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Rho-kinase as a key participant in the regulation of vascular tone in normal circulation and vascular disorders

O. S. Tarasova, D. K. Gaynullina
Lomonosov Moscow State University, Moscow, Russia
Institute for Biomedical Problems, Russian Academy of Sciences, Moscow, Russia

Corresponding author:
Olga S. Tarasova,
Lomonosov Moscow State University,
Faculty of Biology, Department of Human and Animal Physiology,
Leninskie Gory 1, bld. 12, Moscow, Russia, 119234
Phone: +7(495)939–14–16.
E-mail: ost.msu@gmail.com

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Abstract

Rho-kinase was shown to regulate the functions of almost all cells of our body. The key activator of Rho-kinase is the small guanosine triphosphate (GTP)-binding protein RhoA, but RhoA-independent mechanisms of Rho-kinase regulation exist as well. In this review we describe the mechanisms affecting Rho-kinase activity in vascular smooth muscle and endothelial cells, Rho-kinase regulatory influences on fundamental physiological processes in these cells, as well as its role in the pathogenesis of vascular disorders in systemic and pulmonary arterial hypertension and diabetes mellitus.

Key words: vascular smooth muscle, myosin regulatory light chain phosphorylation, endothelial nitric oxide synthase, endothelial permeability, arterial hypertension, pulmonary hypertension, diabetes mellitus

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Rho-киназа как ключевой участник регуляции тонуса сосудов в норме и при сосудистых расстройствах

О. С. Тарасова, Д. К. Гайнуллина
Федеральное государственное бюджетное образовательное учреждение высшего образования «Московский государственный университет имени М. В. Ломоносова», Москва, Россия
Государственный научный центр Российской Федерации — Институт медико-биологических проблем РАН, Москва, Россия

Контактная информация:
Тарасова Ольга Сергеевна,
ФГБОУ ВО МГУ
им. М. В. Ломоносова,
Ленинские горы, д. 1, стр. 12,
Москва, Россия, 119234.
Тел.: +7(495)939–14–16.
E-mail: ost.msu@gmail.com

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Резюме

Rho-киназа участвует в регуляции функций практически всех клеток нашего организма. Ключевым активатором Rho-киназы является малый гуанозинтрифосфат (ГТФ)-связывающий белок RhoA, но существуют и RhoA-независимые механизмы регуляции этого фермента. В данном обзоре рассмотрены механизмы, влияющие на активность Rho-киназы в гладкой мышце и эндотелии сосудов, ее роль в регуляции фундаментальных физиологических процессов в этих клетках, а также участие в патогенезе сосудистых расстройств при таких заболеваниях, как системная и легочная артериальная гипертензия и сахарный диабет.

Ключевые слова: гладкая мышца сосудов, фосфорилирование регуляторных легких цепей миозина, эндотелиальная синтаза оксида азота, проницаемость эндотелия, артериальная гипертензия, легочная гипертензия, сахарный диабет

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Introduction

Functioning of smooth muscle and endothelial cells is regulated not only by intracellular calcium concentration ($[Ca^{2+}]_i$) but also by numerous signaling pathways acting independently on $[Ca^{2+}]_i$. Many of these pathways involve Rho-kinase (RhoK), which regulate the functions of almost all cells of the organism. Multiple functionality of RhoK is defined by its influence on cytoskeleton organization [1, 2]. High level of RhoK activity is typical for proliferative and migrating cells [3]. In differentiated cells RhoK participates in the regulation of specific cellular functions, such as smooth muscle contraction and endothelial secretion. In healthy individuals RhoK serves as an important unit of dynamic balance between vasoconstrictor and vasodilator signaling pathways. RhoK is constitutively active and can be additionally activated or deactivated by different vasoactive stimuli. On the other hand, unwarrantably high RhoK activity accompanies many vascular pathologies. This review focuses on the mechanisms regulating RhoK activity in vascular smooth muscle and endothelial cells, as well as

the role of RhoK in the regulation of basic physiological processes in these cells and pathogenesis of some vascular diseases.

Mechanisms of RhoK activation in vascular wall

RhoK is a serine/threonine protein kinase. It is expressed in two isoforms: RhoKα (ROCK2) and RhoKβ (ROCK1) [1]. RhoKα is the key functionally important isoform in smooth muscle and endothelial cells [4, 5].

