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REVIEW

RhoA/Rho-kinase and vascular diseases: what is the link?

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Abstract RhoA/Rho-kinase pathway plays an important role in many pathological conditions. RhoA participates in the regulation of smooth muscle tone and activates many downstream kinases. The best characterized are the serine/threonine kinase isoforms (Rho-kinase or ROCK), ROCKα/ROCK2 and ROCKβ/ROCK1. ROCK is necessary for diverse functions such as local blood flow, arterial/pulmonary blood pressure, airway resistance and intestinal peristalsis. ROCK activation permits actin/myosin interactions and smooth muscle cells contraction by maintaining the activity of myosin light-chain kinase, independently of the free cytosolic calcium level. The sensitization of smooth muscle myofilaments to calcium has been implicated in many pathological states, such as hypertension, diabetes, heart attack, stroke, pulmonary hypertension, erectile dysfunction, and cancer. The focus of this review is on the involvement of RhoA/Rho-kinase in diseases. We will briefly describe the ROCK isoforms and the role of RhoA/Rho-kinase in the vasculature, before exploring the most recent findings regarding this pathway and various diseases.

Keywords RhoA · Rho-kinase · Cardiovascular diseases · Diabetes · Pulmonary hypertension · Erectile dysfunction · Cancer

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Introduction

Rho-kinase is a serine/threonine protein kinase that contains an N-terminal catalytic kinase domain. It has been identified as the downstream effector of RhoA which mediates calcium (Ca^{+2}) sensitization [1]. RhoA small GTPase (a member of the Rho subfamily within the RAS superfamily of monomeric GTPases) is the molecular switch for various extracellular signals and is implicated in a variety of biological cellular functions, including contraction, migration, adhesion, cell cycle progression, and gene expression. These functions are regulated by RhoA through Rho-kinase or ROCK, one of the best characterized Rho effectors, which exists in two isoforms: ROCK 2 (also called ROKα) and ROCK1 (also known as ROKβ or p160ROCK) [2, 3]. Rho-kinase is activated not only by RhoA but also by arachidonic acid, which is released from smooth muscle in response to various agonists [4].

Many of the targets for Rho-kinase have been indentified, including the myosin-binding subunit (MBS) of myosin phosphatase, ezrin-radixin-moesin (ERM) family, adducin, vimentin (an intermediate filament), $Na^{+}H^{+}$ exchanger, and LIM-kinase, which phosphorylates cofilin. Among these, one of the main substrates of Rho-kinase is MLCP (myosin light chain phosphatase), which is physiologically responsible for the dephosphorylation of the light chains of myosin II (MLC_{20}) [5]. Thus, MLCP phosphorylation is believed to be a hallmark of Rho-kinase activation [6]. Rho-kinase can also phosphorylate MLC directly [7]. Since the inactivation of MLCP is associated with increased phosphorylation of MLC, the net effect of Rho-kinase activation is consistent with increased phosphorylation of MLC. Phosphorylation or activation of myosin enables the molecular interaction with actin, leading to muscle contraction.

Since the contraction of a smooth muscle cell (SMC) occurs through two main mechanisms, Ca^{+2} signaling cascades and RhoA/Rho-kinase signaling pathways, Rho-kinase has been shown to be substantially involved in this process. In addition, RhoA/Rho-kinase can alter the Ca^{+2} sensitivity of the contractile system [8], and its activation inhibits endothelial nitric oxide synthase (eNOS), thereby altering nitric oxide (NO) production. The impairment of both processes has been shown in human and animal studies to be involved in pathological conditions, mainly vascular diseases and other pathologies, such as hypertension, stroke, vasospasm, atherosclerosis, heart failure, pulmonary hypertension, and more recently, cancer [5, 9–13]. Many of these pathologies demonstrate a common theme: the rapid and dynamic reorganization of the actin cytoskeleton in which Rho-kinase signaling has now emerged as a major switch control.

The RhoA/Rho-kinase pathway has been largely investigated in the last decade, but many aspects regarding this signaling cascade are still unclear. Considering that RhoA mediates important cellular functions and has already been implicated in the regulation of vascular tone, along with inflammation and oxidative stress, the inhibition of this pathway may have significant clinical implications. Some compounds have been studied to inhibit Rho-kinase [14] and have been proposed to have therapeutic benefits regarding multiple diseases. The most widely used experimentally are two non-selective inhibitors, Y27632 and H1077 or fasudil. However, these inhibitors cannot distinguish between ROCK isoforms or the differential mechanisms of ROCK in individual cell components [15], so the precise role of ROCK in the vasculature has thus far been limited by a lack of specific pharmacological inhibitors. Nevertheless, these inhibitors of Rho-kinase have contributed greatly to elucidate altered mechanisms in vascular diseases and have helped to highlight them as therapeutic targets. This review summarizes the current status of this pathway and illustrates its role in multiple diseases, including some speculations on the therapeutic benefits of Rho-kinase inhibitors. The role of RhoA/Rho-kinase in the vasculature, as well as its isoforms and expression, will also be briefly described.

RhoA/Rho-kinase activity, isoforms, and expression

Rho-family proteins have lipid modifications that target them to cell membranes and they can cycle between GTP- and GDP-bound states [6]. Like other GTP-binding proteins, RhoA exhibits both GDP/GTP-binding activity and GTPase activity, and functions as a molecular switch, cycling between a GDP-bound inactive state (GDP-Rho) and a GTP-bound active state (GTP-Rho). The activity of RhoA is cyclically regulated [6]. When cells are stimulated with various agonists, GDP-Rho is converted to GTP-Rho through the action of guanine nucleotide exchange factors (GEFs) that stimulate the GTP–GDP exchange reaction. GTP-Rho is then targeted to the cell membrane through its C-terminal geranyl-geranylated tail and interacts with its specific targets (Fig. 1). In resting cells, Rho GDP dissociation inhibitor (Rho GDI) binds to GDP-Rho and extracts GDP-Rho from the membrane to the cytosol [16–18].

