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Rho Kinase: An Important Mediator of Atherosclerosis and Vascular Disease

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Abstract

Atherosclerosis is a complex inflammatory process characterized by the cross-talk between excessive inflammation and lipid accumulation. In the past few years, compelling evidence suggests that statins can decrease vascular inflammation and attenuate the development of atherosclerosis through their so-called “pleiotropic effects”. These cholesterol-independent effects are predominantly due to their ability to inhibit isoprenoid synthesis. In particular, inhibition of geranylgeranylpyrophosphate synthesis leads to inhibition of Rho and its downstream target, Rho-kinase (ROCK). Thus, one of the beneficial effects of statin therapy could be due to inhibitory effects on ROCK. ROCK is involved in mediating diverse cellular functions such as smooth muscle contraction, cell migration and proliferation. While increased ROCK activity is associated with endothelial dysfunction, cerebral ischemia, coronary vasospasms and metabolic syndrome, the inhibition of ROCK by statins or selective ROCK inhibitors leads to up-regulation of endothelial nitric oxide synthase (eNOS), decreased vascular inflammation, and reduced atherosclerotic plaque formation. This review will focus on the impact of ROCK in cardiovascular disease and its contributory role to vascular inflammation and the atherosclerosis.

Keywords

Rho-kinase; inflammation; atherosclerosis; statin

Introduction

Atherosclerosis is a complex inflammatory process that is characterized by the cross-talk between excessive inflammation and lipid accumulation [1]. Its development is partly initiated by local endothelial cell dysfunction leading to the activation of endothelial cells and recruitment of proinflammatory cells. Local inflammation then promotes the adhesion of leukocytes and recruitment of activated platelets to the damaged endothelium, leading to increased permeability of blood vessels for lipid components in the plasma [2]. Subsequently, monocytes that are loaded with cell-activating lipids, accumulate in the arterial intima and acquire the morphological characteristics of macrophages leading to the transformation into foam cells [3,4]. Following the accumulation of additional inflammatory cell subsets and extracellular lipids, these early plaques, also known as fatty streaks progress into mature atherosclerotic plaques. Plaque cells promote their own growth by secreting cytokines and growth factors resulting in further deposition of extracellular matrix components and progression of plaques and stenosis. At the same time, matrix-degrading proteases and

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cytokines are secreted by plaque cells and result in thinning of the fibrous cap, eventually leading to disintegration of the cap and plaque erosion [5].

In the past few years, statins have been shown to prevent or reduce atherosclerosis. In particular, statins have been shown to modulate immune activation by decreasing the number of inflammatory cells in atherosclerotic plaques and to contribute to plaque stability by reducing plaque size or by modifying the molecular composition of the lipid core [6]. Statins exert their extrahepatic effects through its ability to prevent the synthesis of other important isoprenoid intermediates of the cholesterol biosynthetic pathway, such as farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP). As a results, recent research have shifted the focus to isoprenoid intermediates, especially since they serve as important lipid attachments for the post-translational modification of intracellular signaling molecules, such as Rho, Rac and Cdc42 [7]. In particular, the inhibition of Rho and its downstream target, Rho-associated coiled-coil forming protein kinase (ROCK), has emerged as the principle mechanisms, which contributes to the pleiotropic effects of statins.

Rho KINASE

Rho kinases (ROCKs) are protein serine/threonine kinases of 160 kDa and are downstream effectors of the small GTPase Rho [8]. They were initially characterized by their ability to mediate the formation of RhoA-induced stress fibers and focal adhesions through increasing the phosphorylation of myosin light chain (MLC) [9]. ROCKs consist of an amino-terminal kinase domain, followed by a mid-coiled-coil-forming lesion containing a Rho-binding domain (RBD), and a carboxy-terminal cysteine-rich domain (CRD) located within the pleckstrin homology (PH) motif. The two isoforms ROCK1 and ROCK2 share a 65% homology in their amino acid sequence and 92% homology in their kinase domains [8]. The carboxy-terminal regions of ROCKs, which contain the PH domain and the RBD, serve as an autoregulatory inhibitor of the amino-terminal kinase domain [10]. The interaction of GTP-bound RhoA to the RBD of ROCKs increases ROCK activity through repression of the carboxy-terminal RBD-PH domains on the amino-terminal kinase domain, leading to an active “open” kinase conformation. This open conformation can also be formed by the binding of arachidonic acid to the PH domain or by cleavage of the carboxy terminus by caspase-3 [11-13]. Interestingly, ROCKs can also be activated independently of RhoA through amino-terminal transphosphorylation caused by protein oligomerization. Other small GTP-binding proteins such as Gem and Rad specifically regulate either ROCK1 or ROCK2-mediated cell rounding and neurite retraction [14]. Although further studies are needed to uncover the precise mechanism, these results indicate that ROCK1 and ROCK2 may have different physiological roles in cellular function.

