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A Method for Measuring Rho Kinase Activity in Tissues and Cells

Ping-Yen Liu and James K. Liao

Vascular Medicine Research Unit, Brigham and Women's Hospital and Harvard, Medical School, Boston, Massachusetts

Abstract

The Rho-associated kinases (ROCKs) can regulate cell shape and function by modulating the actin cytoskeleton. ROCKs are serine-threonine protein kinases that can phosphorylate adducin, ezrin-radixin-moesin proteins, LIM kinase, and myosin light chain phosphatase. In the cardiovascular system, the RhoA/ROCK pathway has been implicated in angiogenesis, atherosclerosis, cerebral and coronary vasospasm, cerebral ischemia, hypertension, myocardial hypertrophy, and neointima formation after vascular injury. ROCKs consist of two isoforms: ROCK1 and ROCK2. They share overall 65% homology in their amino acid sequence and 92% homology in their amino kinase domains. However, these two isoforms have different subcellular localizations and exert biologically different functions. In particular, ROCK1 appears to be more important for immunological functions, whereas ROCK2 is more important for endothelial and vascular smooth muscle function. Thus, the ability to measure ROCK activity in tissues and cells would be important for understanding mechanisms underlying cardiovascular disease. This chapter describes a method for measuring ROCK activity in peripheral blood, tissues, and cells.

1. Introduction

The small GTP-binding proteins act as molecular “on-off” switches to control multiple biological signaling pathways (Etienne-Manneville and Hall, 2002; Hall, 1998). The Rho-associated kinases (ROCKs) are found to be one of the downstream targets of RhoA (Ishizaki *et al.*, 1996; Matsui *et al.*, 1996). They not only regulate cell growth, migration, and apoptosis via control of the actin cytoskeletal assembly, but also the contraction of different cells through serine-threonine phosphorylation of adducin, ezrin-radixin-moesin (ERM) proteins, LIM kinase, and myosin light chain phosphatase (MLCP) (Riento and Ridley, 2003). Vascular smooth muscle cells (VSMCs) play an especially important role in the modulation of vascular tone and in the pathogenesis of atherosclerosis and vascular proliferative disease. ROCKs increase myosin light chain (MLC) phosphorylation through phosphorylating and inhibiting the myosin-binding subunit (MBS) of MLC phosphatase, thereby increasing MLC phosphorylation and contraction (Kimura *et al.*, 1996; Somlyo and Somlyo, 1994). They can also phosphorylate and inhibit LIM kinase, which phosphorylates the cofilin/actin-depolymerizing factor complex involved in the depolymerization and severing of actin filaments (Maekawa *et al.*, 1999). Overall, the physiological effects of ROCKs are to enhance actin-myosin association through increasing MLC phosphorylation and preventing actin depolymerization.

The Rho-associated kinases are also important regulators of cellular apoptosis, growth, metabolism, and migration via control of actin cytoskeletal assembly and cell contraction (Riento and Ridley, 2003). In the cardiovascular system, the RhoA/ROCK pathway has been shown to be involved in angiogenesis (Hyvelin *et al.*, 2005), atherosclerosis (Mallat *et al.*, 2003), cerebral and coronary vasospasm (Sato *et al.*, 2000), cerebral ischemia (Toshima *et al.*, 2000), hypertension (Uehata *et al.*, 1997), myocardial hypertrophy (Higashi *et al.*, 2003), and neointima formation after vascular injury (Sawada *et al.*, 2000). Increased leukocyte

ROCK activity has been found to be associated with cardiovascular risk factors (Liu *et al.*, 2007), and inhibition of ROCK leads to an improvement in endothelial function (Nohria *et al.*, 2006).

