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Rho kinase and hypertension

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Abstract

Arterial hypertension is a multifactorial disease that is characterised by increased peripheral vascular resistance often accompanied by smooth muscle cell hypertrophy and proliferation. Rho kinases (ROCKs) are the most extensively studied effectors of the small G-protein RhoA and abnormalities in RhoA/ROCK signalling have been observed in various cardiovascular disease including hypertension. The RhoA/ROCK-pathway is a key player in different smooth muscle cell functions including contractility, proliferation and migration. Furthermore, there is extensive crosstalk between RhoA/ROCK- and NO-signalling. Therefore, not only ROCK inhibitors but also NO-donators or pleiotropic agents like statins exert their beneficial effects on the cardiovascular system at least in part via Rho/Rho-kinase.

Keywords: Rho kinase; hypertension; NO-signalling; Rho kinase inhibitors; statins

1. Introduction

Arterial hypertension is a common health problem which represents a major risk factor for a variety of diseases like myocardial infarction or stroke. Since the discovery of Rho-kinases (ROCKs) as major effectors of the small GTPase RhoA, ROCKs have been extensively studied in the field of cardiovascular research. In smooth muscle cells, RhoA and ROCKs not only affect actin polymerisation but regulate a wide range of cellular functions like contraction, migration and proliferation [1]. Upregulation of the RhoA/ROCK signalling cascade has been observed in different cardiovascular disorders like atherosclerosis, pulmonary hypertension and stroke. Furthermore, RhoA/ROCK seem to have an impact on NO-signalling and vice versa.

This review describes the current understanding of ROCK signalling especially in smooth muscle cells and highlights the role of ROCKs as targets for current therapeutic approaches to treat hypertension.

2. General information on ROCKs

2.1. Structure and expression of ROCKs

ROCKs are serine/threonine kinases with a molecular mass of ~ 160 kDa that are expressed in invertebrates (*C. elegans*, *Drosophila*) and in vertebrates (e.g. zebrafish, *Xenopus*, mouse, human). Two different ROCK isoforms encoded by two different genes have been identified [2-4]. In humans the genes coding for ROCK I and ROCK II are located on chromosome 18 (18q11.1) and chromosome 2 (2p24), respectively. In both enzymes the kinase domain is located at the amino-terminus, followed by a coiled-coil forming region that contains the Rho-binding domain (RBD; fig. 1A). At the carboxy-terminal part of the protein a Pleckstrin-homology (PH) domain with an internal cystein-rich domain can be found. The PH domain is supposed to be involved in protein localization.

The amino-acid sequences of the two isoforms ROCK I and ROCK II have an overall identity of 65 %. The identity in the RBD is 58 % and the highest similarity (92 %) is found in the kinase domain [3]. The phylogenetic tree shows that myotonic dystrophy kinase (DMPK), DMPK-related cell division control protein 42 (Cdc42)-binding kinases (MRCK) and citron kinase are most closely related to ROCK I and ROCK II [5].

The mRNAs of ROCK I and II are ubiquitously expressed. While the ROCK II transcript is preferentially found in muscle tissue and in brain, both ROCK isoforms are expressed in vascular smooth muscle and in heart [3, 6, 7]. The regulation of ROCK expression has not been studied in detail but some data show an upregulation of ROCK by angiotensin II via angiotensin II type 1 (AT₁) receptor-mediated pathways. Mice receiving continuous Ang II administration showed increased amounts of ROCK in the coronary arteries [8].

In addition to organ distribution the subcellular localization of ROCKs has been the subject of various investigations. Cell-fractionating studies showed that ROCKs are mainly distributed in the cytoplasm but are partially translocated to the peripheral

membrane by RhoA activation [4]. In addition, ROCK II also localises at the vimentin intermediate filament network [9] and at actin stress fibers [10].

2.2. Regulation of ROCK activity

As already described, ROCKs contain a kinase domain at the N-terminus and regulatory RBD and PH domain at the C-terminal part of the amino acid sequence. The C-terminus seems to function as an autoinhibitory domain because truncated forms of ROCK lacking the PH- and RBD-domain are constitutively active whereas the C-terminus expressed in cells acts in a dominant-negative way [11]. Binding of the activated, GTP-bound form of RhoA to the RBD stimulates ROCK activity by disrupting the self-associative interaction between the catalytic and the inhibitory domain [12] (fig. 1B). However, this stimulatory effect of RhoA on ROCK activity is moderate, not exceeding 1.5- to 2-fold increase [13]. In addition to RhoA, some lipids, such as arachidonic acid can effectively stimulate ROCK activity (5- to 6-fold) independent of RhoA binding [13, 14]. The lipids seem to bind to the regulatory C-terminus of ROCK, disrupt the autoinhibitory interaction and thus lead to kinase activation.

Activation of ROCKs by truncation of the C-terminal autoinhibitory part of the protein is occurring *in vivo*. During apoptosis caspase-3 cleaves ROCK I at the recognition site DETD and gives rise to an constitutively active kinase [14] (fig.1B). This cleavage site is not present in ROCK II. However, granzyme B, a proapoptotic protease, can cleave the C-terminus of ROCK II thus also generating an active enzyme [15]. Again this cleaving site is not present in ROCK I.