ROCKs are important regulators of cellular apoptosis, growth, metabolism and migration *via* control of the actin cytoskeletal assembly and cell contraction. Stimulation of tyrosine kinase and G protein-coupled receptors recruits and activates Rho GEFs, leading to activation of RhoA. ROCKs are pivotal downstream effectors of RhoA in regulating the actin cytoskeleton by phosphorylation and inhibition of MLCP, which increases MLC phosphorylation and cellular contraction. By affecting tight and adherent junctions through actin cytoskeletal contractions, ROCKs can also regulate macrophage phagocytic activity and endothelial cell permeability.

Although ROCK1 and ROCK2 are ubiquitously expressed in mouse tissues from early embryonic development to adulthood, ROCK1 mRNA is preferentially expressed in lung, liver, spleen, kidney and testis, whereas ROCK2 mRNA is highly expressed in the heart, skeletal muscle, adipose tissue, and brain [15-17]. Growing evidence suggests a pivotal role for ROCK in the pathophysiology of cardiovascular diseases, such as hypertension, myocardial hypertrophy, cerebral ischemia, neointima formation and atherosclerosis (Fig. 1). The

emergence of this linkage coincides with the growing acceptance of the pleiotropic effects of statins, as a therapeutic ROCK inhibitor. Indeed, it has become increasingly apparent that the overall benefits observed with statins are not mediated solely by their lipid-lowering properties, but by a cascade of cholesterol-independent or pleiotropic effects [18,19].

Statins and ROCK

Statins have emerged as the leading therapeutic class of lipid lowering agents and are established therapy in the primary and secondary prevention of coronary artery diseases. As potent competitive inhibitors of the 3-hydroxy-methylglutaryl coenzyme A (HMG-CoA) reductase, statins bind to the enzyme's active site and block the substrate-product transition state of the enzyme [20,21]. However, in contrast to the original rationale of the biological effect of statins, it has become increasingly apparent that the overall benefits observed with statins are not mediated solely by their lipid-lowering properties, but by cholesterol independent or pleiotropic effects [18,19]. Indeed, statins prevent the synthesis of other important isoprenoid intermediates of the cholesterol biosynthetic pathway, such as farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP) that are downstream from L-mevalonic acid [22]. These intermediates serve as important lipid attachments for the post-translational modification of proteins, including nuclear lamins, Ras, Rho, Rac and Rap [7]. Through posttranslational modifications, isoprenylation is critical for intracellular trafficking and function of small GTP-binding proteins [23]. In particular, by inhibiting mevalonate synthesis, statins prevent membrane targeting of Rho and its subsequent activation of ROCK. Indeed, *in-vitro* studies suggest that many of the pleiotropic effects of statins are due to alterations in the RhoA/ROCK signaling pathways [24-26]. For example, similar to the effects of statins, the administration of ROCK inhibitors has been shown to prevent cerebral vasospasm after subarachnoidal hemorrhage [27] and to prevent arterial remodeling after vascular injury [28].

The concept of statin pleiotropy is still controversial, because it has been difficult to separate the cholesterol-lowering effects of statins from their pleiotropic effects in humans. Previous data indicate that statin pleiotropy on endothelial function and inflammation appears to be dose related. Recently, a new cholesterol inhibitor ezetimibe, which inhibits intestinal cholesterol absorption, has been shown to reduce cholesterol by 15-20% when used alone [29]. If used in the so-called dual therapy, i.e. in conjunction with statins, it can enhance the cholesterol-lowering effect of statins up to 40% [30]. Comparing high-dose statin mono-therapy with equivalent cholesterol-lowering efficacy of the same statin at a lower dose plus ezetimibe, our group has recently shown that high-dose simvastatin alone improves endothelial function as assessed by decreased ROCK activity and increased flow-mediated dilation (FMD), more than dual therapy of ezetimibe and low-dose simvastatin [31]. This finding underlines the concept that inhibition of ROCK contributes to some of the pleiotropic effects of statins therapy and also supports the hypothesis that ROCK inhibition may elicit protective effects on the cardiovascular system.