Rho-kinase is a widespread and evolutionary conserved downstream effector of RhoA, as well other GTPases, RhoB and RhoC, which are all potential activators. However, there are inhibitory GTPases such as RhoE that directly bind to ROCK and block its kinase activity. ROCK1-induced stress fiber formation is inhibited by RhoE bound to the amino-terminal region [19, 20]. Differently, Rho-A activates ROCK1 through interaction with carboxy-terminal site. RhoE and RhoA are not able to bind ROCK1 simultaneously [21], and RhoE phosphorylation events seem to antagonize RhoA-induced stress fiber assembly [20]. ROCK activation has also been suggested to occur directly by lipids, such as sphingosylphosphorylcholine (SPC) [22]. Rho-kinase was initially identified as a GTP-Rho-binding protein from bovine brain by affinity column chromatography on matrix-bound GTP-Rho [23]. There are two isoforms of Rho-kinase, ROCK1 and ROCK2, that share overall 65% homology at the amino-acid level and 92% homology in their kinase domains [3]. The tissue distribution of ROCK1 and 2 is similar, and relatively few studies have outlined the isoform specific roles of ROCK. Although ROCK1 rather than ROCK2 was suggested as important for stress fiber formation, ROCK2 acts as a counterbalance in the regulation of the microfilament bundle and focal adhesion site [24]. ROCK1, but not ROCK2, is sensitive to caspase 3-mediated cleavage in apoptotic cells [25]. Overexpression of both isoforms increases MLC phosphorylation [26] and, consequently, ROCK isoforms lose their specificity when overexpressed [24]. Analysis of the ROCK2-deficient mouse suggested that there is no compensation for the loss of ROCK2 by ROCK1 [27]. However, in vascular smooth muscle cells (VSMC), silencing of each ROCK isoform leads to upregulation of the other isoform, suggesting that the expression level of the ROCK isoforms is tightly controlled and interrelated. When both ROCK isoforms are silenced, this leads to reduced myosin binding subunit (MBS) and MLC phosphorylation [26].

ROCK isoforms are expressed in invertebrates such as *Caenorhabditis elegans*, *Drosophila*, and mosquito, and vertebrates such as zebrafish, *Xenopus*, chicken, mouse, rat, and human [28]. ROCK1 and ROCK2 are ubiquitously expressed in mouse tissues from youngest embryonic development to adulthood. Evaluation of expression in the

