The cells were labeled with phalloidin and vinculin as above. The scale bars are 30μm.

**Online Figure VII:**  Inhibition of rat aortic smooth muscle cell contraction by Y27632. Untransfected cells were plated on micrfabricated posts, and stimulated with 1μM LPA with and without pretreatment with 10μM Y27632 for 30 minutes. The number of contracting cells per field were counted and averaged over three experiments. Control and Y27632-treated samples contained similar numbers of cells.

**Online Figure VIII:**  Inhibition of rat aortic smooth muscle cell contraction by blebbistatin. Untransfected cells were plated on micrfabricated posts, and stimulated with 1μM LPA with and without pretreatment with 20μM blebbistatin for 30 minutes. The number of contracting cells per field were counted and averaged over three experiments. Control and blebbistatin-treated samples contained similar numbers of cells.

**Online Figure IX:**  Immunofluorescence microscopy of representative primary rat aortic VSMCs following scrambled negative control (Scr, top row), ROCK1 (middle row) and ROCK2 (bottom row) silencing. The cells were plated on fibronectin coated coverslips, serum-deprived for 48 hours and treated with LPA for 30 minutes. Immunofluorescence labeling was performed with phalloidin (left column) to image actin fibers and phospho-MLC (right column). The scale bar is 30μm.
**Online Figure X:** Primary rat aortic VSMCs after silencing of ROCK isoform expression, and treatment with Y27632 for 30 minutes before fixation. The cells were labeled with phalloidin and phospho-MLC as above. The scale bar is 30μm.

**Online video 1:** Primary rat aortic smooth muscle cells, 48 hours after transfection with scrambled dsRNA were plated on microfabricated posts, serum deprived for 48 hours and stimulated with 1μM LPA. The image capturing begins when LPA is added, and continues to capture 1 frame per minute for 30 minutes.

**Online video 2:** Primary rat aortic smooth muscle cells, 48 hours after transfection with ROCK1 specific dsRNA were plated on microfabricated posts, serum deprived for 48 hours and stimulated with 1μM LPA. The image capturing begins when LPA is added and continues to capture 1 frame per minute for 30 minutes.

**Online video 3:** Primary rat aortic smooth muscle cells, 48 hours after transfection with ROCK2 specific dsRNA were plated on microfabricated posts, serum deprived for 48 hours and stimulated with 1μM LPA. The image capturing begins when LPA is added and continues to capture 1 frame per minute for 30 minutes.
Reference List


(3) Surks HK, Richards CT, Mendelsohn ME. Myosin Phosphatase-Rho Interacting Protein: A New Member of the Myosin Phosphatase Complex that Directly Binds RhoA. *J Biol Chem.* 2003;278:51484-93.


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Online Figure IV

MBS-P696

MBS

P-696/Total MBS

Scr  R1  R2a  R2b  2xScr  R1+R2

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Online Figure V

LPA

Phalloidin  Vinculin

Scr RNAi

ROCK1 RNAi

ROCK2 RNAi
Online Figure VI

Y27632

Scr RNAi

ROCK1 RNAi

ROCK2 RNAi

Phalloidin

Vinculin
Online Figure VII

![Bar chart showing the number of contracting cells/field for Control and Y27632. The chart indicates a significant increase in the number of contracting cells for Y27632 compared to the Control.](image-url)
Online Figure VIII

The graph shows the number of contracting cells per field under control and Blebbistatin conditions. There is a significant difference, with the control group having a much higher number of contracting cells compared to the Blebbistatin group.
Online Figure IX

LPA

- Scr RNAi
- ROCK1 RNAi
- ROCK2 RNAi

Phalloidin

p-MLC