Negative regulators of ROCK activity have also been described. The small GTPase RhoE binds to the N-terminal part of ROCK I and interferes with binding of RhoA, thus preventing kinase activation [16, 17]. Two other small G-proteins, Gem and Rad, have also been shown to bind to and thereby inhibit ROCKs but the detailed mechanisms have to be further studied [17].

2.3. ROCK targets

More than 15 ROCK substrates have been described of which some are listed in table 1. The most important targets for cardiovascular function will be discussed in

more detail (fig. 2). A large number of ROCK targets are related to actin-filament dynamics, organization of the cytoskeleton and regulation of contractility.

One of the most important substrates of Rho-kinases is the myosin-phosphatase target unit (MYPT-1) of the myosin light chain phosphatase (MLCP) [18]. The MLCP consists of three different subunits: a catalytic domain (protein phosphatase type 1), a myosin binding subunit (MYPT-1) and a small non-catalytic subunit of unknown function. Native MLCP can be purified as a trimer consisting of these three proteins [19]. Recently a small myosin phosphatase-RhoA interacting protein (M-RIP) has been discovered [20]. M-RIP binds to both RhoA and MYPT-1 and might thereby target RhoA/ROCK to the MLCP [21]. Another recent study showed a direct binding of only ROCK II to MYPT-1 and also a major role of only ROCK II for vascular smooth muscle cell contraction [22]. These are the first results pointing towards a differential function of ROCK I and ROCK II in smooth muscle cells.

Phosphorylation of MYPT-1 by ROCK II at various sites (threonine 696, serine 854 and threonine 853, numbering of the human isoform) results in the inhibition of MLCP activity and a concomitant increase in phosphorylated regulatory light chain of myosin (MLC) and smooth muscle cell contractility ([23, 24]. Under certain conditions, phosphorylation of MYPT-1 induces the dissociation of MYPT-1 and thus MLCP from myosin in an agonist-specific manner thus hindering the phosphatase to dephosphorylate MLC [25]. This also leads to an increased phosphorylation state of MLC and to enhanced contractility of the smooth muscle cell.

ROCK II also phosphorylates MLC directly at serine 19 *in vitro* [26] but the relevance of this phosphorylation event has still to be elucidated because activation of the Rho-Rho-kinase pathway without concomitant activation of MLCK induces only minimal MLC phosphorylation and cell contraction [27, 28].

ROCK also phosphorylates CPI-17 [29], a small regulatory protein that in its phosphorylated state inhibits MLCP activity by binding to the phosphatase subunit of MLCP [30]. Phosphorylation of CPI-17 by ROCK induces a conformational switch of CPI-17 [31] thus increasing the inhibitory effect of CPI-17 on MLCP activity [32]. The relative contribution of CPI-17 phosphorylation to ROCK-dependent contraction of smooth muscle has not been studied in detail yet.

Most ROCK substrates have been identified in *in vitro*-experiments after activation of endogenous ROCK or transfection of one of the two ROCK isoforms, in most cases ROCK II. For a long time it has been assumed that because of the high identity of the

kinase domains of ROCK I and ROCK II both enzymes share the same substrates. RhoE was the first example for a ROCK target that is phosphorylated by ROCK I but not ROCK II [33]. The abovementioned direct binding of ROCK II but not ROCK I to MYPT-1 is another hint pointing to different functions of the two isoforms [22]. Additional data from experiments with mice haploinsufficient for ROCK I or ROCK II in leucocyte recruitment and neointima formation after vascular injury has also shown different functions of the two kinases [34]. Haploinsufficient ROCK I mice showed significantly reduced neointima formation and decreased level of proinflammatory adhesion molecules after carotid artery ligation. Interestingly, while blood pressure and heart rate are unchanged in haploinsufficient ROCK I and ROCK II mice [34, 35], cardiac-specific overexpression of RhoA leads to a dramatically depressed heart rate [36]. Thus, at least in smooth muscle tissue the two ROCK isoforms have distinct roles under different conditions. A detailed analysis of specific roles of individual ROCK isoforms is necessary to selectively target the responsible ROCK enzyme in the therapy of cardiovascular disease.

3. ROCK and smooth muscle cells

The Rho/Rho-kinase pathway is an important regulator not only of vascular smooth muscle cell contraction but also of migration, proliferation and differentiation.

3.1. Contractility

The contractile state of VSMC is mainly determined by the phosphorylation level of MLC. MLC is phosphorylated by the Ca^{2+} /calmodulin-dependent MLCK and dephosphorylated by the Ca^{2+} -independent MLCP. Various vasoactive agonists like angiotensin II, endothelin-1 and thromboxane A₂ activate G-protein coupled receptors and lead not only to an increase in intracellular Ca^{2+} via G-proteins of the G_q/G_{11} -family but also activate G-proteins of the G_{12}/G_{13} -family that stimulate the Rho/Rho-kinase signalling pathway, thereby activating ROCK. Thus, as outlined above the inhibition of MLCP via phosphorylation of MYPT-1 by ROCK leads to increased phosphorylation of MLC and enhanced VSMC contractility [13, 18]. In addition, ROCK might also modulate VSMC contractility by direct phosphorylation of the MLCP-inhibitory protein CPI-17 [29] and MLC [18, 26].