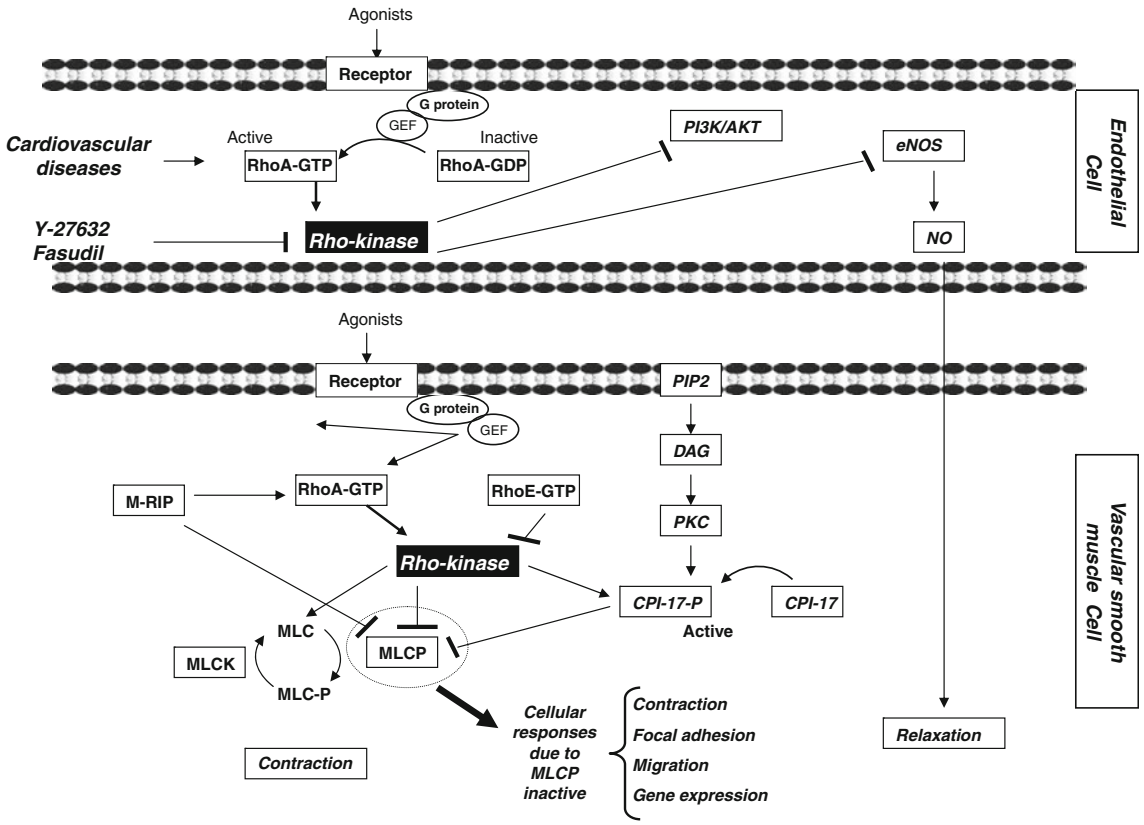

Fig. 1 The RhoA/Rho-kinase pathway. Agonist triggers RhoA (RhoA-GTP) by stimulation of G-protein-coupled receptors leading to Rho-kinase activation. In stimulated cells, RhoA-GDP is converted in RhoA-GTP through the action of GEF. The activated Rho-kinase subsequently inhibits the eNOS and PI3 K/AKT pathways, impairing NO released from endothelial cells. There is also inhibition of MLCP activity in vascular smooth muscle cells leading to contraction. RhoA/Rho-kinase activation is common in cardiovascular diseases. CPI-17 is activated by Rho-kinase and PKC in vascular smooth muscle cells, resulting in MLCP inhibition. The complex M-RIP protein/RhoA/Rho-kinase also inactivates MLCP, which alters the contractile process and many cellular responses as cited in the figure. Y-27632, fasudil and activated RhoE are Rho-kinase inhibitors. ROCK-induced contraction is inhibited by RhoE bound to the amino-terminal region, whereas Rho-A activates ROCK1 through interaction with carboxy-terminal site. Rho-kinase, representing two isoforms of ROCK1 and ROCK2. *GEF* Guanine nucleotide exchange factor, *MLCP* myosin light chain phosphatase, *MLCK* myosin light chain kinase, *MLC* myosin light chain, *MP-RIP* myosin phosphatase-rho interaction protein, *PIP2* phosphatidylinositol 4,5-bisphosphate, *DAG* diacylglycerol, *PI3K* phosphatidylinositol-3-kinase

mouse indicates that ROCK1 mRNA is preferentially expressed in lung, liver, spleen, kidney, and testis, whereas ROCK2 mRNA is highly expressed in the heart and brain [29, 30]. Recently, it has been demonstrated that there are direct increases in neural ROCK2 expression in ischemic brain tissue [31]. Immunolocalization and cell fractionation studies have shown that ROCK2 is distributed mainly in cytoplasm [23, 32]. In contrast, little is known regarding the intracellular localization of ROCK1, which may be colocalized to centrosomes [33] and in the Golgi complex, together with RhoE [19].

RhoA/Rho-kinase in the vasculature

A perfect balance between contraction and relaxation of smooth muscle is critical in maintaining many biological functions and disruptions in this dynamic process can result in various pathologies. Vascular smooth muscle tone plays a fundamental role in regulating blood pressure, blood flow, capillary permeability, and other cardiovascular functions. The Rho-kinase pathway is intrinsically involved with the process of smooth muscle contraction [24] and has been shown to be a regulated determinant of numerous cellular processes [4]. The Ca^{2+} sensitivity of smooth muscle reflects the ratio of activities of MLCP to myosin light-chain kinase (MLCK), resulting in contraction or relaxation. Since the main substrate of Rho-kinase is MLCP, which is physiologically responsible for the dephosphorylation of MLC, this implicates Rho-kinase in mediating Ca^{+2} sensitivity inside the cell, ultimately leading to contraction by inhibition of MLCP activity.

It is now widely accepted that MLCK and the RhoA/Rho-kinase pathway are two major cellular targets for

regulating Ca^{2+} sensitivity of myosin II, and they generally operate in parallel. RhoA is an important messenger of Ca^{2+} sensitization, and agonists can activate RhoA through numerous G protein-coupled receptors: α-adrenergic, muscarinic, purinergic, endothelin, prostanoid, oxytocin, epidermal growth factor, ephrin, semaphorin, angiotensin II, and Edg lysophospholipid receptors [6, 34]. Activation of RhoA and also phospholipase C (PLC) induces inositol 1,4,5-triphosphate (IP_3) production and Ca^{2+} release from the sarcoplasmic/endoplasmic reticulum, and increased Ca^{2+} influx through receptor-operated or voltage-gated channels, and inhibits maxi-K potassium channels resulting in depolarization. Also, the linkage between Ca^{+2}-calmodulin (Ca-CAM) activates the MLCK, which in turn leads to increased phosphorylation of MLC, promoting the actin filament cross-linking activity of myosin II, and resulting in contraction [35].

PLC activation catalyzes the formation of diacylglycerol (DAG), a second messenger, leading to protein kinase C (PKC) activation, which phosphorylates specific target proteins. There are several isozymes of PKC and each has a tissue-specific role [36]. However, in many cases, PKC has contraction-promoting effects, such as phosphorylation of different kinases including Rho-kinase and others such as MLCK, ERK, calmodulin-dependent protein kinase II, transporters, and various ion channels [37]. So, PKC participates in the contractile response mainly by directly activating MLCK and, indirectly, by activating Rho-kinase.

Rho-kinase was first identified to phosphorylate the myosin-binding subunit (MBS) of MLCP, named myosin phosphatase targeting (MYPT1) subunit, inhibiting its activity. Later, many kinases were found to phosphorylate MYPT1, thus promoting the phosphorylated state of myosin. Within MYPT1 or MBS, which are often used synonymously in the literature, the major sites of phosphorylation by Rho-kinase have been indentified as Ser849/854, Thr850/855, and Thr695/697. The Ser849/854 site is specifically phosphorylated by Rho-kinase, but the main site involved in the inhibition of MLCP activity is Thr695/697 [17]. However, recently, Thr850/855 has been implicated as the major ROCK phosphorylation site, whereas Thr695/697 is thought to be phosphorylated by other kinases [38]. The specific phosphorylation sites on ROCK and their activities still need to be elucidated.

