International Journal of Cardiology 215 (2016) 309–317

Contents lists available at ScienceDirect

International Journal of Cardiology

journal homepage: www.elsevier.com/locate/ijcard

Simultaneous Rho kinase inhibition in circulating leukocytes and in cardiovascular tissue in rats with high angiotensin converting enzyme levels

Camila Fierro [a,1], Ulises Novoa [b,1], Veronica González [a,2], María Paz Ocaranza [a,c,1], Jorge E. Jalil [a,*,1]

[a] Division of Cardiovascular Diseases, School of Medicine, Pontificia Universidad Católica de Chile, Chile
[b] Department of Biomedical Sciences, Facultad de Ciencias de la Salud, Universidad de Talca, Chile
[c] Advanced Center for Chronic Diseases (ACCDis), Faculty of Medicine, Pontificia Universidad Católica de Chile, Chile

ARTICLE INFO

Article history:
Received 4 February 2016
Accepted 2 April 2016
Available online 7 April 2016

Keywords:
Rho kinase
Angiotensin
Ezrin
Leukocytes
Fasudil
Remodeling

ABSTRACT

Background: The small guanosine triphosphatase RhoA and its direct target Rho kinase (ROCK) play important roles in cardiovascular pathophysiology. Activated ROCK phosphorylates intracellular proteins with detrimental effects on cardiovascular remodeling. Increased ROCK activity in circulating leukocytes is observed in hypertension and in heart failure, but its relationship with ROCK activation in the myocardium and vessels is unknown. We hypothesized that ROCK activation and phosphorylation/activation of some of its key downstream molecules in the heart and arterial wall are reflected in circulating leukocytes.
Methods: Phosphorylation of MYPT1, ERM and p38-MAPK and levels of p65-NF-κB were determined in the left ventricle (LV), aortic wall and circulating leukocytes in rats with high (Brown Norway, BN) and low (Lewis) angiotensin converting enzyme. A group of BN rats received the ROCK inhibitor fasudil (7 days).
Results: Compared to Lewis rats, in the BN group phosphorylated levels of MYPT1, ERM and p38-MAPK and levels of p65-NF-κB were increased ($P < 0.05$) in the LV (67%, 92%, 52% and 98%, respectively); in the aortic wall (57%, 51%, 68% and 66%, respectively) and in circulating leukocytes (61%, 72%, 49% and 105%, respectively). Fasudil reduced all these levels to those observed in Lewis rats. Phosphorylated MYPT1, ERM, and p38-MAPK and levels of p65-NF-κB in circulating leukocytes were significantly correlated with their respective LV and aortic wall levels (excepting p65-NF-κB in aorta).
Conclusion: ROCK activity in circulating leukocytes reflects activation of this signaling pathway in the myocardium and aortic wall in this model, and supports its value as a potential cardiovascular remodeling marker.

1. Introduction

The small guanosine triphosphatase Rho A and its direct target Rho kinase (ROCK), an intracellular signaling pathway playing important roles in several cellular functions such as motility, migration, grow control and cytokinesis. In the cardiovascular system ROCK does regulate vascular smooth muscle contraction, blood pressure and also remodeling [1–5]. Following Rho A activation by different agonists such as angiotensin II (Ang II) [6] or noradrenalin and also by growth factors through membrane receptors associated to G proteins, Rho A phosphorylates its main associated kinase ROCK [1–5]. In endothelial cells, Ang II leads to subsequent ROCK dependent activation of p38 mitogen-activated protein kinase (MAPK) [7] leading to endothelial dysfunction and vascular remodeling. Additionally, in human monocytic cells, angiotensin II induces NF-κB, JNK and p38 MAPK activation and increases matrix metalloproteinase-9 expression in a ROCK and PKC dependent manner [8].

Activated ROCK does phosphorylate and activate several intracellular proteins with relevant effects on various cellular processes and cardiovascular pathologic conditions [1–5] such as cardiac hypertrophy and ventricular dysfunction, apoptosis, fibrosis, vasoconstriction and endothelial dysfunction [1–5].

