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New Angle of View on the Role of Rho/Rho Kinase Pathway in Human Diseases

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ABSTRACT

Rho-kinase is an effector molecule of RhoA, a monomeric GTP-binding protein, and causes Ca^{2+} sensitization through inactivation of myosin phosphatase. The major physiological functions of Rho/Rho-kinase cascade include contraction, proliferation and migration in cells.

There are some excellent reviews about Rho/Rho-kinase signal pathway, most of which focus on the specific proteins of the pathway including some upstream regulators and its final effects. But few articles cover signal pathways that can activate the signaling concerned, and/or the pathways that Rho/Rho-kinase can exactly activate.

This review hence highlights the two questions after a profound survey of published literatures. Rho/Rho-kinase can exert positive feedback with just another kinase/signal transducers and activator of transcription, receptor tyrosine kinase signal pathways, even reactive oxygen species, which seem to comprise certain signal loops. The authors also presume, accordingly, that the positive feedback suggests a possible reason for exacerbation of some kind of inflammatory diseases including asthma, rheumatoid arthritis, multiple sclerosis, atherosclerosis, etc. This essay, therefore, provides a new angle of view for the therapy of these kinds of diseases.

Keywords: Ca^{2+} mobilization; Ca^{2+} sensitization; Rho/Rho-kinase; Signal pathway; Positive feedback; Signal loops

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INTRODUCTION

Rho/Rho-kinase widely expresses in many types of inflammatory cells, e.g. eosinophil, T-lymphocyte,

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macrophages, schwan, epithelial and endothelial cells as well as smooth muscle cells (SMC) (vascular, coronary, bronchial, cerebral, visceral and gastric),[1-5] which causes migration of inflammatory cells and sustained contraction of SMC through inhibitting myosin phosphatase targeting subunit 1 (MYPT1). This signal pathway is also known as Ca^{2+} sensitization, which means no changing of intracellular Ca^{2+} concentration when SMC contraction increases, compared to the way of Ca^{2+} mobilization. Rho/Rho-kinase has been implicated in numerous diseases associated with inflammatory cell infiltration and SMC contraction. Ca^{2+} sensitization has been well studied in asthma, which plays a significant role in almost all pathophysiological and pathological changes, airway hyperresponsiveness, airway remodeling, inflammatory cell migration and mucus hypersecretion. Inhibiting the pathway, at least partly, reverses these processes above in vivo or vitro trials.[6] Atherosclerosis is a complex pathophysiological process characterized by progressive inflammation, lipid accumulation, arterial wall fibrosis. Inhibiting the post-translational modification of Rho, geranylgeranylation, by statins can exert significant beneficial effects apparently independent of cholesterol lowering.[7] Moreover, the phenomenon of Ca^{2+} sensitization involved in disease of rheumatoid arthritis (RA)[8] and multiple sclerosis (MS)[9,10] implies the unique role of Rho/Rho-kinase in connecting neural system and immune system through migration of inflammatory cells, especially T helper cells.

Also the Rho/Rho kinase pathway has been reported to be involved in angiogenesis,[11] cerebral ischemia,[12] erectile dysfunction,[13] hypertension,[14] myocardial hypertrophy, myocardial ischemia–reperfusion injury,[15] neointima formation,[16] pulmonary hypertension,[17,18] and vascular remodeling.[19] In addition, Rho kinase inhibitors have shown benefits in animal models of Alzheimer's disease, diabetes, obesity, cancers, glaucoma, and osteoporosis.[20-22]

The clinical significance of Rho/Rho-kinase signal pathway has been emphasized in some excellent reviews, and descriptions are on the specific proteins of the signaling cascade and their final effects. However, despite a growing number of reports demonstrating that Ca^{2+} sensitization activity is increased under a variety of pathological conditions, little is known regarding the molecular mechanisms that contribute to increased Rho/Rho-kinase activity and what the downstream targets of the Rho/Rho-kinase cascade are, especially in the context of inflammatory disease based on latest studies. This review highlights both upstream and downstream signaling pathways of Rho/Rho-kinase cascade. Surprisingly, among these signaling pathways, some of them comprise a signal loop with Rho/Rho-kinase in the style of positive feedback, which has not ever been reported thus far.