Modulation of VSMC contractility via ROCK seems to be of particular importance during tonic contractions [37] in different vascular beds including pulmonary artery,

mesenteric artery and portal vein. Another important role for ROCK has been shown for maintaining myogenic tone of small arteries [38, 39].

3.2. Migration

Migration of cells, including smooth muscle cells, depends on the reorganization of the actin cytoskeleton. Involvement of ROCK in cell migration has been extensively studied using pharmacological tools. Depending on cell type and experimental conditions ROCK inhibition can either inhibit or enhance cell migration. As several ROCK targets are implicated not only in actin- but also intermediate filament network- and microtubule- dynamics (see table 1) the final effect on cell migration might depend on the relative contribution of each of these pathways in a particular cell type. Smooth muscle cell migration induced by PDGF and lysophosphatidic acid can be attenuated by ROCK inhibitors or transfection of a dominant-negative form of ROCK [40]. In addition, smooth muscle cell migration in 3D collagen matrices is also inhibited by blocking ROCK signalling [41]. In contrast, motility of differentiated aortic smooth muscle cells is increased by inhibition of ROCK with fasudil, a selective ROCK inhibitor [42]. These contradictory data might reflect a differential importance of ROCKs depending on the differentiation status of the smooth muscle cell.

3.3 Proliferation

Dysregulated proliferation of smooth muscle cells can be one feature of cardiovascular disease. ROCKs are implicated in the regulation of SMC proliferation. As already seen for migration, proliferation stimulated by PDGF is influenced by inhibition of the Rho/ROCK pathway [43]. Inhibition of ROCK abolishes the PDGF-induced activation of extracellular-regulated kinases 1/2 (ERK 1/2). Similarly, ROCKs might also play a role in angiotensin II-induced vascular hypertrophy [44]. Opposite data, that do not reveal a significant role of ROCK in smooth muscle cell proliferation, also exist. Some *in vivo* studies report no effect of ROCK inhibition on smooth muscle cell proliferation after balloon injury [45, 46]. Again, the relative importance of the single ROCK substrates in a distinct experimental setting might affect the final observation after interfering with the Rho/Rho-kinase pathway.

3.4 Differentiation

Smooth muscle cells unlike most other differentiated cells retain the capacity to modulate their phenotype in response to certain stimuli. The contractile phenotype of a smooth muscle cell is characterized by the coordinated expression of several contractile and cytoskeletal genes that are regulated via serum-response factor (SRF) [47]. Under certain conditions, e.g. vascular disease, this gene expression pattern is switched to enhance growth and extracellular matrix production leading to the so called synthetic phenotype of the smooth muscle cell [47]. The Rho/Rho-kinase pathway seems to be critically involved in the regulation of the smooth muscle phenotype. Inhibition of ROCK by Y27632, a selective inhibitor of ROCK activity, decreases the expression of contractile genes [48] whereas expression of constitutively active RhoA increases the activity of smooth muscle-specific promoters [49].

4. ROCK in cardiovascular disease

4.1. ROCK and hypertension

Hypertension is characterized by high arterial pressure resulting from increased peripheral vascular resistance that can be attributed to both enhanced contractility of vascular smooth muscle cells and arterial wall remodelling. Increased activity of the Rho/Rho-kinase pathway has been proposed to play an important role in the development and maintenance of hypertension. In various animal models of experimental hypertension a role of RhoA and ROCK has been demonstrated. Blocking ROCK activity with Y27632 has blood pressure lowering effects in spontaneously hypertensive rats (SHR), deoxycorticosterone-acetate (DOCA)/salt-treated and renal hypertensive rats [50]. In contrast, treatment of normotensive rats with the ROCK inhibitor leads to an only small transient decrease in blood pressure. Similarly, oral administration of fasudil to SHR rats significantly lowered blood pressure [51]. In addition, several studies have addressed changes in RhoA activity in isolated vascular segments from hypertensive animals suggesting that increased RhoA activity is responsible for enhanced ROCK function in the pathology of hypertension. In mesenteric and cerebral arteries from SHR and normotensive rats the relaxation induced by treatment with Y27632 was markedly higher in arteries from SHR rats [52, 53]. This has also been shown for mesenteric arteries from DOCA/salt-treated rats [54]. In animals treated with L-NAME, an inhibitor of NO-synthase, oral administration of Y27632 lowered blood pressure [55] and the level of active, GTP-

bound RhoA was markedly increased in vessels from L-NAME treated rats. Similarly, direct evidence for increased amounts of active RhoA have also been found in stroke-prone SHR, DOCA/salt- and renal hypertensive rats [55-57]. Analysis of the expression levels of RhoA has led to controversial results with some studies showing an increased expression of RhoA under hypertensive conditions [56] whereas in other reports no differences in the expression profile of proteins from the RhoA/ROCK pathway have been detected [55].