Similar to MYPT1, the phosphorylation of the small protein CPI-17, a phosphorylation-dependent inhibitory protein of MLCP, can be catalyzed by multiple kinases including Rho-kinase. Specifically, inhibition of the activity of MLCP involves Rho-kinase by means of phosphorylation of either MYPT1 at Thr850/855 of MLCP or the protein CPI-17, which inhibits the catalytic domain of MLCP [39, 40]. CPI-17 can be phosphorylated by PKC as well as by Rho-kinase (Fig. 1). Therefore, CPI-17 can be seen as a potential mediator of Ca^{2+} sensitization which is independent of MYPT1 phosphorylation.

New members of the MLCP complex have been characterized myosin phosphatase-rho interacting protein, M-RIP, and its murine homolog MP-RIP or $p116^{RIP}$. Both are cytoskeletal scaffold proteins that bind directly to both RhoA and MBS, targeting MLCP via different mechanisms [41, 42]. One targeting function of M-RIP is to localize the MLCP complex to the actinomyosin contractile filament to dephosphorylate myosin leading to inactivation of MLCP activity (42, Riddick). However, it has been described that the binding of MP-RIP (or $p116^{RIP}$) to MBS activates MLCP activity. The elimination of MP-RIP by MP-RIP-specific siRNA consistently increased MLC_{20} phosphorylation [41, 42]. It is postulated that MP-RIP contributes to the decrease in myosin phosphorylation via activation of the myosin dephosphorylation activity of MLCP and the inactivation of the RhoA pathway [42]. Additionally, since ROCK and MP-RIP bind separate domains of MBS, a model where RhoA bound to MP-RIP and ROCK bound to MBS are brought into proximity by MP-RIP/MBS binding has been suggested [26]. The same authors describe that both isoforms of ROCK can bind to MBS, but ROCK1 interacts with MBS twice as much as ROCK2 [26]. Also, in VSMC, ROCK2 is the predominant isoform that regulates contractility and recent studies demonstrated that ROCK2 regulates force production, as well as contraction [26].

Since the RhoA/Rho-kinase pathway is so heavily involved in the cytoskeletal function of the vasculature, it would be expected that there is a link between this pathway and many vascular diseases. However, the involvement of RhoA/Rho-kinase with the cytoskeleton does not just limit it to diseases of the vasculature, as this review will highlight. Nevertheless, many processes regarding the exact role of RhoA/Rho-kinase activation need to be better understood, as well as the consequences of its dysregulation in different parts of the body. This will lead to a total comprehension of this pathway and its involvement in several pathologies.

Connections between Rho-kinase and diseases

The small GTPase RhoA and its target, Rho-kinase, are involved in the sequence of events which stimulates vascular smooth muscle contraction, stress fiber formation, cell migration, and, indirectly, blood pressure regulation. In this way, RhoA/Rho-kinase activation has significant effects on various cardiovascular diseases, mainly arterial hypertension [43], atherosclerosis [44], heart attack [45], stroke [46], and others such as coronary vasospasm [47], myocardial hypertrophy [48], myocardial ischemia–reperfusion injury [49], and vascular remodeling [50].

Compounds that specifically inhibit this signaling pathway can offer clinical benefits regarding the treatment of these diseases, as well as contributing pharmacological tools for vascular studies.

Accumulating evidence indicates that endothelial nitric oxide synthase (eNOS), which is an important mediator of vascular function, is regulated by the RhoA/Rho-kinase pathway (Fig. 1) [51, 52]. For example, dominant-negative mutants of RhoA or inhibitors of ROCK have been shown to increase eNOS expression [46, 52]. Since eNOS is protective in the vasculature, the RhoA/Rho-kinase pathway has been suggested to play a critical pathophysiological role in several aspects of cardiovascular disease. Also, there is increasing evidence that eNOS activity could be regulated in part through association with various protein kinases [53, 54]. For example, inhibition of RhoA or ROCK isoforms leads to the rapid activation of the lipid kinase phosphatidylinositol-3-kinase (PI3 K)/Akt pathway and phosphorylation of eNOS [51, 52]. Studies also indicate that ROCK isoforms are activated in patients with a cardiovascular disorder or with associated risk factors [55, 56]. Furthermore, RhoA mRNA expression and activity is increased in aortas from aged rats, suggesting a role of RhoA in the development of age-related cardiovascular disease [57].

Vascular remodeling is an important component of vascular diseases and may be associated with RhoA/Rho-kinase signaling. Vascular remodeling occurs during normal development and participates in various physiological processes. However, structural changes to the vasculature can be pathologic as well as adaptive, leading to arterial disease development, which can contribute to cardiovascular dysfunctions such as hypertension and atherosclerosis. Angiotensin II (Ang II) is a potent growth factor involved in arterial wall homeostasis. Cardiac inflammation via activation of the cardiac Ang II system is suggested to play a role in cardiac remodeling [58]. Since some of the cytoskeletal changes that occur in vascular remodeling, specifically in VSMCs, are induced by Ang II [59], Rho-kinase has been directly linked to pathologic vascular remodeling through evidence suggesting that Ang II activates the Rho-kinase pathway and regulates the cytoskeleton [59–61].