Characters of Proteins in Rho/Rho-kinase Signal Pathway

Protein Rho

Rho, a monomeric G protein, belongs to the Ras superfamily and the Rho family has at least 20 members, the most extensively characterized of which are the Rho, Rac, and Cdc42 proteins. Rho has isoforms of A-E and G, however, most of the functions of Rho are described, based on the studies of RhoA.[6,23] As compared to other regions, the RhoA C-terminus (amino acids 187-193) shows a low level of similarity between mammals and non-mammals. The human and mouse RhoA genes are localized to chromosomes 3p21 and 9, respectively and RhoA mRNA is constitutively expressed in a wide variety of tissues and cells.[24-26] The RhoA gene encodes 193–amino acid proteins and the sequence of these amino acids has been highly conserved during evolution. The C-terminus of all Rho families is post-translationally processed by prenylation for membrane binding.[26,27] In addition, serine residue 188 of RhoA is phosphorylated by several kinases.[28-30] This phosphorylation leads to the suppression of Rho-mediated stress fiber formation or inhibition of the membrane translocation of RhoA.[31] Like other G proteins, RhoA exhibits both GDP/GTP binding activity and GTPase activity, and acts as a molecular switch between a GDP-bound inactive state (GDP-RhoA) and a GTP-bound active state (GTP-RhoA).[32,33] The rat RhoA promoter sequence exhibits high sequence similarity with the mouse and human RhoA promoter sequences. Database analysis of the rat RhoA promoter region identified several putative transcription factor binding sites: Sp1, CCAAT/enhancer binding protein (C/EBP), signal transducers and activator of transcription (STAT), CdxA, and nuclear factor-kappa B (NF-κB). These regions provide physical basis of interaction with just another kinase (JAK)/STAT and receptor tyrosine kinase (RTK) signaling.[34,35]

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Protein Rho Kinase

Rho kinase acts as a serine/ threonine kinase that is activated by a direct interaction of a C-terminal Rho-binding domain (RBD) with GTP-bound RhoA.[36-38] Rho kinase has two isoforms, Rho kinase a (ROCK2/ROKa) and Rho kinase β (ROCK1/ROKβ). Both isoforms are ubiquitously expressed in various human as well as rodent tissues, they share over 90% sequence homology in the kinase domain, whereas significant differences are observed in the regulatory domains at the C-terminus.[39,40] The molecular weight of Rho kinase is about 160 kDa, and the kinase region is present in the N-terminus, a coiled-coil region at the center, and a pleckstrin homology (PH) region at the C terminus. Rho kinase specifically binds to activated RhoA (GTP-Rho) through the coiled-coil structure.[5,41] Rho kinase activated by RhoA interacts with myosin phosphatase and inhibits myosin phosphatase activity by phosphorylating threonine 696 and 853 of myosin phosphatase targeting subunit 1 (MYPT1), a myosin binding subunit. In addition to (contractile) agonists acting on G-protein coupled receptors, a variety of other stimuli, including cytokines and extracellular matrix proteins,[42] have emerged as inducers of the Rho/Rho-kinase signaling pathway described below.

Regulators of Rho/Rho-Kinase Cascade

Activity of Rho is governed through the action of three upstream groups of proteins: guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and guanine nucleotide dissociation inhibitors (GDIs). Regulator of G-protein signal (RGS) proteins for $G_{12/13}$ proteins are RhoGEFs, four of which are known to be regulated by G_{12}-type proteins: p115-RhoGEF, PSD-95/Disc-large/ ZO-1 homology (PDZ)-RhoGEF, leukemia-associated RhoGEF (LARG) and lymphoid blast crisis (Lbc)-RhoGEF consisted of 927, 1522, 1544, 893 amino acids, respectively in human. Two common motifs of RhoGEF proteins in the C terminal region are the Dbl-homology (DH) and pleckstrin-homology (PH) domains. The DH domain specifically binds to and stabilizes nucleotide- and Mg^{2+}-free RhoA transition states to enhance its nucleotide exchange. The PH domain is essential for full GEF activity and moreover anchors RhoGEF to other signaling proteins to trigger specific subcellular localizations.[43,44] PDZ-RhoGEF and LARG additionally contain an N terminal PDZ domain, which enables coupling to cell surface receptors such as plexins, insulin-like growth factor receptors or GPCRs.[33,45] These receptors make it possible that Rho/Rho-Kinase can be activated by just another kinase/signal transducers and activator of transcription (JAK/STAT), receptor tyrosine kinase (RTK), G protein-Coupled Receptors (GPCR) pathways. Recently, Arhgef15,[46] Ephexin-1,[47] and Arhgef5[48] have been proved to belong to the RhoGEF superfamily.