ROCKs are also involved in spontaneous tone development under hypertensive conditions. Arteries from DOCA/salt-hypertensive rats develop spontaneous tone that is not observed in vessels from normotensive rats [58]. Treatment with Y27632 significantly reduces this tone development in arteries from hypertensive rats but has no impact on basal tone of normotensive rats.

Not only oral but also direct central administration of ROCK inhibitor has an impact on blood pressure [59, 60]. Central application of Y27632 to SHR and L-NAME hypertensive rats lowered blood pressure and reduced enhanced sympathetic activity. Again, both membrane-bound RhoA and Rho-kinase activity in the brainstem of these hypertensive animals was increased.

Alterations in the RhoA/ROCK pathway are most likely the consequence of an upstream event in the hypertensive state. One upstream signalling molecule that seems to be involved is angiotensin II and its receptor type 1 (AT1). Y27632 has been shown to be more potent in relaxing arteries from angiotensin II-induced hypertensive compared to normotensive animals [61] pointing towards an increased sensitivity to ROCK inhibition in these animals. Specific blockade of AT1 receptor prevents the upregulation of RhoA and ROCK activity in different models of experimental hypertension [57, 62]. In addition, in arteries from stroke-prone SHR increased mRNA levels of several RhoGEFs that function as activators for RhoA have been detected thereby linking G-protein coupled receptor signalling to enhanced RhoA and ROCK activity [63]. Indeed, a recent study revealed the essential role of the signalling from heterotrimeric G-proteins via RhoGEF (LARG) to RhoA/ROCK pathway [64]. With an inducible and smooth muscle specific conditional knockout mouse line that allows for the deletion of either $G\alpha_{q/11}$ or $G\alpha_{12/13}$ experimental DOCA/salt-hypertension was analysed. In mice lacking either of the two families of signalling molecules the development as well as the maintenance of

DOCA/salt-induced hypertension was completely abolished. The same was also seen in mice deficient for the RhoGEF LARG [64].

Another upstream event that is linked to activation of RhoA and ROCKs in hypertension is the formation of reactive oxygen species (ROS). Oxidative stress characterizes different forms of hypertension in animals and humans [65, 66]. It has been shown that rat aorta contracted under conditions that induce ROS formation has an increased sensitivity towards Y-27632 [67] pointing towards an activation of the RhoA/ROCK pathway by ROS [62]. Indeed, the amount of membrane bound, active RhoA was increased by ROS. Air pollution is an environmental factor that is known to potentiate hypertension in humans [68]. A recent study could show that exposure of rats to air pollution in combination with angiotensin II-infusion leads to an increase in blood pressure that is accompanied by a ROS-mediated increase in ROCK activity and MYPT-1 phosphorylation [69].

As outlined above, ROCKs not only influence VSMC contraction but have a more general impact on actin dynamics thereby also influencing proliferation and migration of vascular smooth muscle cells. Overexpression of Profilin 1, a small actin binding protein that has been shown to be a ROCK target (table 1) [70], in blood vessels of mice increased stress fiber formation and induced hypertrophy [71]. Furthermore, profilin 1 led to an elevated blood pressure in adult animals that was accompanied by increased ROCK II expression. Thus, at long sight, profound changes in the actin cytoskeleton that primarily induced hypertrophy can directly influence vascular smooth muscle cell contractility.

Other cardiovascular disorders (e.g. atherosclerosis) that often accompany hypertension might also be in part due to changes in RhoA and ROCK activity. Indeed, long-term blockade of ROCK with a nonhypotensive dose of fasudil suppresses medial hypertrophy and perivascular fibrosis in coronary arteries from SHR and angiotensin II-treated hypertensive rats [51, 72].

A role for enhanced ROCK activity in clinical hypertension has also been reported [73]. Forearm blood flow was measured in normotensive and hypertensive subjects. In hypertensive patients, resting forearm vascular resistance was higher compared to normotensive subject. Graded intra-arterial infusion of fasudil increased forearm blood flow and this decrease in vascular resistance was more pronounced in hypertensive patients. If sodium nitroprussid was used instead of fasudil the vasodilator responses did not differ between the two groups of patients. This leads to

the conclusion that enhanced activity of the RhoA/ROCK pathway is involved in the increased vascular resistance in clinical hypertension. Furthermore, in patients with heart failure intra-arterial infusion with fasudil decreases vascular forearm resistance and increases forearm blood flow [74]. Interestingly, in cigarette smokers, a high risk group for developing cardiovascular disease, increased RhoA and ROCK activity in forearm vasculature has been reported [75]. Another evidence for the importance of the RhoA/ROCK pathway for hypertension in humans comes from genetic studies [76, 77]. For ROCK II a polymorphism exists at amino acid position 431 that can be either threonine or asparagines (Asn) [76]. This amino acid is located in the coiled-coil region of ROCK II and might therefore be involved in ROCK dimerization, Rho binding and thus ROCK activity and phosphorylation of ROCK targets. The Asn/Asn genotype has been shown to be associated with a greater resting systolic blood pressure and a higher blood pressure increase after applying environmental (cold) stress. These effects seem to be due to an increased vascular resistance as cardiac output, large artery compliance and vasoactive hormone metabolism are unchanged. A second study provides further evidence that ROCK polymorphism might be involved in blood pressure control. A haplotype block consisting of four SNPs within the ROCK II allele was found to be recessively associated with a lower risk of hypertension [77]. This block is located at the 5'-end of the ROCK II locus and covers exons encoding the kinase domain of the enzyme.