Rho-kinase has also been linked with non-cardiovascular diseases such as diabetes, renal injury, erectile dysfunction, pulmonary hypertension, and cancer. Furthermore, RhoA/Rho-kinase has emerged as a pathway involved in some immuno-pathologies. For example, Rho-kinase has an important function in human immunodeficiency virus (HIV)-1-mediated disruption of the integrity of the blood–brain barrier [62]. Tat is a protein produced and released by HIV-1-infected cells, and circulating Tat can be detected in HIV-1-infected patients [63]. Tat, acting via intact lipid rafts, activates RhoA/Rho-kinase, leading to inhibition of MLCP and increased phosphorylation of MLC. This suggests that Tat-induced RhoA activation may constitute an early signaling mechanism leading to upregulation of efflux transporters on the brain endothelium, thus limiting antiretroviral drug penetration into CNS [62]. Many of these diseases related to Rho-kinase activation presented with damaged vasculature. The involvement of RhoA/Rho-kinase in these pathologies is clear due to an increasing number of experimental studies, along with the evidence that ROCK inhibitors can be used to treat some of those diseases [13, 64, 65]. Nevertheless, there are many points regarding the precise participation of RhoA/Rho-kinase in these disorders that remain unclear. We will explain some of the pathologies that have been shown to be associated with Rho-kinase activation and present the most recent published data.

Hypertension

Arterial hypertension is one of the most common cardiovascular disorders characterized by altered vascular tone and increased vascular contractility resulting in high blood pressure [66, 67]. It is accompanied by proliferation, migration of VSMCs, and varying levels of inflammation of the arterial wall, processes that together constitute vascular remodeling [58]. The Rho-kinase pathway plays a crucial role in the regulation of arterial blood pressure [68]. The role of Rho-kinase signaling in arterial hypertension was first recognized in 1997 [69]. In that study, a Rho-kinase inhibitor was observed to reduce arterial blood pressure in three experimental models of hypertension. In addition, Rho-kinase could also regulate blood flow via direct effects on the central nervous system [70, 71] or indirectly through negative effects on eNOS expression and activity [72, 73]. The Rho-kinase pathway is increased in spontaneously hypertensive rats [74, 75] and hypertensive patients [68]. This pathway has been frequently investigated since many studies demonstrated that Rho-kinase inhibitors, such as Y-27632 and fasudil, could be potential tools to treat hypertension and atherosclerosis, as well as other cardiovascular diseases [76]. Recently, a novel and potent selective Rho-kinase inhibitor, SAR407899, with promising antihypertensive activity, has been demonstrated to have a superior effect to that of fasudil and Y-27632 [77]. However, it is still a long way away from being used to treat hypertension clinically.

Atherosclerosis

Rho-kinase also contributes to the development of atherosclerosis and vascular inflammation [78]. Atherosclerosis is characterized by the cross-talk between excessive inflammation and lipid accumulation. Selective Rho-kinase inhibitors lead to upregulation of eNOS, decreased vascular

inflammation, and reduced atherosclerotic plaque formation [44]. In addition, experiments using ROCK1 (−/−) mice showed that ROCK1 in bone marrow-derived macrophages is critical to the development of atherosclerosis, in part by mediating foam cell formation and macrophage chemotaxis [79]. Recently, it has been suggested that statins, or HMG-CoA reductase inhibitors, which are accepted as first line agents for the treatment of hyperlipidemia to reduce the risk of adverse cardiovascular events [80], induced vascular benefits similar of selective RhoA/Rho-kinase inhibitors in vitro [81, 82], exerting an antiatherogenic effect that is independent of cholesterol reduction. This effect was observed in cultured VSMCs from both animal models and human samples [83, 84]. Furthermore, in the apolipoprotein E knockout mice, a model of accelerated atherosclerosis, lesion development was inhibited by Y-27632 and was associated with inhibition of ERM, a target protein of Rho-kinase [85].

Stroke

Rho-kinase pathway activation has been observed in various disorders of the central nervous system [86] and it seems important in the pathogenesis of several cerebral vascular diseases, such as stroke [46, 87] and cerebral vasospasm [47, 88]. Recent studies showed that Rho-kinase is directly involved in neuronal damage that occurs during a stroke. Rho-kinase plays an important role in neuronal apoptosis and in the execution phase of apoptosis [89]. The Rho-kinase inhibitor, fasudil, protects the brain tissue against ischemic damage in vitro [46, 90, 91] and the ischemia-induced delayed neuronal death when the treatment was started 24 h after induction of ischemia [90]. Moreover, fasudil was reported to prevent ischemic neuronal damage in vivo by increasing cerebral blood flow through upregulation of eNOS and decreasing the inflammatory response [31, 46], which is achieved by an inhibition of neutrophil migration [92]. This is supported by studies showing fasudil was not effective against induction of transient ischemia and reperfusion in eNOS knockout mice [46]. Recently, it has been described that this drug triggers proliferation and differentiation of adult neural stem cells at the subventricular zone in mice following hypoxia/reoxygenation injury, suggesting a direct effect of fasudil in neurons [93].