Canonical RhoK activator is protein RhoA that binds guanine nucleotides guanosine triphosphate (GTP) and guanosine diphosphate (GDP). GTP binding leads to RhoA activation and translocation to the cellular membrane, where RhoA interacts with RhoK and activates it. Three types of proteins regulate RhoA activity [1, 6, 7]. Guanine nucleotide exchange factors (GEFs) catalyze exchange of GDP to GTP leading to RhoA activation. On the contrary, GTPase-activating proteins (GAPs) decrease its activity. At last, guanine nucleotide dissociation inhibitors (GDIs) prevent RhoA translocation to the membrane and thereby prevent its spontaneous activation.

Numerous pathways of GEFs activation can be realized in both vascular smooth muscle and endothelial cells. Initially activation of GEFs-RhoA-RhoK pathway was shown to occur by the ligands of membrane receptors coupled to heterotrimeric GTP-binding proteins [8]. Powerful activators of this cascade are receptors coupled to $G\alpha_{12/13}$, such as thromboxane A_2 receptors [9]. $G\alpha_{q/11}$-associated receptors possess by similar but less pronounced influence. It means that RhoK activation can occur by a variety of vasoactive substances, such as norepinephrine, histamine, serotonin, angiotensin II, endothelin 1, vasopressin, ADP, ATP, some prostanoids, thrombin *etc*. [10]. In addition, activation of RhoK signaling pathway occurs when receptor tyrosine kinases are activated by growth factors and other regulators [6, 11].

Under natural conditions a strong activation of RhoK signaling pathway can occur under exposure of cells to mechanical stimuli. For example, myogenic tone, a smooth muscle contraction in response to transmural pressure-induced stretch, is associated with RhoK activation [2, 12]. Several mechanisms provide the increase of RhoK activity during myogenic tone development. Firstly, it is the increase of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) that occurs at the initial step of myogenic tone development [13, 14]. Secondly, some of G-protein coupled receptors, such as angiotensin II receptors, can be activated by mechanical stimuli even in the absence of ligand stimulation [15, 16]. Moreover, being exposed to mechanical stimuli vascular cells can produce active substances such as ATP, uridine triphosphate, sphingosine-1-phosphate and others, leading to RhoK activation by receptor-dependent mechanism [17, 18].

Another important stimulus for RhoK activation is the increase of reactive oxygen species (ROS) production by mitochondria or membrane NADPH-oxidase [7, 18, 19]. Vigorous provocateurs of oxidative stress are angiotensin II [20] and sphingosine-1-phosphate [18]. Obviously RhoK activation by ROS is the result of cysteine residues oxidation. Under physiological conditions this oxidation is reversible [20].

Besides canonical mechanism of RhoA-associated activation of RhoK, it is also regulated by RhoA-independent pathways. For example, arachidonic acid, which abundance in cells rises during inflammation, directly evokes conformational changes in RhoK molecule by removing its autoinhibition [21]. RhoA-independent RhoK activation can occur during increase in blood contents of cholesterol and low-density lipoproteins [22]. During apoptosis irreversible RhoK activation in cells is induced by its proteolysis by caspase 3 (for RhoKβ) or by granzyme B (for RhoKα) [7]. Finally, vasospasms under anoxia, a condition of pathological reduction of O_2 concentration, is associated with RhoK activation by cyclic inositol monophosphate, which is produced by soluble guanylate cyclase instead of guanosine monophosphate under normal conditions [23].

To conclude, the augmentation of RhoK activity can happen under exposure to different stimuli. One can suggest that under normal conditions RhoA-associated mechanism of RhoK activation is more pronounced, while the disturbance of vascular homeostasis increases the contribution of RhoA-independent pathways.

Key effects of RhoK in vascular smooth muscle cells

In vascular smooth muscle the ratio between $[Ca^{2+}]_i$ and contractile response varies in rather wide range; this is designated as changes in Ca^{2+}-sensitivity of contraction [4, 21]. If the relation between $[Ca^{2+}]_i$ and contractile response becomes steeper, it means that at a particular $[Ca^{2+}]_i$ smooth muscle develops stronger contractile response, thus Ca^{2+}-sensitivity increases. This occurs during the exposure to many vasoconstrictor agents or the development of myogenic tone and is associated with RhoK activation [4].

