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***In vitro* and *in vivo* approaches to assess Rho-kinase activity**

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Running Head: Rho-kinase activity

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i. Summary/Abstract

Increased arterial tone and the resulting rise in peripheral vascular resistance are major determinants of the elevated arterial pressure in hypertension. The RhoA/Rho kinase signaling pathway is now recognized as a major regulator of vascular smooth muscle contraction and arterial tone. Here we describe methods to directly and indirectly assess Rho kinase activity *in vitro*, and in cells and tissues.

ii. Key words

Rho-kinase, myosin light chain phosphatase, *in vitro* kinase assay, immunoblot.

1. Introduction

Arterial hypertension is a major risk factor of myocardial infarction and stroke that affects 25% of the adult population in industrialized societies. Increased arterial tone and the resulting rise in peripheral vascular resistance are major determinants of the elevated arterial pressure in hypertension (1). Since the discovery of the essential role of RhoA/Rho kinase (ROCK) signaling in the regulation of vascular tone (2) and the anti-hypertensive effect of ROCK inhibitors (1, 3), much evidence has accumulated to implicate ROCK activation as a common component for the pathogenesis of hypertension. Accordingly, ROCK inhibitors are now considered as promising future therapies against cardiovascular diseases (4).

Vascular tone is regulated by arterial wall smooth muscle cells contraction that depends on the myosin light chain phosphorylation (2). Activation of the RhoA/ROCK pathway leads to increased myosin light chain phosphorylation through inhibition of the myosin light chain phosphatase activity by phosphorylation of its regulatory subunit MYPT-1 at inhibitory sites T696 and T850 (5). T850 is considered

to be a ROCK-specific phosphorylation site whereas T696 can be phosphorylated by other kinases (5). There are two isoforms of ROCK, ROCK 1 and ROCK2 that are both expressed in many tissues, including in vascular smooth muscle cells.

Nevertheless, although both ROCK1 and ROCK2 regulate myosin light chain phosphatase and myosin light chain phosphorylation, the control of vascular smooth muscle cell contractility essentially depends on ROCK2 (6).

All these data point out the need to assess ROCK activity both *in vitro* as well as in cells or tissues and this chapter describes suitable methods for such ROCK assays.

These methods are dedicated to ROCK2, but similar assays can be performed for ROCK1 with the corresponding specific tools. *In vitro* kinase assay with recombinant ROCK2 allows to directly measure the enzymatic activity and to screen putative ROCK inhibitors while the same assay with ROCK2 immunoprecipitated from vascular cells and tissues from patients or animal models, gives access to the kinase activity in different physiological or pathological situations. This assay consists in measuring, by scintillation counting, the ROCK-mediated incorporation of ³²P into a substrate peptide. The measurement of the level of phosphorylation of MYPT-1 by immunoblotting is an alternative but indirect method to assess ROCK in vascular smooth muscle cells and tissues with the advantage to be cheaper, easier and safer to set up because of the absence of radioactivity.

2. Materials

Water used to prepare all solutions must be deionized and free of trace organic contaminants (ultrapure water). Solutions could be prepared previously and stored at 4°C. For kinase assay, buffers should be used the same day it is prepared.

2.1 Rho-kinase activity assay

1. Kinase buffer: Tris 20 mM, β -glycerophosphate 25 mM, EGTA 1 mM, Orthovanadate 0.1 mM, DTT 1mM. Mix these chemical compounds and adjust pH to 7.5.
2. ROCK2 solution: ROCK2 from Merck Millipore (cat. number 14-338). Prepare a ROCK2 solution at 4 mU of specific enzyme activity/ μ L of kinase buffer (Note 1)
3. $Mg^{2+}Cl_2$ /ATP cocktail (Merck Millipore, cat. number 20-113): 20mM MOPS, pH 7.2, 25mM β -glycerophosphate, 5mM EGTA, 1mM Na_3VO_4 , 1mM dithiothreitol, 75 mM $MgCl_2$, and 500 μ M ATP.
4. Long S6 kinase substrate peptide: Prepare a 1.1 mM S6 solution. Dilute 1 mg of S6 peptide (Merck Millipore, cat. number 12-420) in 250 μ L of water.
5. [$\gamma^{32}P$] ATP solution: 1 mCi of [$\gamma^{32}P$] ATP (Perkin Elmer, cat. number NEG-502A).
6. Phosphoric acid solution 0.75%
7. 24-well/ P81 paper: Cut pieces of P81 paper (Whatman, cat. number 3698915) to cover each well of 24-well plate.

2.2 Immuno-precipitation of ROCK2

1. Protein G sepharose beads CL-4B (Pharmacia, cat. number 17-0618-01)
2. Lysis buffer: 10 mM Tris-HCl (pH 8.0), 1% Triton X-100, 150 mM NaCl, 1% aprotinin, 250 μ M PMSF, 1mM NaF and 100 μ M sodium orthovanadate.
3. Phosphate buffer solution (PBS)

2.3 Immunoblotting

1. Tris buffered saline (TBS): 150 mM NaCl, 100 mM Tris-HCl, pH7.4
2. TBS-Tween solution: TBS containing 0.05% Tween-20
3. Blocking solution: 5% milk in TBS.

3. Methods

3.1 *In vitro* ROCK assay

3.1.1 Purified active ROCK assay protocol

1. Mix 978 μL of kinase buffer, 497 μL of $\text{Mg}^{2+}\text{Cl}_2/\text{ATP}$ cocktail, 72 μL of long S6 kinase substrate peptide and 50 μL of $[\gamma^{32}\text{P}]$ ATP solution.

2. In a 96-well plate, add 15 μL of the mix, 5 μL of ROCK2 solution (20 mU) and 5 μL of putative ROCK inhibitor to be tested dissolved in the kinase buffer per well.