GDIs can prevent membrane anchoring and nucleotide exchange by forming cytoplasmic complexes with GDP-bound Rho and keep the protein soluble by shielding the hydrophobic post-translationally modified moiety.[49] It has become apparent that dissociation of monomeric G-proteins from GDIs is necessary for membrane association and GEF-mediated activation; although the mechanisms involved in dissociation are currently not clear, they are possibly regulated by integrins.[50] GAPs can promote intrinsic activity of Rho-GTPase to accelerate transformation from activated GTP-binding formation to inactivated GDP-binding formation. PKA can inhibit RHOA/RHO kinase directly and PKC can indirectly stimulate Ca^{2+} sensitization through phosphorylated CPI-17, a 17-kDa PKC-potentiated protein phosphatase 1 inhibItor protein, which inhibits MLC-phosphatase and is another potential mediator of Ca^{2+} sensitization.[51] In addition, activation of RhoA is strongly dependent on its post-translational modification (isoprenylation) mediated through the activity of geranylgeranyl transferases and farensyl transferases.[52] This process allows translocation to specific plasma membrane sites that can be blocked by caveolin-1 peptide mimetics in smooth muscle cells,[49,53] and is required for membrane anchoring of the small GTPase and for subsequent association with its effectors. Indeed, statins, which indirectly inhibit posttranslational prenylation by depleting the 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) downstream metabolites farnesylpyrophosphate, geranylgeranylpyrophosphate and subsequent mevalonate cascade, can inhibit translocation of RhoA to plasma membrane, resulting in an inhibition of RhoA-mediated function in cultured tumor cell lines.

In addition to the classic regulators of the Rho/Rho-kinase pathway described above, a negative regulator of RhoA expression is miR-133a, a endogenous micro-RNA, which can bind complementarily to the 3′ untranslated region (UTR) of RhoA-mRNAs, resulting in mRNA cleavage and/or translation repression[54-56]

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reported in cardiomyocytes.[57] MiR-133a can be suppressed by its inhibitor, called antagomir-133a, and precursor MiR-133a, in cultured human bronchial smooth muscle cells (hBSMCs). Besides miR-133a, recent studies also proved that RhoA protein expression was negatively regulated by MiR-155[58] and MiR-31[59,60] in cancer cells, however, whether these miRNAs also down-regulate RhoA protein in the bronchial smooth muscle cells need to be clarified. To sum up, at least nine intracellular upstream regulators of Rho/Rho-kinase signal pathway in both protein and gene level have been reported to date.

Pathways to Affect Rho/Rho-kinase

Receptor-Dependent Pathways

G Protein-Coupled Receptors (GPCRs)

GPCRs, a well-known and classic way to activating Rho/Rho-kinase, represent a large family of seven transmembrane receptors, which communicate extracellular signals into the cellular lumens. The human genome contains 720–800 GPCRs, and their diverse signal characteristics are determined by their specific tissue and subcellular expression profiles, as well as their coupling profile to the various G protein families (G_s, G_i, G_q, $G_{12/13}$), as shown in table 1. The G protein coupling pattern links GPCR activation to the specific downstream effector pathways. $G_{12/13}$ signaling of GPCRs has been studied in more detail, and involves activation of RhoGTPase, and nucleotide exchange factors (RhoGEFs). Four mammalian RhoGEFs regulated by $G_{12/13}$ proteins are known: p115-RhoGEF, PSD-95/Disc-large/ZO-1 homology-RhoGEF, leukemia-associated RhoGEF and lymphoid blast crisis-RhoGEF described above. These link GPCRs to activate the small monomeric GTPase, RhoA, and subsequent downstream effectors. P115-RhoGEF localizes throughout the cytosol, and rapidly translocates to the plasma membrane upon GPCR-activation of $G_{12/13}$.[61,62] Mutant $Ga_{12/13}$ supresses p115-RhoGEF interaction and recruitment to the plasma membrane.[63]