In the mammalian system, ROCKs consist of two isoforms: ROCK1 and ROCK2. ROCK1, which is also known as ROCK- β or p160ROCK, is located on chromosome 18 and encodes a 1354 amino acid protein (Ishizaki *et al.*, 1996). ROCK2, which is also known as ROCK- α , is located on chromosome 12 and contains 1388 amino acids (Riento and Ridley, 2003). ROCK1 and ROCK2 share overall 65% homology in their amino acid sequence and 92% homology in their kinase domains. ROCK1 and ROCK2 are expressed ubiquitously in mouse tissues from early embryonic development to adulthood. However, these two isoforms have different tissue distribution and exert disparate biological functions (Noma *et al.*, 2007). For example, ROCK1 is expressed preferentially in the lung, liver, spleen, kidney, and testis, whereas ROCK2 is highly expressed in the heart and brain (Nakagawa *et al.*, 1996; Wei *et al.*, 2001). In addition, immunolocalization and cell fractionation studies have shown that inactive ROCK2 is distributed mainly in the cytoplasm but could translocate from the cytoplasm to membranes when activated by GTP-bound RhoA (Matsui *et al.*, 1996). In contrast, ROCK1 colocalizes within or near the centrosomes (Chevrier *et al.*, 2002). Functionally, ROCK2 phosphorylates Ser¹⁹ of MLC, the same residue that is phosphorylated by MLC kinase and thus increases cellular contractility via dual effects on MLC kinase and MLCP. Indeed, ROCK2 can alter the sensitivity of VSMC contraction in response to changes in Ca²⁺ concentration (Amano *et al.*, 1996). Furthermore, ROCKs could also phosphorylate ERM proteins, which serve as cross-linkers between actin filaments and membrane proteins at the cell surface. ROCK-mediated phosphorylation of ERM proteins, namely Thr⁵⁶⁷ of ezrin, Thr⁵⁶⁴ of radixin, and Thr⁵⁵⁸ of moesin, leads to the disruption of the head-to-tail association of ERM proteins and actin cytoskeletal reorganization (Matsui *et al.*, 1998).

2. Downstream Targets of ROCK

The Rho-associated kinases phosphorylate various targets and mediate a broad range of cellular responses that involve the actin cytoskeleton in response to GTP-bound RhoA by activators of RhoA such as lysophosphatidic acid or sphingosine-1 phosphate, which stimulate Rho GEFs. The consensus amino acid sequences for phosphorylation are R/KXS/T or R/KXXS/T (R, arginine; K, lysine; X, any amino acid; S, serine; T, threonine) (Kawano *et al.*, 1999; Sumi *et al.*, 2001). ROCKs can also be autophosphorylated, suggesting that the function of ROCKs may be dependent in part on autoregulation (Maekawa *et al.*, 1999). MBS on MLCP is an important downstream target protein of ROCKs. Phosphorylation of MBS on MLCP by ROCKs leads to the phosphorylation of MLC and subsequent contraction of VSMCs (Somlyo and Somlyo, 2000). The MLCP holoenzyme is composed of three subunits: a catalytic subunit (PP1), a MBS composed of a 58-kDa head and a 32-kDa tail region, and a small noncatalytic subunit, M21.

As mentioned previously, ROCK2 can phosphorylate MBS at Thr⁶⁹⁷, Ser⁸⁵⁴, and Thr⁸⁵⁵ (Kawano *et al.*, 1999). The functional significance of MBS phosphorylation at Ser⁸⁵⁴, however, is not known. Phosphorylation of Thr⁶⁹⁷ or Thr⁸⁵⁵ attenuates MLCP activity and, in some instances, the dissociation of MLCP from myosin (Feng *et al.*, 1999; Velasco *et al.*, 2002). In addition, MLC is one of the major downstream target proteins of ROCKs. However, it is still not known whether phosphorylation of MBS on MLCP or ERM proteins is specific to ROCK isoforms. Nevertheless, ROCK1 phosphorylates LIM kinase-1 at Thr⁵⁰⁸ and LIM kinase-2 at Thr⁵⁰⁵, which inhibits cofilin-mediated actin filament disassembly by phosphorylating cofilin (Maekawa *et al.*, 1999; Ohashi *et al.*, 2000; Sumi *et al.*, 2001). Adducin, which is a membrane skeletal protein that associates with and promotes the association of spectrin with F-actin, is also a downstream target of ROCK2 (Fukata *et al.*, 1999; Kimura *et al.*, 1998). Adducin is

localized at cell-cell contact sites and is thought to participate in the assembly of the spectrin-actin network by capping the fast-growing ends of actin filaments and recruiting spectrin to the filament ends. The phosphorylation of α -adducin by ROCK2 enhances the binding activity of α -adducin to F-actin, thereby increasing the contractile response.

In order to assess ROCK function *in vitro* and *in vivo*, it is important to accurately measure ROCK activity in tissues and cells. This chapter provides a method for the preparation and analysis of total ROCK activity in cultured cells and tissues, as well as leukocytes isolated from peripheral blood of humans or mice.