4.2. ROCK and NO signalling

Nitric oxide (NO) derived from the endothelium is a potent vasodilator. NO, after diffusing into the smooth muscle cells of the vessels, activates soluble guanylate-cyclase and thus stimulates the formation of cyclic GMP (cGMP) and the subsequent activation of cGMP-dependent protein kinase (cGK). The vasodilation induced by the NO/cGK signalling cascade results from a decrease in intracellular Ca^{2+} due to several mechanisms like phosphorylation of ion channels and activation of the sarcoplasmic Ca^{2+} -ATPase [78] as well as a reduced Ca^{2+} release from intracellular stores via phosphorylation of IRAG [79] and phosphorylation and activation of myosin phosphatase [80].

Impaired endothelial function and decreased NO-production has been implicated in the etiology of hypertension. Decreased expression of endothelial nitric oxide

synthase (eNOS) is found in aortas from SHR rats [81] and eNOS-deficient mice have an elevated blood pressure [82].

Interestingly, there seems to be extensive crosstalk between NO- and RhoA/ROCK-signalling (fig. 3). There is compelling evidence that the NO/cGK pathway leads to an inhibition of RhoA/ROCK signalling. The NO-donor sodium nitroprussid and constitutively active cGK are able to inhibit the phenylephrine-induced translocation of RhoA from the cytosolic fraction to the membrane in rat aorta [83] thus abolishing subsequent ROCK activation. Furthermore, NO-mediated vasorelaxation has been demonstrated to be in part mediated by inactivation of RhoA/ROCK signalling in rat aorta [84]. Chronic and acute inhibition of NOS increases α 2-adrenoreceptor-stimulated activation of RhoA and ROCK [85] showing once more the capability of NO-signalling to inhibit the RhoA/ROCK cascade.

On the other hand, the RhoA/ROCK cascade seems to reciprocally influence NO-signalling. Inhibition of RhoA function by HMG-CoA reductase inhibitors ("statins"), like fluvastatin or mevastatin, upregulates NOS expression [86, 87] and leads to increased NO production and reduced vascular contractility [88]. Blocking ROCK function with fasudil also results in increased NO production [89]. The mechanism by which ROCK influences NO production seems to be the regulation of eNOS mRNA stability. Hypoxia and thrombin, two stimuli that increase ROCK activity, downregulate eNOS expression via destabilization of eNOS mRNA [90, 91]. Furthermore, direct inhibition of ROCK by e.g. Y27632 increases eNOS mRNA half life and expression [92].

Taken together, increased NO production will lead to an inhibition of the RhoA/ROCK-cascade thus protecting against the enhanced contractile and proliferative effects of RhoA/ROCK signalling. On the other hand, stimulated RhoA/ROCK-activity appears to diminish NO production thus even amplifying the increase in contractility induced by RhoA/ROCK-signalling.

5. Targeting the RhoA/ROCK signalling cascade in the treatment of cardiovascular disease

5.1. ROCK inhibitors

Several compounds have been developed and described to specifically inhibit ROCK activity with some specificity. The most widely used inhibitor is the pyridine derivative Y27632 that competes for the ATP binding site on the ROCKs thus inhibiting the

kinase activity [93]. Thereby it prevents MYPT-1 phosphorylation and inhibits smooth muscle contraction. Y27632 is transported into the cells via a carrier-facilitated diffusion mechanism so that extra- and intracellular concentrations of the compound are similar. Y27632 has no specificity for ROCK isoforms but inhibits both ROCK I and ROCK II. Although Y27632 is reasonably selective for the Rho-kinases and has much lower inhibitory effects on PKC, PKA and MLCK it shows a similar potency for the also Rho-dependent PCK-related kinase I (PRK 2) *in vitro* [94]. Nevertheless Y27632 remains an important pharmacological tool for analysing the involvement of ROCK in cardiovascular processes under normal and pathological conditions.