Just like $G_{12/13}$, the same manner happens to another subunit of G-protein, G_q, agonist of which can activate Rho/Rho-kinase signaling and antagonist has inverse result.[64-70] Referring to G_i activating ROCK, there are some controversies about these trial protocols. In these trials, G_i was proved to be connected with Rho by adding pertoxin, inhibitor of G_i, which inhibited agonist-induced (sphingosine-1-phosphate, S1P) airway hyperresponsiveness. The point is here that G_i can activate phosphodiesterase and suppress adenylate cyclase resulting in reducing of cAMP casuing contraction of smooth muscles. Blocking G_i can cause smooth muscle relaxation instinctly rather than inducing the conclusion that Gi can activate RhoA/Rho kinase pathway, especially without control group.[2,68,71,72] $G_{\beta\gamma}$ binds directly to Arhgef5 in HEK293T cells by using Immunoprecipitation and luciferase reporter gene assay. As a member of the Rho-GEF superfamily, Arhgef5 possesses a DH-PH tandem domain and a C-terminal SH3 domain and has been shown to activate RhoA. Point mutation technology reveals residues Val281–Arg311 and Trp312–Glu424 of Arhgef5 binds to $G_{\beta\gamma}$ and the site is located in and near the PH domain. Arhgef5-null mice study proves that Arhgef5 is a significant regulator of RhoA in immature dendritic cells and contributes to the reduction in eosinophil infiltration resulting in attenuation of allergic airway inflammatory responses. Other studies show $G_{\beta\gamma}$ has direct interaction with Cdc42 and Rac2 members of Rho superfamily.[73-75] All subunits of G-protein described above can activate Rho/Rho-kinase, but G_s suppresses the cascade directly (cAMP-independent) and indirectly (cAMP-dependent) through PKA which inactivates RhoA and MYPT1.[76,77]

Together, these proteins mentioned above indicate that all subunits of G-protein are involved in affecting Rho/Rho-kinase, which further proves Ca^{2+} mobilization activated by G_q and Ca^{2+} sensitization has intimate relationship.

JAK/STAT Signal Pathway

Chiba's team proved that interleukin(IL)-13, one of the central mediators of allergic asthma, could directly up-regulate RhoA in bronchial smooth muscle BSM cells and induce an augmented contraction. Using leflunomode or AS1517499, STAT6 inhibitor, could abolish STAT6 phosphorylation and subsequent RhoA up-regulation induced by IL-13 in human smooth muscles. Further research using the TFSEARCH program shows the upstream genomic DNA sequence of human RhoA contains several STAT-binding sites-for example, 277 (from the transcription start) to 269 (score 85.6), 2270 to 2261 (score 86.5), 2417 to 2409 (score 78.8) and 2518 to2510 (score 84.6).[34,78] The presumed mechanism is that IL-13 activates JAK/STAT and then the phosphorylated STAT can

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bind with the mentioned DNA site, eventually causes up-regulation of RhoA. This mechanism explains the up-regulation of RhoA by repeating antigen-challenge in animal asthmatic model. NF-κB may be another key protein in crosstalk between the two signal pathways. Kudo and co-workers found antibody of NF-κB can inhibit RhoA activation and airway hyperresponsiveness induced by IL-17A as well as antibody of IL-17A and Y27632. These results suggest the member of JAK-STAT pathway, STAT/NF-κB, can activate RhoA/Rho kinase independently. Recent studies have shown that differential requirements for Rho- and Rho kinase-dependent migration vary according to cell type and the environment.[79-81] Our knowledge of the role of Rho GTPases during migration in a complex environment is unclear. GM-CSF, CCL11, and C5a can elicit chemokinetic motility of eosinophils in a dose-dependent manner in 3D collagen matrices, but in GSM-CSF stimulation, C3 transferase and Y27632 can not suppress the mobility of eosinophils and the result is reversed in CCL-stimulation. Furthermore, other findings have shown dominant-negative constructs of Rho blocked migration in endothelial cells, but not in fibroblasts or epithelia.[80] Therefore, the use of the Rho pathway by JAK/STAT is both cell context and environment context-dependent (Figure 1).[82]

ROS can function in cell signaling pathways as well as reactive nitrogen species (RNS),[83,84] particularly in those pathways involving phosphatases.[85] In vitro, oxidizing agents can directly regulate the activity of certain GTPases, such as Ras[86,87] and Campbell's group described a radical-based mechanism for the stimulation of nucleotide exchange on Ras by NO.[88] Recent evidence suggests that there is extensive crosstalk between NO/cGMP/cGK and RhoA/Rho kinase signaling underlying their antagonistic actions in endothelial cells and vascular smooth muscle cells VSMCs.