Heart failure

Rho-kinase is involved in the regulation of myofibrillar Ca^{+2} sensitivity in cardiac muscle [94] and contributes to irreversible myocardial damage [45]. Rho-kinase is also involved in the pathogenesis of cardiovascular remodeling [95], and its inhibition plays a significant role in treatment of the failing heart [96] by limiting infarct size [97], which is the major contributor to the development of heart failure [45]. The cardioprotective effect of Rho-kinase inhibition involves PI3 K/AKT and NOS activation [49, 52]. However, Rho-kinase inhibitor compounds need to be evaluated more closely for their efficacy during varying index ischemia periods, a wide dose range, and in vivo animal models mimicking the clinical setting [98]. Recently, it has been suggested that statins, specifically pitavastatin, could improve cardiac function and remodeling via eNOS production associated with the Rho-kinase pathway [99]. This could be explained using data showing that statins lower the intracellular levels of various proteins, such as RhoA [100]. In addition, statins block the activity of RhoA and prevent the activation of ROCK [101], which can regulate eNOS mRNA stability [51].

Diabetes

Type II diabetes is often associated with a collection of abnormalities including obesity, hypertension, various vasculopathies associated with the Rho-kinase pathway, and insulin resistance [102]. Impaired endothelium-dependent relaxation is a consistent finding in blood vessels from diabetic animals and patients [103, 104]. An increasing body of evidence intensifies the idea that the injured endothelium in diabetes is due, at least in part, to alterations in RhoA/Rho-kinase signaling. Increased vascular permeability is also a major characteristic of diabetic vasculopathy, and, in this condition, evidence indicates that advanced glycation end products (AGEs) activate RAGE, a major receptor for AGEs, and modulate various cell functions by multiple pathways, including Rho-kinase signaling [105]. RhoA and RAGE can unexpectedly form a complex called RhoA/RAGE, which has been suggested to induce Rho-kinase activation, resulting in reorganization of the actin cytoskeleton, leading to endothelial cell hyperpermeability in diabetes [106]. In the renal cortex of the streptozotocin (STZ)-induced diabetic rat, RhoA is highly expressed suggesting its involvement in diabetic renal injury [107]. The kidney is a major site of diabetic microvascular complications leading to renal failure. In kidney mesangial cells, which upregulate matrix protein synthesis in response to high glucose, it has been shown that RhoA/Rho-kinase is required for the fibrotic effects of high glucose in diabetes induced by STZ [108]. Also, activation of RhoA and Rho-kinase is greater in aortas from diabetic mice compared with non-diabetic mice [109]. These studies demonstrate an important role for the RhoA/Rho-kinase pathway in the pathogenesis of diabetic renal disease.

Rho-kinase is also responsible for the impairment of insulin signaling in Zucker obese rats, and its inhibition

corrected glucose levels [110, 111]. RhoA/Rho-kinase inhibition improved the symptoms of diabetic nephropathy reinforcing the participation of this pathway in diabetes complications [112]. However, chronic treatment of obese db/db mice with fasudil was reported to have no effect on blood glucose levels [112]. In contrast, in normal mice, acute treatment with Y-27632 causes insulin resistance in vivo [113]. Since ROCK1-deficient mice exhibited systemic insulin resistance via impaired insulin signaling in skeletal muscle, it has been suggested that ROCK1 can affect glucose homeostasis and insulin sensitivity [114]. In alloxan-induced diabetic rabbits and STZ-induced diabetic rats, the ROCK1 gene and protein were upregulated in penile tissue [115, 116]. The involvement of Rho-kinase in diabetes and its complications is becoming clear due to the increased amount of results showing upregulation of the Rho-kinase pathway in diabetic models, both in vivo and in vitro. The possibility of using Rho-kinase inhibitors to prevent progression of some diabetic problems, such as diabetic nephropathy, has been suggested as a promising novel therapy [108]. However, it is necessary to first clarify all the implications of Rho-kinase signaling alterations during the development of diabetes before employing this therapy in these patients.

Cancer

Recently, it is becoming clear that the Rho proteins play an important role in several aspects of cancer development, and each member of the Rho family (RhoA, RhoB, and RhoC) may be engaged at a different level at various tumor progression stages [117–119]. One of the first studies connecting Rho and malignancy showed that RhoA and a related molecule, Rac2, are overexpressed in head and neck squamous cell cancers [120]. Since tumor cell invasion and metastasis require a complex cascade of events, including changes in the cytoskeleton, it is predictable that the RhoA/Rho-kinase pathway would be involved in cancer development [119]. Also, Rho-GTPases could be early markers for tumor progression [118]. There are studies suggesting that Rho-kinase inhibitors would be useful to prevent cancer progression [121–123]. Nevertheless, some of these studies indicate that reduced Rho-protein function contributes to the morphological changes observed in tumor cells. This elevates the risk that inhibition of Rho proteins might promote a more aggressive tumor phenotype [117]. It has been reported that RhoA/ROCK is involved in progression of human gastric cancer [124]. This kind of cancer displays high expression of RhoA, which has been correlated with aggressive metastasis. In addition, gastric cancer cell invasion can be induced via activation of the RhoA/ROCK pathway by IL-6. Then, RhoA expression has been suggested useful as a prognostic factor in patients with gastric adenocarcinoma [124]. In addition, active RhoA has oncogenic potential which promotes the invasiveness of rat hepatoma cells via the activation of the endogenous RhoA pathway [125]. Also, it was demonstrated that cells transformed by Rho oncogenes do have metastatic potential in vivo [126]. RhoA and RhoC siRNA gene therapy, mediated by adenovirus, has been suggested as useful for inhibition of growth and invasion of human gastric carcinoma by the PI3/Akt pathway [127]. However, it has been suggested that RhoC is most involved in the cancer process, mainly in metastasis formation, more so than RhoA and RhoB [128].