Another group of compounds that are derived from isoquinolinesulfonamide shows a similar selectivity for inhibition of ROCK. Fasudil, or HA1077, also competes with ATP for binding to the kinase [94]. In addition to Y27632 fasudil also exerts inhibitory effects on PKA and PKC but the IC₅₀-values are approximately 5- and 30-times (~5 µmol/l and 37 µmol/l, respectively) higher than for the ROCKs (IC₅₀ ~ 1 µmol/l) [95]. It has also been demonstrated that hydroxyfasudil, a major active metabolite of fasudil after oral administration, has a more specific inhibitory effect on ROCK with the IC₅₀ being ~ 0.7 µmol/l [72, 96]. Fasudil hydrochloride is in clinical use in Japan for several years for the treatment of cerebral vasospasm following subarachnoid haemorrhage [97]. In addition, an anti-anginal effect of fasudil in patients with stable angina pectoris has been observed in clinical trials [98, 99]. Both studies have proven that long-term oral treatment with fasudil is well tolerated and does not cause major adverse events. In patients suffering from vasospastic angina, intracoronary application of fasudil attenuates both arterial spasm and the extent of subsequent myocardial ischemia [100, 101]. Intravenous administration of fasudil in the acute phase of stroke has demonstrated beneficial effects of ROCK inhibition on ischemic neuronal damage [102] In patients with severe pulmonary hypertension intravenous administration of fasudil significantly lowers pulmonary vascular resistance [103]. Furthermore, fasudil suppresses the excessive proliferation of pulmonary artery smooth muscle cells, a key feature in the development of pulmonary hypertension, *in vitro* [104]. Currently clinical phase III trials for the therapy of Raynaud's syndrome (NCT00498615) and carotid stenosis (NCT00670202) with fasudil are ongoing. More recently a novel class of compounds has been developed. This aminofurazan-based inhibitors (GSK269962A and SB772077B) show similar inhibitory effects on ROCK I and ROCK II but with a higher potency than Y27632 [105]. Both drugs act as

vasodilators and lower blood pressure in animal models of hypertension. Other new developed ROCK inhibitors like Wf536 [106] and Y39983 [107] are supposed to be effective in treating cancer or glaucoma.

Efforts are underway to develop more specific and potent ROCK inhibitors that can be used for clinical applications. Taking in account that the two ROCK isoforms seem to have different functions in smooth muscle cells under different conditions [22, 34] the development of isoform specific inhibitors is of interest and may allow for a more targeted intervention. Modification of fasudil led to the compound H1152P, a potent and specific inhibitor of ROCK II with weaker inhibitory effects on other kinases, e.g. PKA, PKC [108]. In addition, SLx-2119, a selective ROCK II inhibitor, has been described [109, 110]. This compound has shown promise in cancer models as well as models of fibrosis without adverse hemodynamic side effects.

5.2. Statins

HMG-CoA reductase inhibitors or “statins” are used clinically to lower elevated cholesterol plasma levels. However, even in normocholesterolemic individuals statins can exert beneficial cardiovascular effects. This is due to pleiotropic effects of statins on cardiovascular function including improvement of endothelial function, reduction of oxidative stress and anti-inflammatory actions and have been reported in animal as well as clinical studies [111]. Indeed, one study in humans could prove pleiotropic effects of statins independent from cholesterol lowering [112]. Treatment of patients with simvastatin improved endothelial function at least in part by reducing oxidative stress without changing LDL cholesterol levels. Furthermore, early beneficial effects of statins on oxidative stress, proinflammatory cytokines and endothelial activation have been observed very rapidly (after 2 h) before changes in the lipid profile could occur [113].

By blocking cholesterol biosynthesis, statins also prevent the formation of isoprenoid intermediates like geranyl-geranyl pyrophosphate (GGPP). Isoprenylation of RhoA with GGPP is essential for activation of RhoA by facilitating membrane localisation of the GTPase where GDP-GTP exchange takes place [114]. The prevention of this membrane interaction leads to inactivation of RhoA and thus ROCKs. Indeed, the comparison of a high-dose statin monotherapy with a combination of low-dose statin plus ezetimibe could show that even though both treatments lead to comparable

changes in the lipid profile only the high-dose statin monotherapy has a direct impact on RhoA/ROCK signalling and lowers ROCK activity [115].

As mentioned above NO- and RhoA/ROCK-signalling display an extensive crosstalk and antipodal influence on their activities. Indeed, treatment with statins leads to increased eNOS expression and increased endothelial NO levels, an effect that can be blocked by GGPP [86]. Statins not only exert effects on eNOS expression levels but also rapidly influence eNOS activity. Statins promote the phosphorylation and thus activation of eNOS in endothelial cells [116], an effect than can be mimicked by RhoA/ROCK inhibition [89].

Indeed, several of the cardiovascular effects of statins are eNOS dependent and are absent in eNOS-deficient mice [117] or after eNOS inhibition by L-NAME [88].

As abnormal activity of the RhoA/ROCK signalling cascade is supposed to be critically involved in several cardiovascular diseases, the ability of statins to inhibit RhoA activity might be a key factor in the beneficial pleiotropic effects of statins for cardiovascular function.

6. Concluding remarks

There is compelling evidence for the Rho/ROCK signalling cascade to be critically involved in regulating smooth muscle contractility and numerous studies have shown that abnormalities in ROCK activity play an important role in cardiovascular disease and especially hypertension. There has been much effort to understand ROCK signalling in vascular smooth muscle cells in detail and several inhibitors of ROCK have been synthesised. One of them, fasudil, already reached the clinic for treatment of different cardiovascular disease and tests for additional indications are ongoing. The Rho/ROCK cascade seems to be interacting with other signalling cascades, like NO-signalling, that are also important regulators of vascular smooth muscle contractility. Therefore, targeting the critically involved ROCK isoform under specific cardiovascular pathophysiological conditions might have multiple beneficial effects on vascular function.