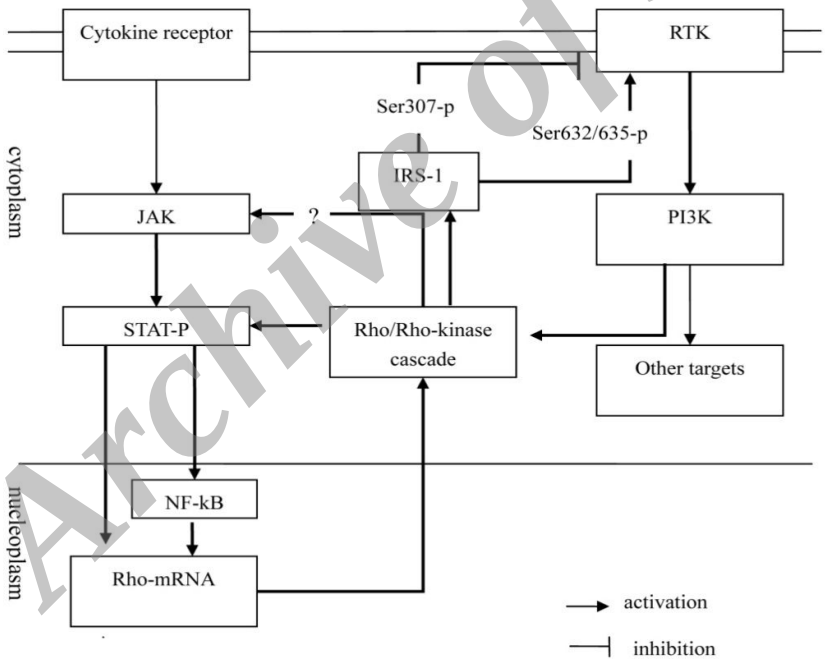

Figure 1. Positive and negative feedback (thick line) among Rho/Rho kinase, JAK/STAT and RTK signal pathways. Experimental evidence in cultured cells and mice show conflicting results, between different models, for the effect of Rho/Rho-kinase on insulin sensitivity. While Rho kinase phosphorylation of IRS-1 at Ser307 negatively impacts insulin signaling, Rho kinase phosphorylation of IRS-1 at Ser632/635 enhances insulin signaling. '?' means whether Rho/Rho-kinase can directly stimulate JAK still needs more evidences. IRS-1, insulin receptor substrate 1; RTK, receptor tyrosine kinase, PI3K, phosphatidylinositol-3-kinase. JAK, just another kinase; STAT, signal transducers and activators of transcription.
Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS)

Table 1. Upstream Effector of Rho/Rho Kinase Cascade

Signal pathway		Effect	References
	GEF, GAP, GDI, Geranylgeranyltransferases	activate/inhibit	43, 44, 49, 51, 52, 53
Regulators			
	PKA, PKC	activate/inhibit	51
	MiR-133a/155/31	inhibit	58-60
	$G_{12/13}$	activate	63-65
	G_i	activate	2, 70, 73, 74
Gprotein	G_q	activate	66-72
	Gβγ	activate	48-50, 76-78
	G_S	inhibit	79, 80
JAK/STAT		activate	82-85
RTK		activate/inhibit	101-104
ROS		activate	94-100
RNS		inhibit	88-92
Stretch		activate	105-111
High K^+		activate	112-114
High Glucose		activate	115-117

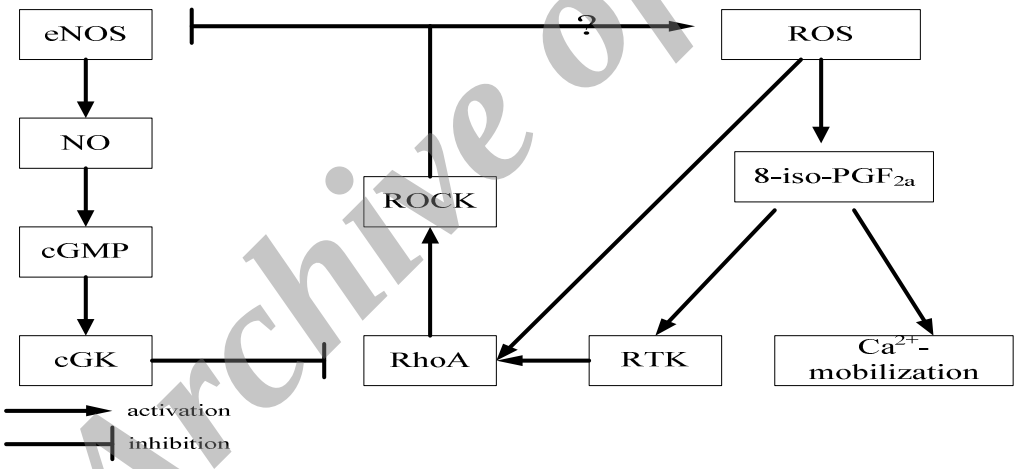

Figure 2. Interaction among Rho/Rho kinase, RNS, and ROS signal pathway. Endothelium-derived NO stimulates synthesis of cGMP and leads to the activation of cGK. cGK blocks Rho/Rho cascade by phosphorylating GTP and GDP-bound RhoA at Ser188, which increases Rho binding to Rho GDI and results in the sequestration of RhoA into the cytosol. At the same time Rho/Rho kinase inhibits eNOS expression and activity leading to endothelial dysfunction that underlies vascular diseases, such as atherosclerosis. ROS can upgrate Rho/Rho cascade via direct and indirect ways. '?' means whether Rho kinase can directly stimulate ROS needs more trials to prove. cGK, cGMP-dependent protein kinase; cGMP, cyclic GMP; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; ROS, reactive oxygen species; 8-iso-PGF2a, an isomer of prostaglandin F2a.