Each molecule should be assayed at a minimum of two dilutions $5 \cdot 10^{-7}$ M and $5 \cdot 10^{-5}$ M. Each dilution should be tested in duplicate (Note 2). Each experiment must contain a minimum of two blanks (15 μL of the mix and 10 μL of kinase buffer), two positive controls (15 μL of the mix, 5 μL of ROCK2 solution and 5 μL of kinase buffer) and two wells with a reference inhibitor at 10 μM (15 μL of the mix, 5 μL of ROCK2 solution and 5 μL of 50 μM fasudil (Sigma, cat. number H139) or Y27632 (Sigma, cat. number Y0503) solution in kinase buffer (Note 3).

3. Heat the plate at 30°C for 30 min.

4. Transfer 20 μL of each well in 24-well/ P81 paper and incubate 8 min at room temperature.

5. Add 1 mL of phosphoric acid solution for 6 min at room temperature and empty the wells. Repeat two times this step (Note 4).

6. Dry and place P81 paper in plastic tube. ROCK activity is measured by the radioactive label counting of $[\gamma^{32}\text{P}]$ ATP in a scintillation counter.

3.1.2 Immuno-precipitated ROCK assay protocol

1. Cell culture: For each condition to be analyzed, a 10 cm culture dish with vascular smooth muscle cells at 80% confluency is necessary (Note 5). Wash the dish with ice-cold PBS, aspirate and add 1 mL of ice-cold lysis buffer onto the dish (Note 6).

Incubate 5 min and scrape cells off the surface with a cell scraper or a rubber

policeman. Dissociate the mixture by pipetting up and down, and transfer to a 2 mL tube.

Tissue samples: Frozen tissues are dropped into 5 volumes of lysis buffer (5 mL/g of tissue). Homogenize the sample on ice using a Polytron-type homogenizer.

2. Centrifuge lysates at 1500 x g for 10 min at 4°C.
3. Collect the supernatant and determine protein concentration of the supernatant.
4. Collect 1000 µg of total protein of the supernatant and bring the total volume to 1 mL using lysis buffer.
5. Add 2 µL of ROCK2 antibody (Santa Cruz, cat. number sc1851) and incubate 12 h at 4°C.
6. Add 20 µL of Protein G Sepharose and incubate at 4°C for 30 min.
7. Centrifuge tubes for 30 sec at 1000 x g. Aspirate the supernatant and wash the beads with 1 mL of kinase buffer. Vortex and collect the beads again by centrifugation. Repeat this step three times. After the last centrifugation, resuspend the beads in 20 µL of kinase buffer.
8. In wells of a 96-well plate, incubate 20 µL of beads with 30 µL of the mix described in the section 3.1.1 (step 1).
9. Heat the plate at 30°C for 30 min.
10. Transfer 40 µL of each well in 24-well/ P81 paper and incubate 8 min at room temperature.
11. Add 1 mL of phosphoric acid solution for 6 min at room temperature and empty the wells. Repeat two times this step (Note 3).
12. Dry and place P81 paper in plastic tube. ROCK activity is measured by the radioactive label counting of [$\gamma^{32}\text{P}$] ATP in a scintillation counter.

3.2 Evaluation of ROCK activity by western blot analysis

1. Total cellular lysates are obtained from primary vascular smooth muscle cells or vascular tissues as described in the section 3.1.2 (steps 1-3).
2. Dilute 30 µg of protein lysates in concentrated Laemmli buffer to finally obtain 25-50 µL of protein samples in 1X Laemmli buffer. Denature the proteins by heating the sample to 95°C for 10 min.
3. Load protein samples in each lane of 7% SDS-polyacrylamide gel. Migrate at 30 mA till the dye front reaches the bottom of the gel.
4. Proceed to protein transfer on nitrocellulose membrane using semi-dry transfer method. (Note 7)
5. Block the membrane with the blocking solution for 30 min.
6. Incubate the membrane with pMYPT-1 antibody [rabbit antiphospho-specific T850-MYPT-1 antibody (Merck Millipore, Cat. Number 04-773) and/or goat antiphospho-specific T696-MYPT-1 antibody (Santa Cruz, cat. number sc-17556)] and rabbit anti-MYPT-1 polyclonal antibody, for 12 h at 4°C. The final concentration of the antibodies is 1/1000 in blocking solution.
7. Wash three times with TBS-Tween solution (5 min each time) at room temperature.
8. Incubate the membrane with the appropriate secondary antibody conjugated to horseradish peroxidase for 1h at room temperature. Dilute the secondary antibody in blocking solution.
9. Wash as in step 7 and detect the immuno-complex using a chemiluminescent method (Pierce Biotechnology, cat. number 32132). (Note 8)
10. After quantification of the immunoreactive bands, ROCK activity is expressed as the ratio of pMYPT-1:MYPT-1 in each sample.

4. Notes

Note 1: To prevent decrease activity of ROCK2, avoid freeze and thaw. Prepare aliquots of ROCK2 enzyme and stock them at -70°C.

Note 2: Triplicate is strongly recommended for statistic analysis.

Note 3: Positive and negative controls are crucial for the interpretation of the experiment. Quadriplate is recommended.

Note 4: Avoid splash during washes to prevent radioactive contamination.

Note 5: Culture of vascular endothelial cells or leucocyte suspension can be used in the same way.

Note 6: Keep always the plate on ice to stop all enzymatic activities.

Note 7: The efficiency of transfer can be evaluated by Ponceau stain.

Note 8: Low or no signal could mean that the induction of the phosphorylation is not sufficient. Run a positive control with your samples to confirm the validity of the immunoblotting.

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