Smooth muscle contraction is triggered by regulatory myosin light chain (MLC) phosphorylation, MLC kinase is activated by Ca^{2+}-calmodulin complex [6, 24]. Functional antagonist of MLC kinase is MLC phosphatase, which activity is not directly dependent on $[Ca^{2+}]_i$. Thus, the level of smooth muscle contraction depends on the balance between MLC kinase and phosphatase activities. RhoK inhibits MLC phosphatase that results in a higher content of phosphorylated MLC and, therefore, in a higher contractile response at given level of $[Ca^{2+}]_i$ [6, 24]. RhoK inhibits MLC phosphatase by phosphorylation of its regulatory subunit MYPT1. MYPT1 can be phosphorylated at two sites — Thr-850 and Thr-696 [6, 24]. Phosphorylation at Thr-850 leads to dissociation of MLC phosphatase from myosin, the decrease of phosphatase activity and, therefore, smooth muscle contraction [25]. MYPT1 phosphorylation at Thr-696 augments contraction due to reduction of MLC phosphatase catalytic activity [26].

Besides direct influence on MYPT1 RhoK is able to reduce MLC phosphatase activity by CPI-17 phosphorylation [6]. This protein of 17 kDa molecular weight was initially described as smooth muscle mediator of MLC phosphatase inhibition by protein kinase C, thus it was named as C-kinase-Potentiated Inhibitor [27]. Now it is clear that protein kinase C-induced CPI-17 phosphorylation occurs only at initial stage of the contractile response, while maintained smooth muscle contraction is associated with CPI-17 phosphorylation by RhoK [28]. RhoK phosphorylates CPI-17 at Thr-38, the same residue as protein kinase C [28].

In addition, RhoK assists to maintain tonic contraction via its influence on smooth muscle cells cytoskeleton. Long-lasting vascular smooth muscle contraction was shown to be associated with the decrease in G-actin content and the increase in F-actin content, as a result of actin filaments polymerization with participation of RhoK [2, 29]. Such smooth muscle cytoskeleton reorganization improves force transmission from contractile machinery to the membrane and intercellular matrix.

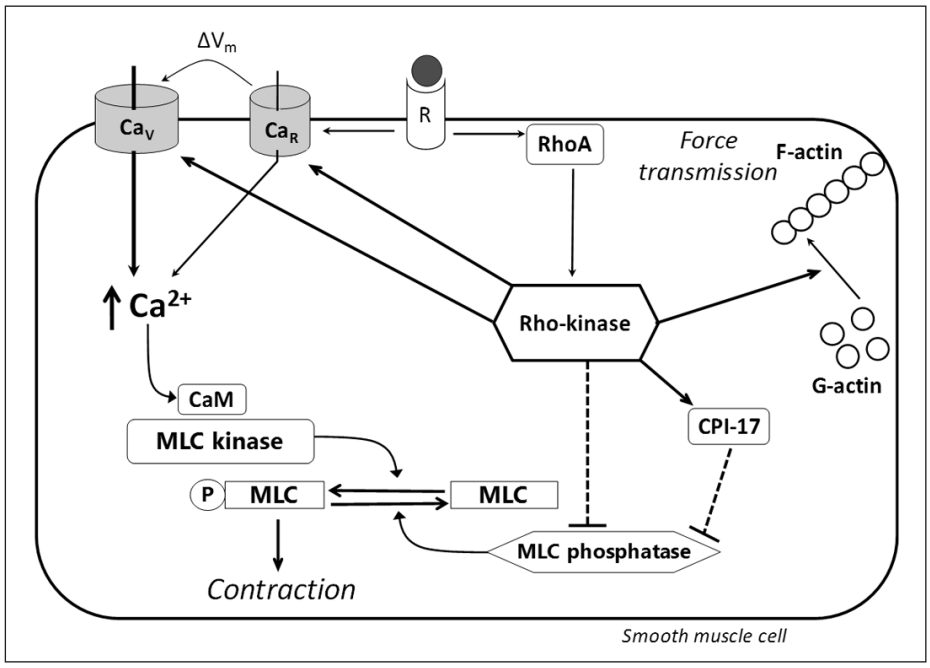
Besides its influences on contractile apparatus RhoK was shown to participate in the regulation of Ca^{2+} homeostasis of smooth muscle cells. RhoK can increase the activity of voltage-gated Ca^{2+} channels [30] and nonselective cation channels [31–33]. Thus, RhoK signaling pathway strengthens smooth muscle contraction via two mechanisms: (i) Ca^{2+}-independent (MLC phosphatase inhibition) and (ii) Ca^{2+}-dependent (augmentation of Ca^{2+} entry into the smooth muscle cells) (Fig. 1).