ROCK in the Vasculature

ROCK and Endothelial Cells

The vascular endothelium forms the inner lining of blood vessels and serves as a physical barrier in the healthy vasculature. In addition, the endothelium is also a secreting organ, releasing vasoactive substances such as NO, prostacyclin, endothelium-derived hyperpolarizing factor and endothelins [32]. Endothelial dysfunction, defined by decreased bioavailability of endothelium-derived nitric oxide (eNOS), is one of the earliest manifestations of atherosclerosis [33,34]. In particular, eNOS plays an important role in the regulation of vascular tone, inhibition of platelet aggregation [35], suppression of smooth muscle cell

proliferation [36] and prevention of leukocyte recruitment to the vessel wall [37]. Increased bioavailability of NO is partly dependent on increased expression and activity of eNOS as well as on decreased inactivation of NO by reactive oxygen species (ROS). Although various conditions and factors such as laminar shear stress, oxygen tension and TGFβ can regulate eNOS expression at the transcriptional level, eNOS expression also can be regulated at the posttranscriptional level. For example, chronic hypoxia, tumor necrosis factor (TNF), thrombin, oxLDL and cellular proliferation are known to decrease eNOS mRNA stability. Chronic hypoxia and cellular proliferation are known to activate RhoA and ROCK. In contrast, statins, which have been shown to increase eNOS mRNA stability, inhibit RhoA geranyl-geranylation and ROCK activity. Thus, RhoA/ROCK inversely regulates eNOS expression through alteration in eNOS mRNA stability [24,38].

The involvement of ROCK in the regulation of endothelial nitric oxide synthase (NOS) has been profoundly demonstrated in several studies. In human endothelial cells, ROCK negatively regulates phosphorylation of eNOS through inhibition of protein kinase B/Akt [39]. Moreover, inhibition of ROCK leads to a rapid phosphorylation and activation of Akt *via* the phosphatidylinositol 3-kinase (PI3K), leading to increased NO production [40]. These data suggest an important role of ROCK in the regulation of eNOS in the peripheral circulation of healthy subjects. Previous data in human umbilical vein endothelial cells (HUVEC) and human coronary artery endothelial cells (HCAE) have demonstrated that native lipoproteinA (Lp(a)) elicits re-arrangement of the actin cytoskeleton through its apo(a) component. This re-arrangement is characterized by increased central stress fiber formation, dispersion of vascular endothelial (VE)-cadherin, and increased cell permeability, whereas treatment with LDL or plasminogen had no effect [41]. Interestingly, this effect was mediated by increased MLC phosphorylation through a ROCK-dependent signaling pathway. In a follow-up study the same group has identified that the strong lysine binding sites (LBS) in apo(a) mutants has a key functional role in mediating a Rho/ROCK/MYPT1 signaling transduction pathway to enhance MLC phosphorylation *via* inactivation of MLCP, which thereby increases endothelial cell contraction and permeability [42].

ROCK and Vascular Smooth Muscle Cells

In vascular smooth muscle cells (VSMC), the Rho/Rho kinase system is involved in proliferation and migration [43,44]. Furthermore, ROCK mediates angiotensin II-induced expression of monocyte chemoattractant protein-1 (MCP-1) [45] and plasminogen activator inhibitor-1(PAI-1) [46]. In addition several *in-vivo* studies have identified ROCK activation in experimental models of vascular inflammation or injury [47-49]. In terms of atherogenesis, smooth muscle cells (SMC) from the vessel wall respond to growth factors and migrate and proliferate throughout the intima [50]. Besides the production of collagen and other extracellular matrix proteins, SMCs secrete vascular endothelial growth factor, TNF-α, IL-1 and other pro-inflammatory molecules [51]. Fibrous tissue and proliferating SMCs overlay the mature lipid core, which becomes rich in necrotic debris. The phenotype of SMC migration and proliferation is a critical component of plaque stability, and, interestingly, the lack of SMCs may paradoxically increase the occurrence of arterial thrombosis [52].