One of the main ROCK direct targets is the subunit 1 of the myosin phosphatase (MYPT1), an enzyme that in its active form

Abbreviations: ROCK, Rho kinase; ACE, angiotensin converting enzyme I; Ang II, angiotensin II; BN, Brown Norway strain; ERM, ezrin, radixin and moesin; p-ERM, phosphorylated ERM; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GRK2 or β-ARK1, G protein-coupled receptor kinase 2; HF, heart failure; HRP, horseradish peroxidase; ICAM-1, intracellular adhesion molecule 1; IL-6, interleukin 6; JNK, c-Jun N-terminal kinase; LVH, left ventricular hypertrophy; MAPK, mitogen-activated protein kinase; MBS, myosin binding subunit; p-MBS, phosphorylated myosin binding subunit; MLC-2, myosin light chain 2; MYPT1, subunit 1 of the myosin phosphatase; p-MYPT1, phosphorylated MYPT1; NF-κB, nuclear factor κB; PKC, protein kinase C; VCAM-1, vascular cell adhesion molecule 1.

* Corresponding author at: Division of Cardiovascular Diseases, School of Medicine, Pontificia Universidad Católica de Chile, Lira 85 Piso 2, Santiago, Chile
E-mail address: jjalil@med.puc.cl (J.E. Jalil).

[1] This author takes responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.

[2] Contribution: laboratory and animal work and data analysis and interpretation.

http://dx.doi.org/10.1016/j.ijcard.2016.04.004
0167-5273/

(phosphorylated), inactivates myosin light chain through phosphorylation and thus induces contraction of muscular fibers [9,10]. Another ROCK direct substrate is a protein family complex formed by ezrin, radixin and moesin (ERM), which in its active form modulates polarization and cell migration [11]. Thus, the current standard method to determine ROCK activity is by measuring phosphorylation levels of its direct substrates, MYPT-1 [12–14] and ERM [15–18].

In humans, higher ROCK activity levels in circulating leukocytes have been reported in hypertensive patients compared to normotensive subjects [13,19]. Additionally, increased ROCK activity in circulating leukocytes was observed in hypertensive patients with left ventricular hypertrophy (LVH) compared with hypertensive patients without LVH, and even higher ROCK activity levels when eccentric LVH was present [13]. Furthermore, ROCK activity in circulating leukocytes is markedly increased in patients with heart failure (HF) under medical treatment [14] and it is associated with pathologic LV remodeling and systolic dysfunction [14]. However, mechanisms and dynamics of ROCK activation in patients with cardiac remodeling or HF, its role in the progression of the disease and the direct effect of ROCK inhibition need further investigation.

One important issue in the abovementioned studies in humans is related to the biologic significance of ROCK activation in circulating leukocytes, its relation with cardiovascular remodeling and specifically with ROCK activation in the myocardium and in the vessels. Few information are available in the literature on these specific aspects, but circulating lymphocytes have been used to assess β-adrenergic receptor signaling and to make extrapolations to the cardiac β-adrenergic receptor system, representing a valuable and reliable marker of the functional state of cardiac β-adrenergic receptor signaling [20–23]. In addition, the G protein-coupled receptor kinase 2 (GRK2 or β-ARK1) regulates β-adrenergic receptors in the heart, and its cardiac expression is elevated in human HF. A direct correlation between myocardial and circulating lymphocyte GRK2 activities has been found in patients with HF, implying that myocardial GRK2 expression and activity are mirrored by lymphocyte levels of this kinase in human HF [23] which might also be the case with ROCK activation determined in circulating leukocytes, but it needs to be proven [13,14].

Thus, relevant questions are raised by the current data on ROCK activation in human circulating leukocytes as related to cardiovascular remodeling and risk, such as the pathological significance of ROCK activation in leukocytes, the possible mechanisms responsible for its activation during pathological cardiovascular remodeling, or which mechanisms are responsible for much higher ROCK activation in heart failure than in cardiac hypertrophy.

We hypothesized that ROCK activation as well as phosphorylation and levels of some of its key downstream pro-remodeling molecules in the heart and in the arterial wall, are reflected consistently in circulating leukocytes in order to understand the significance of ROCK activation in circulating leukocytes (as a possible mirror of cardiovascular ROCK activity). Thus, phosphorylation of its key targets was simultaneously determined in circulating leukocytes, in the aorta and in the left ventricle in normotensive rats with genetically determined high (Brown Norway, BN) and low (Lewis) angiotensin converting enzyme I (ACE) levels. In this experimental model, ROCK is considerably activated in the aortic wall in rats with genetically high ACE activity and angiotensin II levels, which increases gene expression and protein levels of molecules that promote vascular remodeling and oxidative stress [24,25].