With participation of RhoK vascular smooth muscle is able to develop and maintain contraction at low $[Ca^{2+}]_i$, which is energetically efficient because of low ATP demands for MLC phosphorylation. Of note, RhoK influence leads to the development of vascular tone, which can be modified according to tissue demands in blood supply.

Of note, the role of RhoK in vascular tone regulation is considerably different during early ontogenesis and in adult organism. RhoK content in arteries and its contribution to contractile responses was shown to be significantly higher in arteries of 1–2-week-old rats than in adults [34, 35]. This is consistent with more pronounced role of RhoK in immature cells in comparison to differentiated ones [3]. RhoK-mediated increase of vascular contractility in newborn animals partially compensates the lack of neurogenic tone, as sympathetic innervations is not developed at this stage of ontogenesis [34].

At early stages of ontogenesis RhoK is an important regulator of pulmonary circuit as well. Fetal lungs are not functional, they are deflated and have low blood supply. Tonic contraction of fetal pulmonary vessels is mediated by RhoK, which activity and expression rapidly declines after birth [36, 37]. Finally, postnatal uncoupling of pulmonary and systemic circulations in birds and mammals by the closure of the arterial duct is also mediated by RhoK. Increased O_2 blood content was shown to induce ROS production in arterial duct smooth

Figure 1. Key mechanisms of smooth muscle contraction mediated by Rho-kinase (RhoK)



Note: Solid and dotted lines show stimulating and inhibitory influences, respectively. R — receptor; Ca_R — receptor-operated Ca^{2+} channels (ensure Ca^{2+} entry into the cell and activate Ca_V via membrane depolarization); CaV — voltage-gated Ca^{2+} channels; CaM — calmodulin; MLC — myosin light chains; P — phosphate; RhoA — small GTP-binding protein; CPI17 — inhibitor of MLC phosphatase.

muscle cells [37, 38]. ROS stimulate RhoK, that leads to smooth muscle contraction and duct lumen occlusion [37, 38].

Key effects of RhoK in vascular endothelium

Vascular endothelium plays a number of functions, a barrier and regulatory functions to be the most important. Barrier function of the endothelium consists in its selective permeability for circulating substances [39] and regulatory function is associated with synthesis of a variety of vasoactive substances. Under normal conditions endothelium produces predominantly vasodilatory factors, nitric oxide (NO) is a key factor among them. In vascular endothelium NO is constitutively synthesized by endothelial NO-synthase (eNOS) [40].

eNOS activity and NO production in endothelial cells are comprehensively regulated by changes in $[Ca^{2+}]_i$ (to be increased with the elevation of $[Ca^{2+}]_{i)}$ and site-specific phosphorylation by a variety of protein kinases including RhoK [41, 42]. RhoK can diminish eNOS activity via direct phosphorylation and via suppression of stimulating signaling pathways. Direct RhoK influence includes eNOS phosphorylation at Thr-495 [42], that leads to the decrease in eNOS activity. Indirect RhoK influence is mediated by the reduction of activity of protein kinases aimed at the main activation site of eNOS, Ser-1177; this also leads to the suppression of eNOS activity. Firstly, RhoK is able to diminish the activity of protein kinase Akt, which strongly stimulates eNOS via phosphorylation at Ser-1177 [43]. Secondly, RhoK inhibits AMP-activated protein kinase [44], which also phosphorylates eNOS at Ser-1177 [41]. Besides that the level of eNOS phosphorylation at Ser-1177 is negatively regulated by PTEN phosphatase (phosphatase and tensin homolog deleted on chromosome 10) [45], which activity is stimulated by RhoK [46]. Thus, in endothelial cells RhoK can suppress eNOS activity directly or indirectly, leading to the decline in NO production and vasoconstriction (Fig. 2). In addition to the suppression of eNOS activity, RhoK can influence eNOS gene expression. For example, long-term exposure to thrombin negatively regulates eNOS expression level due to RhoK activation [47] and reduction of eNOS mRNA stability [1].