The involvement of ROCK in vascular inflammation and remodeling has been demonstrated in several studies. In L-NAME treated rats, ROCK inhibitors attenuate the inflammatory response and vascular remodeling [48,53]. In addition, ROCK activity is increased in the neointima following balloon-induced vascular injury, which is suppressed by ROCK inhibitors or gene transfer of a dominant-negative mutant of ROCK [28,49,54]. Most of these studies however, utilized pharmacological inhibitors of the Rho kinase: Fasudil or Y-27632. Both inhibitors function through inhibition of the ATP-dependent kinase domain, which is highly homologous between the two ROCK iso-forms [55,56]. Therefore, neither Fasudil, nor

Y-27632 can distinguish between cellular processes mediated by ROCK1 and ROCK2. Furthermore, when applied *in-vivo* for prolonged periods and at higher concentrations, these pharmacological inhibitors could also inhibit other serine-threonine kinases such as PKA and PKC.

Recently, several groups have successfully generated mutant mice with deletion of the ROCK1 and ROCK2 allele. While mice harboring homozygous deletion of both ROCK1 or ROCK2 alleles are embryonic and postnatal lethal, haploinsufficient mice are fertile and phenotypically normal [57-60]. Investigating the effect of ROCK on neointima formation after vascular injury, we have found that VSMC proliferation was decreased in the neointima of $ROCK1^{+/-}$ mice following carotid artery ligation [61]. Interestingly, VSMC proliferation in response to serum or PDGF was not different between wildtype and $ROCK1^{+/-}$ mice, while the migration of VSMC in response to PDGF was substantially reduced in $ROCK1^{+/-}$ mice, suggesting that ROCK1 may contribute to increase VSMC migration and survival following vascular injury.

ROCK and Atherosclerosis

Pharmacological inhibition of ROCK activity has been demonstrated to protect against atherosclerosis. In a porcine model, long-term inhibition of Rho-kinase results in a regression of arteriosclerotic coronary lesions [62]. In $LDLr^{-/-}$ mice on high-fat diet, application of the Rho kinase inhibitor Y-27632 significantly reduced atherosclerotic lesion size by 35% compared to control mice fed with high-fat diet [63,64]. Interestingly, the expression of macrophage, smooth muscle cell and collagen in the plaque did not differ between the Y-27632 treated and saline treated animals. But instead, the number of CD3-positive T-lymphocytes per lesion was reduced by 40% in Y-27632 treated mice. The authors conclude that Rho kinase may have direct effects on T-lymphocytes and indeed, Y-27632 was found to exert a profound inhibitory and dose-dependent effect on conA-induced proliferation of spleen-derived T-lymphocytes. In accordance to this study, several other studies have previously demonstrated modulatory role of the Rho/Rho kinase system in T-lymphocyte homeostasis [64-66]. In another study with ApoE knockout mice treated with or without ROCK inhibitor, a cell type-selective distribution and phosphorylation of ROCK target proteins ERM and MLC could be demonstrated [67]. Y-27632 inhibited ERM phosphorylation in the plaque, but, however, failed to demonstrate dose dependence. MLC and phospho MLC were associated with SMC and did not respond to the Y-27632 treatment.

A recent study from our group showed that macrophage ROCK1 is involved in the pathogenesis for atherosclerosis [68]. Transplantation of bone marrow-derived macrophages from $ROCK1^{-/-}$ mice to $LDLr^{-/-}$ mice demonstrates that ROCK1 in bone marrow-derived macrophages is critical to the development of atherosclerosis. Specifically, lipid accumulation and atherosclerotic lesions were reduced in atherosclerosis-prone $LDLr^{-/-}$ mice, whose bone marrows have been replaced with bone marrows derived from $ROCK1^{-/-}$ mice. Indeed, bone marrow-derived ROCK1-deficient macrophages exhibited impaired chemotaxis to MCP-1, reduced ability in lipid uptake and subsequent transformation into foam cells. These findings indicate that bone marrow-derived ROCK1 may be an important therapeutic target for vascular inflammation and atherosclerosis. However, it remains to be determined whether and how ROCK2 is involved in this system. There is evidence that ROCK1 expression rather than ROCK2 is up-regulated upon macrophage adhesion [69]. At the same time, a study by Yoneda *et al.* showed that phagocytic uptake of fibronectin-coated beads was down-regulated in ROCK2-depleted cells, but not in ROCK1-depleted cells [70].

As mentioned above, endothelial permeability is controlled by a variety of chemical stimuli and abrogation of the integrity of the endothelial monolayer contribute to pathological conditions such as atherosclerosis. Several studies have demonstrated the importance of Rho family GTPases in the controlling and maintaining of endothelial barrier function [71,72]. Also,

extravasation of leukocytes has been shown to induce stress fiber formation and increased MLC phosporylation and increased endothelial permeability [73,74].