3. Measurement of ROCK Activity

For accurate and reproducible measurements of ROCK activity, careful attention should be paid to tissue preparation in order to avoid cell lysis and thus prevent overphosphorylation of MBS. ROCK inhibitors could be added during preparation to stop further phosphorylation in some tissues that are sensitive to ongoing activators of ROCK signaling. For human studies, a protocol is provided showing the proper method for collecting tissues or leukocytes from peripheral blood.

4. Cultured Cells

To avoid degradation of target proteins by cellular enzymes, cells must be prepared rapidly in the presence of various protease inhibitors. Thus, we suggest that the number of cell culture dishes be kept at a minimum in order to conserve time. At the appropriate time point, the culture medium is removed rapidly and completely, and the monolayer of adherent cells is washed twice with ice-cold phosphate-buffered saline (PBS) before the addition of cell fixatives and ROCK inhibitor. Approximately 300 μ l of fixative solution (see later) is added at room temperature to each 10-mm culture dish, followed by the addition of 1 mM of hydroxyfasudil (ROCK inhibitor) to stop further MBS phosphorylation after cell lysis. A cocktail of proteinase inhibitors (see later) is also added to avoid protein degradation. All culture dishes should be kept on ice during isolation. Scrape the cell contents and transfer them to a microcentrifuge tube on ice. After vortexing and centrifuging the samples at 4° for 5 min at 14,000 rpm, the supernatant is removed and the pellets are used for ROCK assay. Pellets are stored at -80° until use.

4.1. ROCK activity as determined by the level of MBS phosphorylation

Making two 7.5% separating gels with 1.5-mm spacers requires a 20-ml solution consisting of 9.6 ml H₂O, 5 ml 30% acrylamide/bisacrylamide, 5 ml Tris (1.5 M, pH 8.8), 200 μ l 10% SDS, 200 μ l 10% ammonium persulfate, and 12 μ l tetramethylethylenediamine (TEMED). After the separating gels solidify (approximately 30 to 60 min), the stacking gel solution (5% acrylamide) is added to the top of the separating gel. For two gels, prepare a 5-ml solution consisting of 3.44 ml H₂O, 833 μ l 30% acrylamide/bisacrylamide, 625 μ l Tris (1 M, pH 6.8), 50 μ l 10% SDS, 50 μ l 10% ammonium persulfate, and 5 μ l TEMED.

We use SDS-PAGE buffer as the electrophoresis buffer (for recipe, see later). After centrifugation, pellets are dissolved in 10 μ l of 1 mol/liter Tris and mixed with 100 μ l of extraction buffer (8 mol/liter urea, 2% SDS, 5% sucrose, 5% 2-mercaptoethanol, 0.02% bromophenol blue). A SDS-PAGE protein standard (i.e., Bio-Rad, Richmond, CA) should be loaded on each gel in a separate lane. To avoid the interference by different exposure durations and variable membrane conditions, we use lipopolysaccharide-pretreated NIH/3T3 cell lysates as a positive control and also to standardize results between different experiments. Standard size gels are electrophoresed at 130 V at 23° for 1.5 h. After complete electrophoresis, the proteins are then transferred to polyvinylidene fluoride membranes (Immobilon P, Millipore

Bedford, MA). The membrane is then soaked for 5 s in methanol and washed briefly in H₂O. The gel, transfer membrane, and filter paper are then soaked in transfer buffer (for recipe, see later) for 5 min.

For transferring, mount the following layers in order from bottom (anode of transfer apparatus) to top (cathode of transfer apparatus): one buffer-soaked thick filter paper, the transfer membrane, the gel, and one buffer-soaked thick filter paper. Air bubbles between these layers should be avoided and removed by gently rolling a glass pipette over the transfer membrane. Negatively charged proteins will move downward (from the gel into the membrane). The proteins are transferred at 105 V for about 105 min at 4°. Blocking of unspecific binding sites is achieved by incubating the membrane in PBS with 0.1% Tween and 5% milk for 0.5 h at room temperature or overnight at 4°.

The membranes are then incubated with rabbit antiphospho-specific Thr⁸⁵³-MBS polyclonal antibody (Biosource) and rabbit anti-MBS polyclonal antibody (Covance). Bands are visualized using the ECL detection kit (Amersham Corp./New England Nuclear). ROCK activity is expressed as the ratio of pMBS in each sample per pMBS in each positive control divided by MBS in each sample per MBS in each positive control (Fig. 14.1).

4.2. Reagents

Fixative solution: 50% trichloroacetic acid (Sigma), 50 mM dichlorodiphenyltrichloroethane (Sigma), protease inhibitors (Calbiochem, EMD Biosciences, Inc., Darmstadt, Germany), 1 mM phenylmethylsulfonyl fluoride, and 1 mM NaF. The last three substances should be added immediately before use.