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; thus, abolishing subsequent Rho kinase activation.[89] The inhibitory effect of cGMP signaling on Rho/Rho kinase pathway is at least, in part, exerted through direct phosphorylation by cGK type I of RhoA at serine 188 close to its c-terminus, which results in increased binding of GTP-bound RhoA to Rho GDP-

dissociation inhibitor that sequesters RhoA into cytosol. [30,89]

Interestingly, studies identified a distinct redox-active motif located in the phosphoryl-binding loop in another subset of GTPases, the Rho family (RhoA, Rac1, and Cdc42). Previously published in vitro observations have shown that RhoA has a redox-sensitive motif (GXXXCGK(S/T)C) containing two cysteine residues in the phosphoryl binding loop.[90,91] Aghajanian's team mutated both cysteine 16 and 20 to alanine residues for transfecting REF52 cells and used miRNA targeted against a conserved region of RhoA to prevent interference of endogenous RhoA. They found that while the C16/20A RhoA mutant is unresponsive to ROS treatment, it is still capable of being activated and inactivated by other physiological stimuli. This finding further proves that ROS can activate RhoA directly through redox-sensitive motif.[92] Another trial suggested ROS can also activate RhoA through 8-iso-PGF2a, an isomer of prostaglandin F2a and useful biomarkers for oxidative stress in vivo, which has contractile effects via binding to thromboxane A2 receptors (TP receptor) in airway smooth muscle.[93,94] Comparing to these trials, some studies have reached different conclusions. Isoprostanes and hydrogen peroxide produced by oxidative stress contract airway smooth muscle by increasing intracellular concentration of Ca^{2+}. In other words, ROS can activate Ca^{2+} mobilization.[95,96] The seeming conflict conclusions imply ROS induce SMC constraction through both Ca^{2+}-sensitization (directly) and Ca^{2+}-mobilization (indirectly) (Figure 2).

Receptor Tyrosine Kinase Pathway (RTK)

Long-term treatment (8 d) with insulin has been demonstrated to increase expression of specific contractile phenotype markers in bovine tracheal smooth muscle (BTSM) cells and strips.[97] Laminin competing peptides, YIGSR and RGDS, can attenuate airway remodeling and hyperresponsiveness induced by insulin. Moreover, suppressing RhoA/Rho-kinase by Y27632, selective inhibitor of Rho kinase, can inhibit amount of laminin and subsequent airway remodeling and hyperresponsiveness. Findings demonstrate insulin can promote laminin expression through RhoA/Rho-kinase.[98] Another evidence is TGF suppresses IFN-γ induced NO production in macrophage, accelerates iNOS protein degradation[99] and regulates iNOS gene expression posttranslationly.[100] Moreover, pretreatment TGF-2 prior to IL-1/TNF/IFN stimulation in airway epithelial causes decreasing of nitric oxide (NO), but if incubate it with Y27632, decreasing of NO can be reversed.[3] To sum up, these trials suggest a close relationship between the two signalings. Receptor tyrosine kinase pathway activates Rho/Rho-kinase, however the key protein of the crosstalk is not elucidated (Figure 1).