Table 1

Selected targets of ROCK

Targets	Effect	Outcome	Reference
Regulation of contractility			
<i>MYPT-1</i>	Inhibition of MLCP activity	Enhanced contractility/ stress fiber formation	[18]
<i>MLC</i>	Stimulation of actin- myosin ATPase activity	Enhanced contractility/ stress fiber formation	[26]
<i>CPI-17</i>	Inhibition of MLCP activity	Enhanced contractility/ stress fiber formation	[29]
<i>Calponin</i>	Inhibition of calponin binding to actin	Enhanced contractility	[118]
Regulation of the cytoskeleton			
<i>Adducin</i>	Enhanced adducing/F- actin assembly	Enhanced cell motility	[119]
<i>LIM-kinases 1 and 2</i>	Stimulation of kinase activity	Decreased actin-filament disassembly (via inactivation of cofilin)	[120, 121]
<i>RhoE</i>	Increased RhoE stability	Enhanced stress fiber disruption and Ras- induced transformation	[33]
<i>ZIPK</i>	Activation of ZIPK activity	Enhanced stress fiber formation	[122]
<i>Profilin</i>	Inhibition of Profilin binding to actin	Decreased Profilin- induced actin remodelling	[70]
Regulation of intermediate filaments			
<i>Vimentin</i>	Enhanced filament disassembly	Cytokinesis?	[123]
Regulation of signalling proteins			
<i>PTEN</i>	Stimulation of phosphatase activity	Decreased intracellular PIP3 levels; tumor suppression	[124]

Figure legends

Fig. 1. ROCK structure and regulation of activity. A) The structure of ROCKs. The kinase domain of ROCK is located at the amino terminus of the protein followed by a coiled-coil forming region containing the Rho-binding domain (RBD). In the carboxyl terminus a pleckstrin-homology (PH) domain with an internal cystein-rich domain (CRD) is situated. B) Mechanisms of ROCK activation. In the inactive form the PH and RBD domain bind to the amino terminus of the enzyme, forming an autoinhibitory loop. Upon binding of active, GTP-bound RhoA to the RBD the conformation opens and gives rise to an active kinase. Similarly, binding of arachidonic acids leads to an open and active conformation of ROCK. Cleavage of the C-terminus by caspase-3 (ROCK I) or granzyme B (ROCK II) removes the autoinhibitory part of ROCK and generates a constitutively active kinase.

Fig. 2. ROCK signalling in smooth muscle cells. The contractile state of smooth muscle cells is mainly determined by the phosphorylation level of the regulatory light chain of myosin (MLC) which is regulated by the two enzymes myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP). Binding of vasoconstrictors to G-protein coupled receptors leads to activation of heterotrimeric G-proteins of the $G_{q/11}$ - and $G_{12/13}$ family. While $G_{q/11}$ via activation of PLC- β increases intracellular Ca^{2+} concentration and concomitantly stimulates MLCK activity, activation of $G_{12/13}$ leads to enhanced RhoA/ROCK activity and thus MLCP inhibition. Another mechanism for attenuating MLCP activity is the phosphorylation and thereby activation of CPI-17 by ROCK. In addition, ROCK can directly phosphorylate MLC. Arachidonic acid is also capable of activating ROCK and inducing the abovementioned signalling cascades leading to enhanced contractility. PLC, phospholipase C; Rho GEF, guanine nucleotide exchange factor; MYPT-1, myosin binding subunit; PP-1c, protein phosphatase 1c;

Fig 3. NO and statins in ROCK signalling. Endothelium-derived nitric oxide (NO) stimulates synthesis of cyclic GMP (cGMP) and leads to activation of cGMP-dependent protein kinase (cGK) in smooth muscle cells. Active cGK interferes with the translocation of RhoA from the cytosol to the membrane thereby preventing RhoA and subsequently ROCK activation. Statins increase eNOS activity and expression.

Furthermore, by blocking the synthesis of geranylgeranyl-pyrophosphate statins prevent RhoA binding to the membrane. In contrast, RhoA/ROCK has an inhibitory impact on NO-signalling leading to decreased NO-levels.