There is increasing evidence regarding the role of Rho-kinase in cell proliferation and survival. The requirement for survival signals to prevent apoptosis in normal cells is reduced in tumor cells. Rho family proteins including RhoA have been described in both pro- and anti-apoptotic signaling [129]. In studies involving cancer tissues and cell lines, it was reported that there was a direct relationship between cell proliferation and the level of ROCK2 expression in both malignant and nonmalignant cells [130, 131]. Besides cell proliferation, Rho-kinase has been implicated in the drug resistance observed in liver cancer patients [132]. In ovarian cancer, which is very invasive, the lysophosphatidic acid (LPA)/RhoA/Rho-kinase pathway is intimately involved in the course of ovarian cancer progression, and fasudil administration attenuated the invasiveness of the cells by inhibition of this pathway [122]. Pharmacological modulators of several steps of the Rho-kinase signaling pathway have been investigated, and a new chemical compound, CCG-1423, has demonstrated inhibition downstream of RhoA, which can be used as a pharmacological tool to disrupt the Rho-kinase pathway in cancer [133].

Pulmonary hypertension

Convincing evidence indicates that Rho-kinase is also involved in pulmonary hypertension (PH) [10, 64, 134, 135], a disease characterized by progressive elevation of pulmonary arterial pressure and vascular resistance due to pulmonary vasoconstriction and vessel remodeling, as well as increased inflammation [136, 137]. In isolated human lung tissue from PH patients, Rho-kinase expression and reactivity were significantly increased compared with controls, and relevant correlations were observed between Rho-kinase activity and the severity and duration of PH [138]. Also, in patients with moderate PH, treatment with intravenous fasudil had beneficial effects, such as decreased pulmonary artery blood pressure and pulmonary vascular resistance [139]. Additionally, PH patients that did not respond to oxygen inhalation, NO inhalation, or nifedipine, 30-min intravenous fasudil treatment

significantly decreased elevated pulmonary vascular resistance [134]. Also, fasudil inhalation for 10 min significantly reduced mean pulmonary arterial pressure in patients with PH [140]. Rho-kinase signaling inhibitors have been suggested as a viable therapeutic agent for PH treatment. However, there is no study showing the long-term effects of Rho-kinase inhibitor administration, such as fasudil, in patients with severe PH, even though, in both human and animals, it is reasonable to argue that Rho-kinase has an important role in PH due to its involvement in the sustained vasoconstriction, vascular remodeling, and inflammation that occurs in this pathology. Accumulating evidence from several laboratories indicates that RhoA/Rho-kinase signaling plays an important role in the pathogenesis of many experimental models of PH, including chronic hypoxia [141–143], monocrotaline [143, 144], VEGF receptor inhibition [137], and mild hypoxia-induced PH in neonatal fawn-hooded rats [145]. Furthermore, Rho-kinase signaling mediated vasoconstriction in severe occlusive PH in rats [137], and small pulmonary arteries exhibit Rho-kinase-dependent increases in myogenic tone in chronic hypoxic PH [146].

In mouse models of PH, treatment with Y-27632 decreased the muscularization of distal pulmonary arteries and upregulated eNOS expression [147]. The beneficial effect of sildenafil on PH is mediated, at least in part, by the inhibition of the RhoA/Rho-kinase pathway [141]. It has been demonstrated that serotonin (5-HT), also synthesized by pulmonary endothelial cells, is internalized in SMCs through the 5-HTT (5-HT transporter) and is linked to RhoA by intracellular type 2 transglutaminase, leading to constitutive RhoA activation [148]. Recent studies showed that enhancement of RhoA and Rho-kinase activities in PH is associated with increased RhoA serotonylation, suggesting direct involvement of 5TT/RhoA/Rho-kinase in proliferation of pulmonary artery smooth muscle cells (PA-SMCs) and platelets, during PH progression [149]. Finally, in addition to atherosclerosis, research indicates that statins improve PH in several rat models. New studies have supplied evidence that reversal of hypoxic PH by simvastatin decreased lung expression and activity of both ROCK1 and ROCK2 [150].

Erectile dysfunction

Another ailment that has been associated with the involvement of RhoA/Rho-kinase in its pathologic mechanism is erectile dysfunction (ED). The penis becomes erect with the relaxation of arteriolar and sinusoidal smooth muscle. NO produced by eNOS [151, 152] is fundamental for smooth muscle relaxation and, consequently, normal erectile function [153]. Since the main function of Rho-kinase is the regulation of smooth muscle tone [154], the upregulation of the Rho-kinase pathway increases cavernosal smooth muscle contraction, leading to ED [155, 156]. In addition, RhoA/Rho-kinase-mediated Ca^{+2} sensitization has an important role in the regulation of corpora smooth muscle tone and keeps the penis in the flaccid state [157]. Further, in the absence of arousal, the penis remains in the non-erect state by cavernosal vasoconstriction induced mainly by norepinephrine and endothelin 1 (ET-1), which are Rho-kinase-mediated responses [115, 158].

ED is associated with cardiovascular diseases, mainly hypertension [159] and other diseases, such as diabetes [116] and hypogonadism [160]. Cardiovascular diseases, mainly atherosclerosis and hypertension, have been considered a risk factor for ED [161]. In spontaneously hypertensive rats (SHR) and DOCA-salt rats (a mineralocorticoid-dependent hypertensive rat), increased activity of the RhoA/Rho-kinase pathway was observed, and could be the cause of ED in these hypertensive models [158]. However, over the past few years, many studies have suggested that ED may be an early marker in the development of cardiovascular pathologies [162], reinforcing the involvement of Rho-kinase activity in these diseases.