Clinical Implication of ROCK and ROCK Inhibitors

ROCK has undoubtedly attracted significant interests as a potential target for the treatment of cardiovascular disease and numerous clinical studies have already demonstrated a linkage for ROCK and cardiovascular disease in human. In Japan, the isoquinoline derivative fasudil has been used in clinics since 1995 and proven to be successful in preventing vasospasm associated with subarachnoid hemorrhage (SAH) [75], acute ischemic stroke [76], angina pectoris [77, 78], coronary artery spasm [79,80], pulmonary arterial hypertension [81] and atherosclerosis [82,83].

ROCK in Endothelial Dysfunction

A significant relationship between endothelial dysfunction and increased ROCK activity in young, current smokers has been previously demonstrated, showing a significant relationship between ROCK activity with age, systolic blood pressure, serum concentration of total cholesterol, and number of pack-years smoked in healthy male subjects [84,85]. In a multivariate analysis the authors demonstrated that age and number of pack-years smoked were independent predictors of ROCK activity among these candidates. In addition, the concentration of serum malondialdehyde-modified (MDA)-LDL, a marker for oxidative stress and impaired aortic stiffness as assessed by pulse wave velocity (PWV) also correlated with ROCK activity [83]. Indeed, oxidative stress has been linked with impaired aortic stiffness and has been shown to be an independent marker to estimate subjects with cardiovascular disease [86-89]. Furthermore, our group has previously demonstrated a correlation between elevated ROCK activity and impaired endothelial function in coronary artery disease (CAD) patients [82]. Treatment with the ROCK inhibitor fasudil reduced the over-activity of ROCK in patients with atherosclerosis and improved endothelium-dependent vasodilation as well as flow-mediated, endothelium-dependent dilation. Of note, this finding was only present in patients with CAD, but not in healthy individuals, where ROCK is presumably not “overactive”. Most interestingly, endothelium-dependent vasodilation in healthy subjects tended to worsen with fasudil therapy compared with placebo.

In animal models, Rho expression is regulated by a negative feedback mechanism mediated by the actin cytoskeleton [90]. Under physiological conditions, such as in healthy individuals in whom basal ROCK activity is not up-regulated, inhibition of ROCK may lead to decreased negative feedback and thus to increased transcription of Rho, which in turn may lead to a subsequent compensatory increase in the downstream effects of Rho including suppression of endothelial NO production. On the other hand, ROCK inhibition in healthy individuals may also lead to an excess of NO production, resulting in the formation of peroxynitrite, which might lead to eNOS uncoupling and worsening endothelial function [91]. From our clinical study, it appears that some basal ROCK activity is probably required for the maintenance of vascular homeostasis.

ROCK in Metabolic Syndrome

In a previous paper by Liu *et al.*, a correlation between ROCK activity and metabolic syndrome in the Taiwanese population has been demonstrated [92]. The authors showed that increased levels of ROCK activity independently predicted the diagnosis of metabolic syndrome, and that in addition, higher ROCK activity was also associated with greater body mass index (BMI), waist circumference, greater hs-CRP as well as lower adiponectin levels. This clinical finding is supported by several *in vitro* studies, where increased ROCK is involved in the phosphorylation of insulin receptor substrate (IRS)-1, which prevents insulin signaling and

glucose transporter-4 translocation leading to altered glucose metabolism [93-95]. Interestingly, the insulin sensitivity is ameliorated by ROCK inhibition in obese, but not in lean rats, supporting the clinical finding of elevated ROCK activity in obese patients [93]. Also, ROCK has been demonstrated to mediate high glucose-induced NF-κB activation, resulting in PAI-1 expression in endothelial cells [96].

Conclusions

Growing evidence from animal and clinical studies suggest the importance of ROCK in the pathogenesis of cardiovascular disease. Indeed, many of the pleiotropic or cholesterol-independent effects of statins may be due to their ability to block isoprenoid synthesis and inhibit the Rho/ROCK pathway. Thus, targeting ROCK with specific inhibitors may be an important therapeutic strategy in the prevention and treatment of cardiovascular disease in patients with normal or low lipid levels. As ROCKs are involved in various aspects of vascular function and disease, understanding their role in the vascular wall may provide key insights into how the vasculature as a whole is regulated under normal and pathophysiological conditions. However, despite a growing number of reports demonstrating that ROCK activity is increased under a variety of pathologic conditions, little is known regarding the molecular mechanisms that contribute to increased ROCK activity or what the downstream targets for ROCK are. Also, determining the precise role of ROCK in the vascular wall for each isoform will certainly provide more insights into the molecular function of ROCK.