In addition to the influence on eNOS RhoK was shown to affect NO production by modulating

the activity of arginase-2 that competes with eNOS for the substrate. RhoK participates in arginase-2 translocation from mitochondria to cytoplasm in endothelial cells [48], leading to the augmentation of arginase-2 activity and the decrease in bioavailability of L-arginine for eNOS. As a result, NO production declines and vascular tone rises.

RhoK influence on endothelial permeability is associated with cytoskeleton reorganization and contraction of endothelial cells [39]. Changes in actomyosin cytoskeleton play an important role in endothelial response to mechanical stimuli caused by alterations in blood flow and blood pressure [49]. Mechanical factors may cause RhoA activation with subsequent increase in RhoK activity [19]. As in vascular smooth muscle, RhoA/RhoK activation in endothelial cells leads to inhibition of MLC phosphatase, myosin phosphorylation, assembly of myosin filaments and contraction [49]. This results in increased rigidity of endothelial cells, the rise of endothelial permeability and the development of inflammatory processes. Besides that, RhoK regulates actin polymerization in endothelial cells [50], that provides actin stress fibers formation and plays an important role in the regulation of endothelial rigidity and permeability.

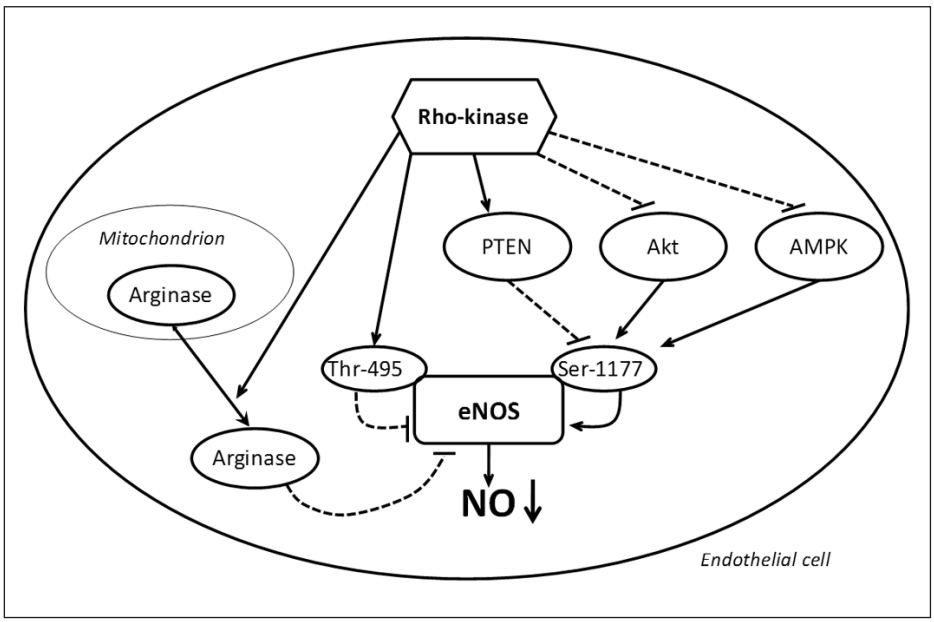
Thus, RhoK is involved into the regulation of both barrier and regulatory endothelial key functions. One can suppose that under normal conditions RhoK is a part of regulatory balance between endothelial activity and current physiological demands. However, pathological RhoK activation can cause unwanted increase of endothelial permeability and hypercontractility of vascular smooth muscle.

The role of RhoK in the development of vascular pathologies

RhoK is involved in the pathogenesis of vascular disorders associated with systemic hypertension, pulmonary hypertension, diabetes mellitus (DM) and many others [7, 19].

