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ROCK2 primes the endothelium for vascular hyperpermeability responses by raising baseline junctional tension

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ABSTRACT

Rho kinase mediates the effects of inflammatory permeability factors by increasing actomyosin-generated traction forces on endothelial adherens junctions, resulting in disassembly of intercellular junctions and increased vascular leakage. In vitro, this is accompanied by the Rho kinase-driven formation of prominent radial F-actin fibers, but the in vivo relevance of those F-actin fibers has been debated, suggesting other Rho kinase-mediated events to occur in vascular leak. Here, we delineated the contributions of the highly homologous isoforms of Rho kinase (ROCK1 and ROCK2) to vascular hyperpermeability responses. We show that ROCK2, rather than ROCK1 is the critical Rho kinase for regulation of thrombin receptor-mediated vascular permeability. Novel traction force mapping in endothelial monolayers, however, shows that ROCK2 is not required for the thrombin-induced force enhancements. Rather, ROCK2 is pivotal to baseline junctional tension as a novel mechanism by which Rho kinase primes the endothelium for hyperpermeability responses, independent from subsequent ROCK1-mediated contractile stress-fiber formation during the late phase of the permeability response.

1. Introduction

Numerous pathological conditions including life-threatening sepsis and acute respiratory distress syndrome (ARDS) are characterized by endothelial barrier failure [1]. The sequelae include uncontrolled fluid extravasation and edema [1,2]. Under acute inflammatory conditions, the post-capillary venules in particular become leaky, whereas in ARDS the alveolar–capillary barrier is the major site of leakage. Despite increasing incidence of sepsis [3] and high mortality rates in ARDS, no treatment is currently available to combat endothelial barrier disruption in these diseases [1].

Endothelial barrier function is controlled principally by cytoskeletal elements that –in addition to local signaling events that weaken the junctions– orchestrate intercellular junctional complexes, and facilitate cell–matrix adhesion [2]. Dysfunction of the endothelial barrier can be elicited through activation of specific receptors by vasoactive agents such as thrombin and vascular endothelial growth factor (VEGF), as well as by interaction of the endothelium with leukocytes. Thrombin-induced signaling via its receptor PAR1 –which is short for protease-activated receptor 1– involves several signaling mechanisms that are simultaneously activated, including the influx of calcium ions, the activation of small Rho GTPases, the activation of various kinases, and the phosphorylation of (junctional) proteins. Among the small GTPases, RhoA is mainly involved in inducing endothelial hyperpermeability, whereas Rac1, Cdc42 and Rap1 contribute to enforcement of an intact barrier function [4].

A key effector molecule of RhoA in regulating vascular permeability is Rho kinase. It has been well-appreciated that enhanced Rho kinase activity upon stimulation by inflammatory mediators such as thrombin has a strong barrier-disruptive effect [5,6]. However, basal Rho kinase activity is also involved in the maintenance of barrier integrity [7,8]. Initial studies in endothelial cells (ECs) showed that transduction of

Abbreviations: ARDS, acute respiratory distress syndrome; DDAB, dimethyldioctadecylammonium bromide; EBA, Evans blue conjugated albumin; EBAE, EBA extravasation; EC, endothelial cell; ECIS, electrical cell impedance sensing; HRP, horseradish peroxidase; HPMVECs, human pulmonary microvascular endothelial cells; HSA, human serum albumin; HUVEC, human umbilical vein endothelial cell; ICAM, intercellular adhesion molecule; LPS, endotoxin; MLC, myosin light chain; MYPT1, myosin phosphatase target subunit 1; PAR, protease-activated receptor; PH domain, Pleckstrin homology domain; RMS, root mean square; ROCK, Rho kinase; Shrm2, Shroom2; TEER, trans-endothelial electrical resistance; VEGF, vascular endothelial growth factor.

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a dominant-negative Rho kinase markedly reduced thrombin-induced formation of F-actin fibers through inhibition of the myosin phosphatase, serving as a paradigm for Rho kinase-mediated vascular hyperpermeability [9]. Yet, their in vivo relevance remained uncertain and has been debated for the microcirculation [10,11]. In cell models, Huveneers et al. showed that the agonist-induced radial F-actin fibers transmit tension to the endothelial junctions [12]. Indeed, pharmacological inhibition of Rho kinase reduces tension to the junctions, and ablates thrombin-induced hyperpermeability of endothelial monolayers by about 50% [6]. The remaining Rho kinase-independent part of the in vitro endothelial hyperpermeability involves regulation by protein tyrosine kinases and protein kinase C zeta [13,14]. In animal models, inhibitors of Rho kinase reduced vascular hyperpermeability induced by vaso-active agents such as VEGF, endotoxin (LPS) and thrombin even more effectively than in vitro [15,16]. But to the contrary, recent reports indicated that treatment of rats with the Rho kinase inhibitor fasudil, while effective in reducing LPS-induced permeability, improving survival in sepsis and preventing ARDS [17], by itself promoted vascular leakage of macromolecules [18]. Taken together, these data suggest the presence of distinct and sometimes even opposing Rho kinase activities determining endothelial morphology and function.

In an attempt to resolve this discrepancy we turned to two distinct isoforms of Rho kinase, ROCK1 and ROCK2, encoded by two different genes [19]. These isoforms are highly homologous except for their PH domains. Their human forms share 64% sequence identity with 89% identity in the catalytic domain [19]. Given their high sequence similarity it is not surprising that available inhibitors do not distinguish between the two ROCK isoforms, except for the ROCK2 inhibitor SLx-2119, also known as KD025, showing 200-fold higher selectivity toward ROCK2 (IC_{50} 105 nmol/L) compared to ROCK1 (IC_{50} 24 μmol/L) [27].