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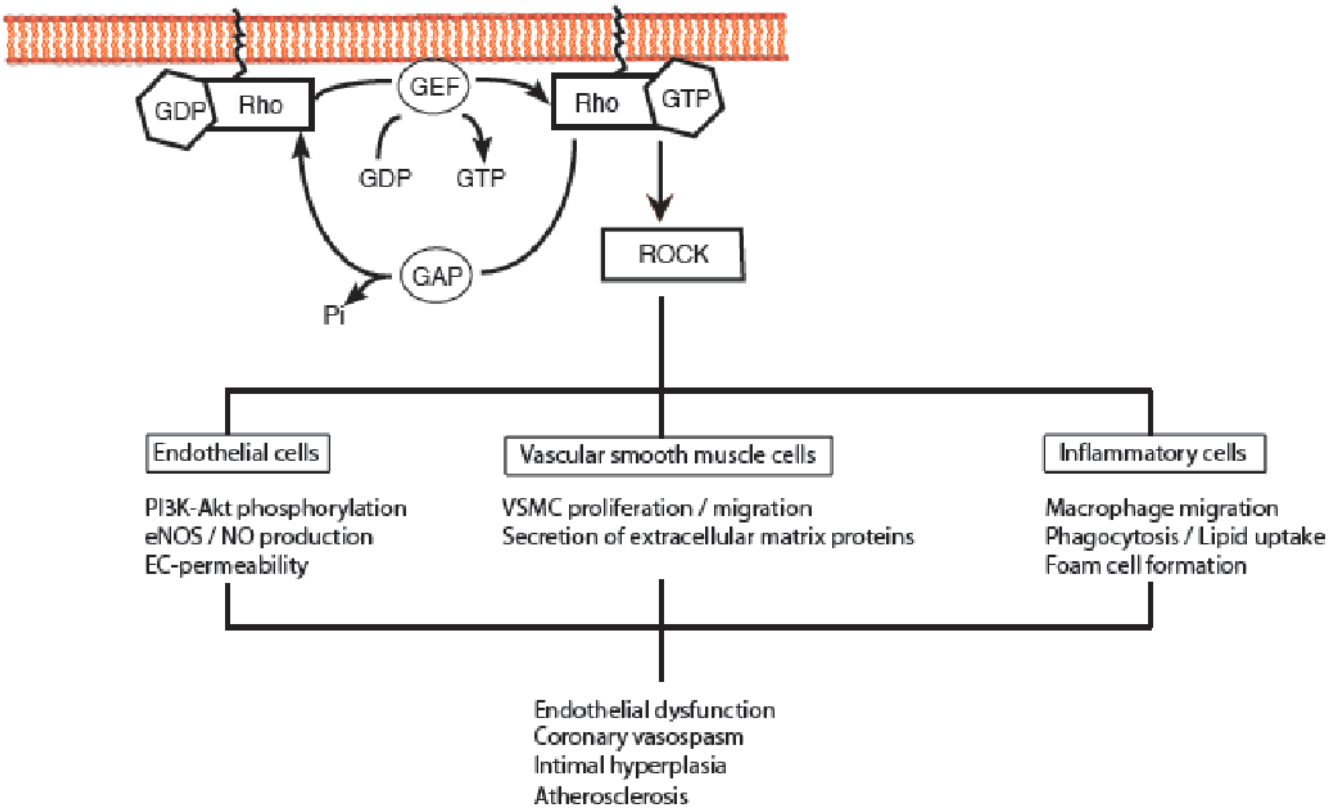

Fig. 1. Biological actions of ROCK in the vasculature

In endothelial cells the inhibition of ROCK leads to a rapid phosphorylation and activation of PI3K/Akt resulting in increased production of NO. In vascular smooth muscle cells ROCK inhibition regulates cell migration and proliferation and is involved in the pathomechanism of vascular inflammation and injury. Finally, the inhibition of ROCK either pharmacologically or genetically prevents the development of atherosclerosis by inhibition altered chemotaxis of macrophages and its transformation into foam cells. (Adapted from Wang *et al.* [97]).