SDS-PAGE buffer, 10× (use dilution 1×): 250 mM Tris, 1.92 M glycine, and 1% SDS, pH 8.3

Western blot transfer buffer, 1×: 25 mM Tris, pH 8.3, 190 mM glycine, and 10% methanol

PBST, pH 7.4: 0.1% Tween 20 dissolved in PBS

5. Leukocytes

To isolate human leukocytes, 20 ml of blood sample is mixed with Hanks balanced salt solution (HBSS) in a 50-ml citrate-containing tube. Ten milliliters of Histopaque (Sigma, Histopaque-1077) is layered with 25 ml of diluted blood in two tubes and centrifuged at room temperature for 30 min at 1400-1500 rpm. The supernatant containing the leukocytes is aspirated, mixed with HBSS, diluted with 2% dextran (1:1 ratio), and allowed to sit at room temperature for 30 min. The top layer is then aspirated, mixed with HBSS, and centrifuged at room temperature for 5 min at 1400-1500 rpm. The supernatant is discarded, and the pellet containing the leukocytes is resuspended in 3 ml of cold PBS. After swirling the tubes for 30 s, HBSS is added to stop the lysis. After centrifugation, the supernatant is discarded and the pellet is resuspended with 5 ml of M199. After determining cell yield and viability by using the Trypan blue exclusion test (usually $4-8 \times 10^6$ viable cells with a viability of more than 95%), the suspension is diluted with HBSS to achieve 5×10^6 cells/ml. Then, 400 μ l of the leukocyte suspension is transferred to four sterile 1.5-ml tubes. We add 100 μ l of fixative solution (see earlier discussion) to each tube. To avoid overphosphorylation, 1 mM of hydroxyfasudil is added to the TCA fixative solution. After vortexing and centrifuging the samples at 4° for 5 min at 12,000 rpm, the supernatant is removed and HBSS is added. The samples are centrifuged again at 4° for 1 min at 12,000 rpm. The supernatant is removed with a micropipette. The remaining leukocyte pellets are stored at -80° until use.

6. Tissues

Similar to cell isolation, extended lysis of cells during preparation is to be avoided. First, HBSS solution is added to the tissue container with proteinase inhibitors and 1 mM hydroxyfasudil in order to avoid protein degradation and continued phosphorylation after lysis, respectively. In addition, a 50% fixative solution (see earlier discussion) is added and the samples are homogenized without sonication. The samples are then centrifuged at 4° for 10 min at 1500 rpm. The supernatant is discarded and the pellet is resuspended in 3 ml of cold PBS. After swirling the tubes for 30 s, HBSS is added to stop the lysis. Then, 400 µl of the suspension is transferred to four sterile 1.5-ml tubes. After vortexing and centrifuging the samples at 4° for 5 min at 12,000 rpm, the supernatant is removed, HBSS is added, and the samples are centrifuged again at 4° for 1 min at 12,000 rpm. Finally, the supernatant is removed with a micropipette. The pellets are stored at -80° until use. ROCK activity is determined by immunoblotting of pellets with the Phospho-Thr⁸⁵³ antibody as described (see Fig. 14.1, right panel with heart and lung tissue as an example).

7. Summary

By using peripheral blood leukocytes to assess ROCK activity, a less invasive method could be used to monitor ROCK activity *in vivo*. Given that ROCK activity contributes to vascular tone and VSMC contractility, such a method may be useful in assessing the role of ROCK in cardiovascular disease. Further studies are required with antibodies from distal targets of ROCK in order to determine whether methods could be developed that can distinguish ROCK1 and ROCK2 activities from total ROCK activity.

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Figure 14.1.

Rho-associated kinase activity as determined by the immunoblotting method in different cells and tissues. (Left) Example of phosphorylation levels of MBS (pMBS) and total MBS (tMBS) in cultured endothelial cells from mouse lung (MLEC) and peripheral blood leukocytes. Positive control (PC) represents cell lysates from 3T3 fibroblasts stimulated with lysophosphatidic acid. ROCK activity is expressed as a relative blot density ratio, which is standardized to the PC blot as $(\text{pMBS sample density}/\text{tMBS PC density})/(\text{tMBS sample density}/\text{tMBS PC density})$. (Right) Example of ROCK activity in tissues from mouse heart and lung, respectively.