Receptor Independent Pathways

Mechanical Stretch Pathway

Stretch has been demonstrated to activate fetal gene expression, increase protein synthesis, induce myofilament organization, and activate MAPK signal pathways, indicative of myocyte hypertrophy.[101-103] Just as GPCR, stretch can activate RhoA. Study demonstrated that stretching of SMC leads to the activation of ERK1/2, JNK1, and p38 MAP kinase.[104] In their subsequent research, they found cyclic mechanical stretch promote TGF-β1 expression , which could be blocked by Y27632 or miRNA-RhoA in cultured human airway smooth muscle cells (HASMCs). Using TGF-β1 promoter-reporter plasmid and site-directed mutagenesis of TGF-β1 promoter, they found that stretch increases TGF-β1 mRNA expression and protein release through de novo RNA synthesis mechanism in HASMCs. Further research found that stretch can activate small RhoA GTPase and its downstream targets Rho kinase1/2 and AP-1 transcription factor. The study also demonstrated that AP-1 played important roles in the mechanical regulation of TGF-β1 expression and its promoter activity. The presence of two adjacent AP-1 cis-acting elements on the TGF-β1 promoter region was essential for AP-1 mediated TGF-β1 promoter activity in response to stretch. Nevertheless, lacking specific mechanosensitive cell surface receptors, it is still not clear how mechanical forces are transmitted into intracellular signals that underlie gene expression. Possible mechanism is that mechanical force could trigger the deformation of the cell membrane, which may directly or indirectly induce conformational changes in protein (and subsequent activation of them) that are anchored to the inner surface of cell membranes or in transmembrane proteins. Rho/Rho-kinase are example of membrane-bound proteins that might be affected by mechanical forces.[105]

High K+ Inducing Depolarization

Some investigations using vascular smooth muscle have revealed a novel mechanism whereby Ca^{2+} sensitizaton can be triggered by depolarization and/or elevation of concentration of intracellular Ca^{2+} ($[Ca^{2+}]i$).[106,107] Janssen' s group demonstrated high-millimolar KCl could stimulate Rho/Rho-kinase activities and Rho kinase inhibitors markedly suppressed KCl induced contraction in ASM of the pig, cow, and human. Further study found that KCl induced changes in tension and Rho/Rho-kinase activities were largely inhibited by nifedipine or removal of external Ca^{2+}, which suggested that membrane depolarization alone was not sufficient for enhancement of Rho/Rho-kinase activities, since those interventions should not affect the change in membrane potential per se. Presumed mechanism is that Rho/Rho-kinase activities is modulated by changes in $[Ca^{2+}]i$ such as those secondary to any voltage dependent Ca^{2+} influx elicited by KCl, which could explain the effects of nifedipine and removing external Ca^{2+}. The specific mechanism about the contraction induced by high-millimolar KCl is not clear and there is a obvious question about the contraction including in elevating $[Ca^{2+}]i$ - whether other stimuli that elevating Ca^{2+} influx mimic these effects on Rho/Rho-kinase signaling?[108]

High Glucose Inducing Activation

Sawada's research group demonstrated that hyperglycemia increases Rho kinase activity in endothelial cells in a PKC and ROS-dependent fashion.[109] Another group reported a similar result showing that high glucose activated RhoA through activation of PDGFR-beta. Because induction of RhoA was abolished by PDGFR inhibitor.[110] Moreover, evidence indicates that advanced glycation end products (AGEs) activate RAGE, a major receptor for AGEs, and modulate various cell functions by multiple pathways, including Rho/rho kinase cascade.[111]

RhoA and RAGE can form a complex called RhoA/RAGE, which has been suggested to induce Rho kinase activation, resulting in reorganization of the actin cytoskeleton, leading to endothelial cell hyperpermeability in diabetes.[110] There is controversial on this conclusion that whether activate Rho/Rho kinase signaling by PDGF or by insulin induced by high glucose (Figure 3)

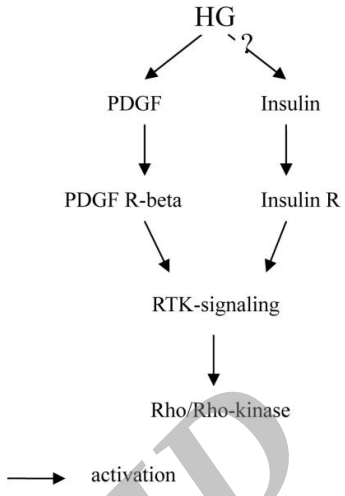

Figure 3. HG interacts with RTK signaling. HG can simulate Rho/Rho kinase through activation of PDGFR-beta, however insulin induced by HG can also promote Rho/Rho kinase activation. HG, high glucose; PDGFR, platelet-derived growth factor receptor; RTK, receptor tyrosine kinase.

Effect of Rho/Rho-kinase activation

Effect on Dynamic Apparatus

Rho kinase can directly phosphorylate myosin light chain phosphotase, myosin light chain, actin filament associated protein kinase,[38] filament associated protein calponin and actin causing cell contraction and changing of cytoskeleton, as shown in table 2.[112] By affecting organization of filamentous actin structures, balance of phosphorylated and dephosphorylated myosin light chain, and cell polarity they can directly influence cellular migration[113] including endothelial cell, eosinophil, neutrophil, macrophage, epithelial cell, schwan cell, and smooth muscle cell. These function have been well described by many nice reviews, so here does not describe in details (Figure 4).