An analysis of Rho kinase knockout-mice suggests that there is no compensation for the loss of either isoform by the other: ROCK2 −/− mice show a high fetal death rate [20] while ROCK1 −/− die early after birth [21], but ROCK1 null animals that survive develop normally. The developmental effects associated with ROCK1 and ROCK2 deficiencies have limited the use of these animals to evaluate the functions of these molecules in physiology and disease in an isoform-specific manner. Also, most pharmacological inhibitors lack isoform specificity.

Rho kinase is involved in many basic vascular activities such as cellular migration, angiogenesis, and development of tone. Evidence has accumulated over the past decade that enhanced activity of Rho kinase plays an important role in many vascular pathologies including (pulmonary) hypertension, atherosclerosis, diabetes, and vascular leak [22,23]. ROCK isoform-specific regulation has been indicated for some of them [24,25].

Given their discrete form and function, we hypothesized that specifically targeting a single Rho kinase isoform in anti-vascular leak therapy would be an effective and novel strategy. Accordingly, using a combination of in-vitro and in-vivo experiments, we investigated the individual contributions of ROCK1 and ROCK2 to the regulation of endothelial barrier permeability.

2. Results

2.1. Efficacy of ROCK1 and ROCK2 depletion by siRNA treatment in vitro and in vivo

In vitro experiments were performed throughout this study with human umbilical vein endothelial cells (HUVECs). Key findings were verified in primary human pulmonary microvascular endothelial cells (HPMVECs) and presented in the online supplement.

The efficacy of siRNAs to downregulate ROCK protein expression in HUVECs was monitored by Western blotting of cell lysates obtained at 48 h after transfection. A net decrease in ROCK protein expression in each experiment of at least 90% was observed in ROCK1-silenced ECs and of 75% in ROCK2-silenced ECs (see Fig. 1A for representative Western blot).

To test whether the siRNAs directed at human ROCK sequences were also effective in downregulating mouse ROCK1 and ROCK2, mouse NIH3T3 cells were used as a model cell system. The siRNAs directed to human ROCK1 and ROCK2 were less effective in downregulating mouse ROCK protein expression (data not shown). Therefore, new siRNAs were designed directed to mouse ROCK sequences (see the Materials & methods section), resulting in a >80% downregulation of mouse ROCK1 and ROCK2 protein expressions in vitro (see Fig. 1B for a representative Western blot).

Immuno-histochemical staining of both ROCK1 and ROCK2 revealed their abundant expression in endothelial cells of large pulmonary arterioles as well as in the small capillaries of the mouse lung (Fig. 1C). Their expression was selectively downregulated 48 h after retro-orbital injection of the corresponding siRNAs as evidenced by semi-quantitative immuno-histochemical analysis of arteriolar ROCK1 expression (Fig. 1D, E).

2.2. Silencing of ROCK1 and ROCK2 reveals differential involvement in endothelial permeability

To evaluate the contributions of ROCK1 and ROCK2 to permeability changes of human endothelial monolayers, HUVECs were grown in vitro on porous filters, and permeability for the tracer HRP was measured. The thrombin-induced endothelial hyperpermeability of these monolayers is for about 40–60% dependent on Rho kinase, representing the in vivo PAR1-mediated vascular permeability, whereas the remainder is independent of Rho kinase, involving other signaling pathways as outlined in the Introduction [2,6].

Thrombin elicited a 7.2 ± 0.7-fold increase in the passage of the tracer molecule HRP (Fig. 2A). It is known that PAR1-activating peptides recapitulate the pro-inflammatory effects of thrombin in endothelial cells [26], which we confirmed in vitro where PAR1-peptide and thrombin evoked a comparable transient decrease in endothelial barrier function (Supplementary Fig. S1). Silencing of ROCK1 did not affect thrombin-induced permeability, whereas silencing of ROCK2 significantly reduced thrombin-induced permeability (Fig. 2A). Simultaneous silencing of ROCK1 and ROCK2 further reduced the absolute or net thrombin-induced increase in permeability, and showed an almost similar effect as pharmacological inhibition by Y-27632.

To evaluate the contribution of ROCK1 and ROCK2 to PAR1-mediated vascular hyperpermeability in vivo, ROCK1 or ROCK2 siRNA-transduced mice were stimulated with PAR1-peptide for 30 min. The pulmonary microvascular permeability following PAR1-stimulation was determined by measuring Evans blue conjugated albumin (EBA) extravasation (Fig. 2B). None of the siRNA treatments affected baseline permeability. PAR1-peptide evoked a 2-fold increase in EBA extravasation. Silencing of ROCK1 had no effect on PAR1-mediated EBA leakage. Silencing of ROCK2 decreased the PAR1 permeability response by about 50%, while silencing of both ROCK1 and ROCK2 almost completely prevented the increase in EBA extravasation upon treatment with a PAR1-peptide. The potent pharmacological pan-Rho kinase inhibitor Y-27632 was equally effective as silencing of both ROCK1 and ROCK2. These findings were supported by comparable changes in lung wet–dry weight ratios (data not shown).

Together, these data indicate that ROCK2 primarily regulates the thrombin receptor-mediated vascular hyperpermeability response both in vitro and in vivo. ROCK1 is dispensable for this hyperpermeability response, but silencing of ROCK1 enforced the attenuating effect of silencing of ROCK2. These data point to distinct roles of ROCK1 and ROCK2 in the regulation of vascular hyperpermeability.

2.3. Specific pharmacological inhibition of ROCK2 attenuates endothelial hyper-permeability

The role of ROCK2 in regulating the endothelial barrier function was further explored by using the ROCK2-selective inhibitor SLx-2119 [27,