Systemic hypertension. Blood pressure rise in systemic hypertension is associated with altered activity of numerous regulatory mechanisms, including RhoK signaling pathway. Augmented vasomotor RhoK activity was demonstrated in different animal models of arterial hypertension such as hereditary (SHR rats), vasorenal, DOCA salt, *etc*; administration of RhoK inhibitors caused the decline in arterial pressure to the normal level [51, 52]. Increased activity of RhoA/RhoK signaling pathway in vascular system plays a causative role

Figure 2. Key mechanisms of Rho-kinase (RhoK) inhibitory influences on endothelial NO-synthase (eNOS) activity



Note: Solid and dotted lines show stimulating and inhibitory influences, respectively. eNOS activity depends on phosphorylation level at Thr-495 (inhibitory site) and Ser-1177 (activating site). Akt — protein kinase Akt; AMPK — AMP-activated protein kinase; PTEN — phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome 10).

in pathogenesis of hypertensive diseases with different etiology (reviewed in [53]). Also during hypertension RhoK expressional level in vessels rises [51]. Taking together, these data point to the key function of RhoK in development of systemic hypertension.

Pulmonary hypertension. Pulmonary hypertension is characterized by the increase in RhoK abundance and activity in smooth muscle and endothelial cells of pulmonary circulation. In rat models of this disease RhoK inhibition almost completely normalizes pulmonary blood pressure and significantly decreases the contractility of pulmonary arterial smooth muscle [54, 55]. In cultured pulmonary endothelial cells, hypoxia, a key factor of pulmonary hypertension pathogenesis, strongly increases content and activity of RhoK in parallel to the decrease in content and activity of eNOS due to the reduction in its mRNA half-life [56]. Importantly, RhoK inhibitor administration prevents the drop of eNOS expression level under the hypoxia [56]. Similarly, pulmonary arterial endothelial cells of newborn piglets with lesser circuit hypertension demonstrate persistent pathological phenotype and increased RhoA activity in association with elevated actin stress fibers formation and endothelial hyperpermeability [57]. RhoA inhibition leads to complete normalization of endothelial cells phenotype and permeability in hypertensive animals [57]. In monocrotaline-induced rat pulmonary hypertension the endothelium-dependent relaxation of pulmonary arteries is weakened as well; this is associated with endothelial dysfunction and reduced smooth muscle responsiveness to vasodilatory stimuli [58]. In such case endothelial dysfunction is accompanied by significant decrease in eNOS protein abundance, while chronically administrated RhoK inhibitor increases eNOS protein content and improves endothelium-dependent relaxation [58]. In addition, the efficiency of RhoK inhibitor regarding pulmonary pressure reduction was demonstrated in hypoxic model of pulmonary hypertension in newborn rats [59].

Data on RhoK contribution to pathogenesis of pulmonary hypertension in humans are rather sparse, but they also point to the significant role of RhoK in the increase of pulmonary resistance during this disorder [60]. Intravenous administration of RhoK inhibitor significantly reduces pulmonary blood pressure in patients with this form of hypertension [61]. Also patients with pulmonary hypertension demonstrate impaired endothelium-dependent dilation of pulmonary arteries [60], which can be associated with RhoK activation.

To conclude, pathogenesis of pulmonary hypertension involves augmentation of RhoK activity, on one hand, and endothelial dysfunction, suppression of eNOS abundance and activity and elevation of endothelial permeability, on another hand.

Type I diabetes mellitus. Increased RhoK activity serves as one of the reason for vascular disorders during type I DM [62]. In rat model of type I DM RhoKα abundance and eNOS phosphorylation at Ser-1177 in aortic endothelium increase in 5 weeks after diabetes induction. Aorta of these animals demonstrates augmented relaxation to RhoK inhibitor in comparison to control animals, the difference between groups is eliminated after NO-syntase inhibition [63]. Similar data on impaired endothelium-dependent relaxations due to reduced eNOS abundance and elevated RhoK content in type I DM were also obtained by another research group [64]. Importantly, they have shown that chronic oral administration of RhoK inhibitor fasudil normalizes endothelial regulatory function [64].

The development of type I DM is also associated with the RhoK-mediated increase in microvascular endothelial permeability. In rat model of type I DM the rise of endothelial permeability induced by platelet-activating factor can be almost completely abolished by RhoK inhibitor [65]. Similar data were obtained on cultured human umbilical vein endothelial cells, where high glucose level activated RhoA/RhoK pathway and increased endothelial permeability eliminated by RhoK inhibitor [66]. Increased content of both RhoK isoforms and impaired endothelium-dependent dilation was also observed in aorta of type I diabetic mice [67]. In this case endothelial dysfunction was associated with the suppression of NO production due to the increase in arginase activity/expression, as arginase inhibition improved endothelial function [67]. Such alterations were not observed or highly suppressed in RhoKα or RhoKβ knockout mice in comparison to wild type animals [67]. Thus, type I DM causes RhoK pathway activation in endothelial cells, followed by increased endothelial permeability, reduced NO synthesis and impaired endothelium-dependent vasodilation.