2. Methods

The current experimental work was conducted following the "Guide for the Care and Use of Laboratory Animals" published by the "National Health Institute" (NIH No. 85-23, revised 1996) and it was reviewed and approved by the Ethics Committee on Animal Welfare of the Faculty of Medicine, Pontificia Universidad Católica de Chile. All efforts were made in order to reduce animal numbers and avoid their suffering.

2.1. Genetic high versus low angiotensin converting enzyme model

Homozygous adult male F2 rats (weight 391 ± 41 g, aged 12 weeks) with genetically low ACE levels, Lewis (n = 23) and high ACE levels, Brown Norway (BN, n = 37) [24–27], were obtained from our animal facility. The homozygous condition from the BN and Lewis groups was confirmed by RT-PCR as previously described [26,27] and their respective phenotypes were assessed by measuring plasma ACE levels. Rats were kept under a 12-h light/dark cycle with regulated temperature and humidity, and free access to food and water ad libitum as well. Additionally, BN rats were randomly assigned to any of the two groups: one group received oral fasudil (BN + F, n = 14), a widely used pharmacologic ROCK inhibitor (100 mg kg^{-1} day^{-1} by gavage during 7 days) [24]. The other BN group received only solvent by gavage during 7 days (n = 23).

One week after administering fasudil or solvent, systolic and diastolic blood pressures were measured by the tail cuff method with a CODA 2 system (Kent Scientific, Torrington, CT) [28]. Measurements were obtained in conscious rats restrained in a thermal plastic chamber. Afterwards the animals were euthanized by deep anesthesia (ketamine HCl/xylazine 35/7 mg kg^{-1} i.p.).

2.2. Protein extraction from the aorta and left ventricle

The heart and aorta from each rodent were both carefully removed, washed in saline, weighted, frozen with liquid nitrogen and stored at −80 °C until further processing. Protein extraction from the aorta and left ventricle was performed from fresh-frozen tissue. Samples were homogenized and lysated with a lysis buffer containing low detergent concentrations (Hepes 50 mM, NaCl 150 mM, $MgCl_2$ 2 mM, EGTA 1 mM, Triton 1% and glycerol 10%) supplemented with a protease inhibitor cocktail (aprotinin 2 μg/ml, leupeptin 10 μg/ml, PMSF 1 mM) and phosphatase inhibitors (NaP_2O_7 4.46 mg/ml, NaF 10 mM and Na_3VO_4 1 mM) on ice. Subsequently, samples were centrifuged at 4 °C for 15 min and supernatant protein content was determined by Bradford assay using 0.1–0.8 mg/ml bovine serum albumin (BSA) as protein standard [18,24].

2.3. Protein extraction from circulating leukocytes

After animals were euthanized, blood was collected by cardiac puncture in EDTA-containing tubes. For isolating circulating leukocytes, 0.5 vol of whole blood containing EDTA was slowly poured over a 0.5 vol of density gradient cell separation medium (Ficoll and sodium diatrizoate, Histopaque-1077, Sigma Chemical Co., St Louis, MO) and centrifuged at 850 × *g* during 45 min at 28 °C. White cells were separated, resuspended and washed in 1× phosphate buffered saline (PBS). Upon isolation, cells were resuspended in lysis buffer containing 150 mM NaCl, 1% NP40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS) and 500 mM Tris. Lysis buffer was also supplemented with a protease inhibitor cocktail (1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM PMSF and 10 μg/ml pepstatin) and phosphatase inhibitors (1 mM Na_3VO_4, 10 mM NaF, 5 mM NaP_2O_7). Protein content was determined by Bradford assay.

2.4. Western blot analysis

For western blot analysis, soluble protein fractions were heated during 5 min at 95 °C with 0.33 vol of 6× SDS sample buffer (375 mM Tris–HCl pH 6.8, 6% SDS, 48% glycerol, 9% 2-mercaptoethanol and 0.03% bromophenol blue).

Equal amounts of protein (20–40 μg) were loaded and separated on a 5% stacking and 8, 12 or 18% resolving SDS-PAGE gel (80 V), and transferred into a nitrocellulose membrane (Bio-Rad) at 400 A during 2 h on