In corpus cavernosum tissue from diabetic rats, it was observed that there was an enhancement of Rho-kinase activity [115]. In streptozotocin-induced diabetic rats, in vivo injection of Y-27632 into the penis increases intracavernosal blood pressure, membrane-fraction RhoA protein expression, and MYPT1 phosphorylation levels, supporting observations that alterations in the Rho-kinase pathway occur in the diabetic rat penis [116]. Conversely, in diabetic mice, even though acetylcholine-induced corpus cavernosal tissue relaxation and erectile function are considerably less than in non-diabetic mice, Rho-kinase inhibitors produce a similar relaxation response, suggesting that RhoA/Rho-kinase signaling may not be altered in the diabetic mouse penis [163]. Regarding hypogonadism-associated ED, it has been suggested that upregulation of the RhoA/Rho-kinase pathway in the penis may be an associated mechanism [164]. The same work demonstrated that testosterone replacement restored erectile function and reduced RhoA and Rho-kinase protein expression in castrated rats, although the expression levels were still significantly increased compared with intact rats. These results could be explained due to multi-factorial effects of testosterone, including NO stimulation [164]. In a castrated rat model of ED, it has been demonstrated that Rho-kinase inhibition was able to improve androgen ablation-induced ED [116]. Also, in diabetes related with ED, the overexpression of RhoA/Rho-kinase signaling was observed in penile tissue from diabetic rats, and ROCK1 protein expression is increased but not ROCK2. In this study, the administration of testosterone in diabetic rats was able to treat hypogonadism and maintain erectile function by

normalizing RhoA/Rho-kinase pathway upregulation [165]. Nevertheless, the role of androgens on the vasoconstrictor action of components of the RhoA/Rho-kinase pathway involved in the erectile process remains to be elucidated.

Since it was demonstrated that Y-27632 improved erectile function in aged rats, its has been suggested that the RhoA/Rho-kinase pathway could be important in the pathology of ED associated with age [166]. However, it is difficult to know exactly the role of RhoA/Rho-kinase in ED because this inhibitor has effects on multiple kinases involved in smooth muscle contraction. Nevertheless, a study used adeno-associated viral gene transfer of dominant-negative RhoA mutant (T19NRhoA) into the rat corpus cavernosum, as a tool to target specifically RhoA. The results indicated increased intracavernosal blood pressure (ICP), suggesting that the ED-associated age process involves increased RhoA/Rho-kinase pathway signaling [167]. The activation of neuronal nitric oxide synthase (nNOS) in corpus cavernosum tissue decreased with ageing. Additionally, it has been suggested that, in old rats, the imbalance between nNOS activity and Rho-kinase could be associated with impaired erectile function [11]. Taken together, it is clear that RhoA/Rho-kinase activity is a fundamental component to keep the penis in the non-erect state, and this pathway is upregulated in ED. Also, the essential balance between contraction and relaxation in the penis, which is maintained by the RhoA/Rho-kinase and NO/cyclicGMP pathways, is modified in this pathology.

Conclusion

Due to the rapidly growing number of studies implicating the RhoA/Rho-kinase pathway in various pathologies, it is undeniable that Rho-kinase is an important therapeutic target. Specific and potent pharmacological modulators of various steps of the RhoA/Rho-kinase signaling pathway are critically needed for treatment intervention in cardiovascular diseases, neurological disorders, and cancer progression. However, a greater understanding of the physiological role of each of the Rho-kinase isoforms and the development of isoform-specific inhibitors are needed to achieve these goals. Taken together, the connection between many diseases and RhoA/Rho-kinase seems to be in the upregulation of this pathway. Increased activation of MLCK is a consequence of RhoA/Rho-kinase pathway upregulation leading to MLCP and eNOS inhibition (Fig. 2). Both events facilitate vascular contraction, along with interfering in other cellular responses, such as focal adhesion, migration, gene expression, and apoptosis signaling, which contribute to the development of several pathologies (Fig. 2) [2, 4, 44, 72, 168]. Nevertheless, many questions about how upregulation of RhoA/Rho-kinase interferes in the each pathology remain unclear.

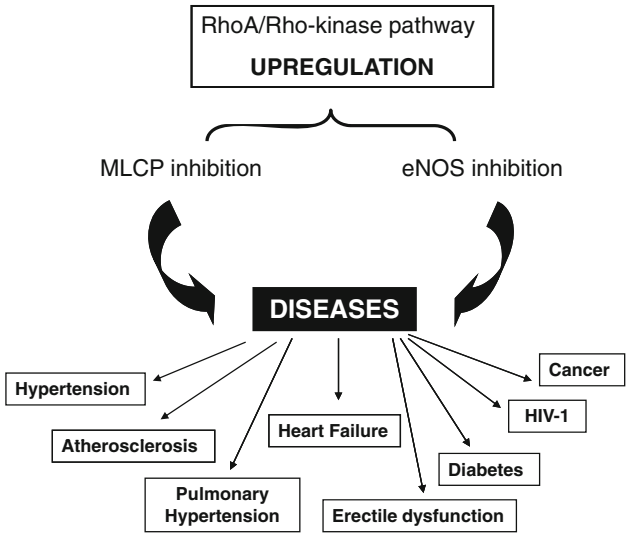

Fig. 2 Upregulation of RhoA/Rho-kinase pathway is a key link among many diseases. MLCP and eNOS activity are targeted by RhoA/Rho-kinase upregulation, leading to alterations in many physiological processes associated with these two molecules, resulting in various disease states

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