Type II diabetes mellitus. RhoK is involved in the pathogenesis of vascular disordes associated with type II DM. Its role appears on systemic level as well as in functioning of individual arteries. RhoK inhibitor normalizes elevated arterial pressure in rats suffered from type II DM, that goes in accordance with increased RhoK contribution to contractile responses of mesenteric arteries [68]. Similarly, higher contribution of RhoK to contractile responses of mesenteric arteries was revealed in *ob/ob* mice with type II DM [69]. This is associated with higher RhoK activity, while expression levels of RhoA and both isoforms of RhoK remain unchanged [69]. Increase in RhoK vasoconstrictor influences during type II DM is typical for cerebral vessels as well. Increased serotonin-induced contractile responses of carotid artery in type II diabetic rats are mediated by RhoK [70]. In cerebral arterioles of type II diabetic mice RhoK inhibitor demonstrates more potent vasodilatory influence in comparison to control animals [71].

At the same time, not all data demonstrate elevated RhoK contribution to contractile responses in type II DM. For example, rats with type II DM shows reduced myogenic tone of cerebral and coronary arteries in comparison to control animals, these differences are abolished after RhoK inhibition [72]. Similarly, contractile responses of mesenteric arteries to α_1-adrenoceptor agonist are impaired in type II diabetic mice and the difference to control animals is abrogated by RhoK inhibition [73].

Therefore, type II DM can be associated with bidirectional alterations in RhoK contribution to vascular tone regulation. Such data diversity can be due to the peculiarities of individual vascular beds or the differences in used experimental models of type II DM; this should be revealed in future studies. However, increased vasomotor contribution of RhoK followed by elevated arterial pressure is most likely scenario in human type II DM [74, 75].

Conclusions

RhoK signaling pathway is essential for normal regulation of smooth muscle and endothelial cells functioning but may become a key pathogenic factor in the development of vascular disorders. The rise in RhoK activity in smooth muscle and endothelial cells increases vascular tone and thereby creates a risk of impaired blood supply to vital organs and arterial pressure elevation followed by severe complications such as stroke, myocardial infarction, pulmonary edema and others.

Increased RhoK activity can be involved in positive feedback mechanism of pathological vascular disorders formation by interaction with cyclophilin A [76]. RhoK was shown to promote the secretion of vesicles with cyclophilin A by smooth muscle cells [76], cyclophilin A activates NADPH-oxydase and ROS production, the latter cause additional RhoK activation.

At present, RhoK is considered as one of the most promising targets for pharmacological correction of smooth muscle hypercontractility and endothelial dysfunction [19]. RhoK inhibitors fasudil and ripasudil are already used in clinics for corrections of regional and systemic circulatory disorders [77, 78]. Given the systemic nature of RhoK effects the diagnostic method based on evaluation of RhoK activity in leucocytes is currently being introduced into clinical practice. In patients with type II DM RhoK activity was shown to correlate positively with hemoglobin glycosylation level [74], *i.e.* can serve as an additional criterion for the severity of the disease. Apparently, the introduction of such novel methods of cardiovascular diagnostics and treatment would help to improve the quality of patients' life.

Abbreviations. ATP — adenosine triphosphate;
GTP — guanosine triphosphate;
GDP — guanosine diphosphate;
MLC — myosin light chain;
$[Ca^{2+}]_i$ — intracellular calcium concentration;
CPI-17 — C-kinase-Potentiated Inhibitor with molecular weight 17 kDa;
eNOS — endothelial NO-synthase;
MYPT1 — myosin light chain phosphatase regulatory subunit;
RhoK — Rho-kinase.

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Conflict of interest
Authors declare no conflict of interest.

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Author information

Olga S. Tarasova, PhD, Doctor of Biological Sciences, Professor, Department of Human and Animal Physiology, Faculty of Biology, Lomonosov Moscow State University;

Dina K. Gaynullina, Candidate of Biological Sciences, Senior Reseacher, Department of Human and Animal Physiology, Faculty of Biology, Lomonosov Moscow State University.