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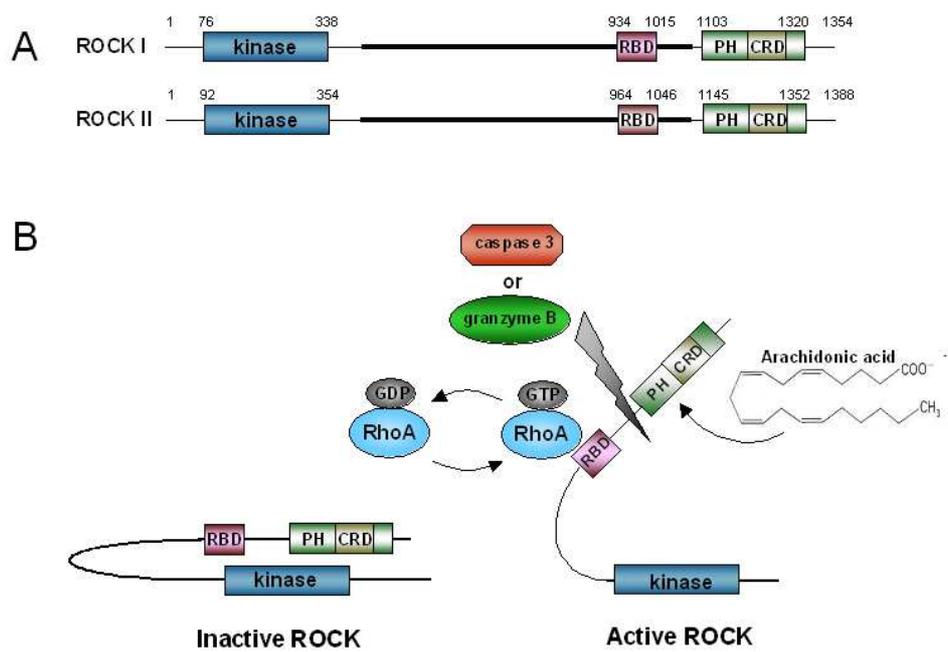
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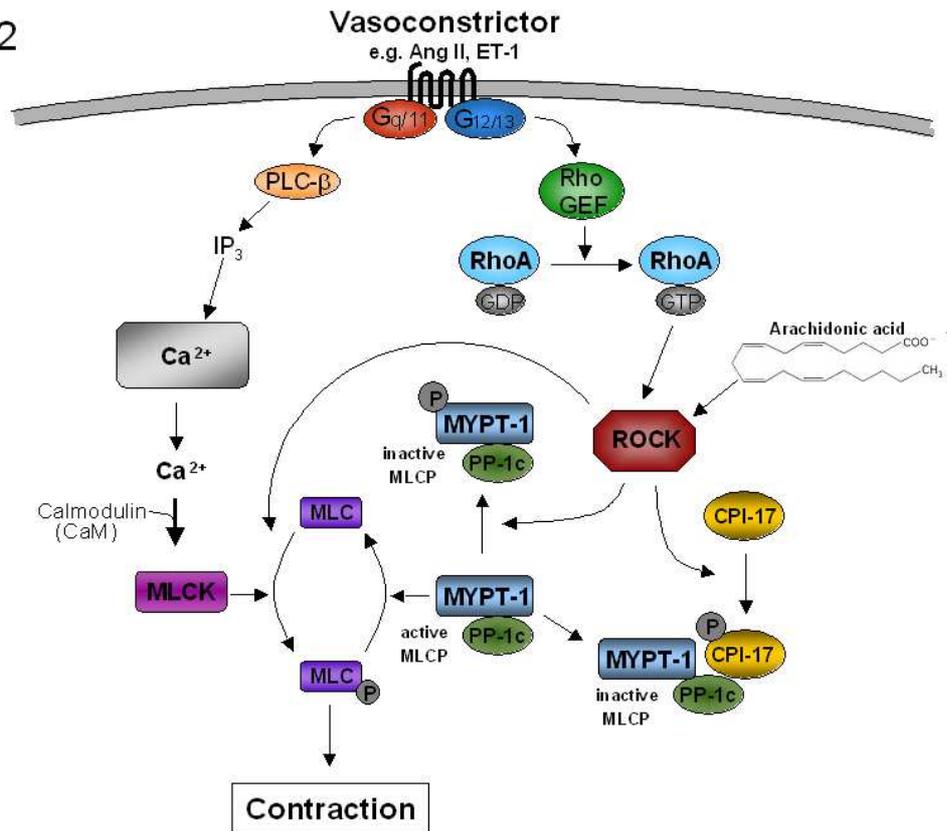
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Fig. 1



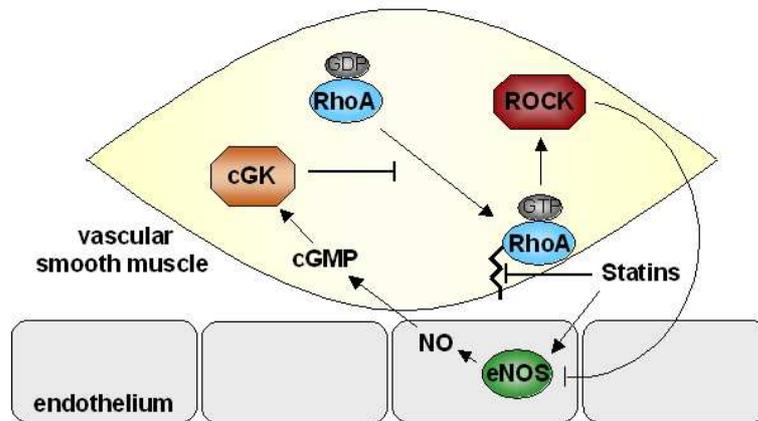
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Fig. 2



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Fig. 3



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