Effect on JAK/STAT Pathway

Pelletier's group found that blocking the function of endogenous Rho with C3 transferase or dominant-negative RhoA prevents the transcriptional activation of STATs by GPCR agonists, without interfering with JAK activity or STAT phosphorylation. This result was consistent with these studies that Ang II and thrombin increase GTP loading of Rac, but not Cdc42, in vascular SMC. More details in that inhibition of Rac

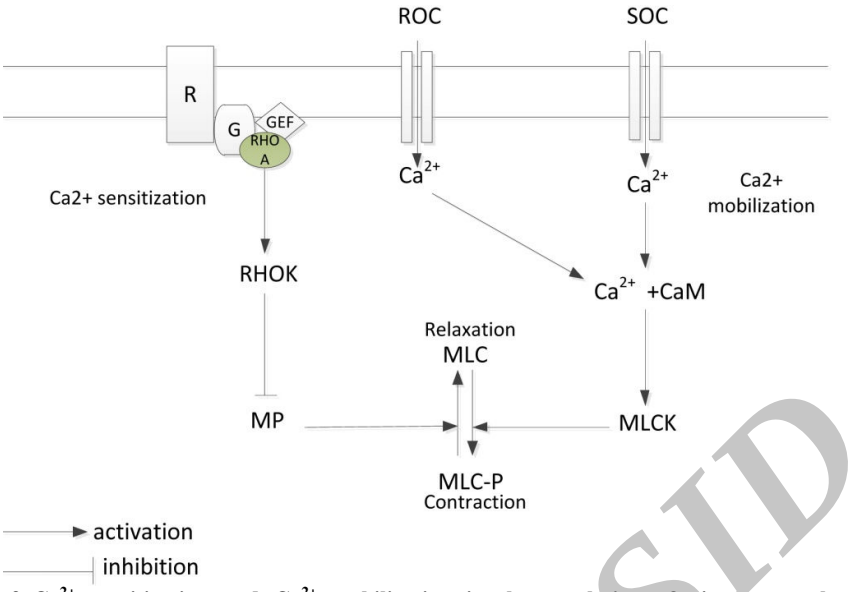

Figure. 4. Role of Ca^{2+} sensitization and Ca^{2+} mobilization in the regulation of airway muscle contraction. MLC phosphorylation (MLC-P), which is regulated by balance between MLCK and MP, is the key point of controlling smooth muscle contraction. Contractile agonist promote intracellular Ca^{2+} concentration via activating SOC and ROC. An increasing of Ca^{2+} concentration can enhance the binding of Ca^{2+} to CaM and then the Ca^{2+}-CaM complex augment MLCK activity, leading to MLC phosphorylation. RhoA activated by G-protein coupled receptors can activate RHOK which subsequently phosphorylates (inactivate) MP, leading to MLC phosphorylation. ROC: receptor operated Ca^{2+} influx, SOC: store-operated Ca^{2+} influx, CaM: camodulin, MLC: myosin light chain, MLCK: myosin light chain kinase, MP: myosin phosphatase.

Table 2. Targets of Rho/Rho-Kinase Cascade

Targets		Effect	References
Dynamic Apparatus	MLC	activate	38, 118, 119
	AFAPK	activate	
	FAPC	activate	
	Actin	activate	
	MP	inhibit	
Proliferation	GATA4	activate	133-135
Apoptosis	Capase	pro or against ?	136-142
JAK/STAT		activate	120-122
RTK		Activate or inhibit	126-131
RNS		inhibit	123
ROS		activate ?	124-125

'?' means needs more evidences. MP, myosin light chain phosphotase; MLC, myosin light chain; AFAPK, actin filament associated protein kinase; FAPC, filament associated protein calponin.

function with toxin B and LTs, or by expression of dominant-negative Rac1 protein, was found to suppress the activating phosphorylation of JAK/STAT and the induction of STAT-dependent transcription in response to GPCRs. Collectively, activated RhoA and Rac1 can signal independently, both are necessary for maximal transcriptional activation of STATs.[114-116] Thus far, almost all of these experiments focus on the phenomenons, but referring to the exactly cell biology and molecular mechanism has not been clarified thoroughly (Figure 1).

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Effect on RNS and ROS

Rho/Rho kinase cascade in endothelium reciprocally influences NO signaling by downregulating eNOS expression and activity. The inhibitory effects of Rho/Rho kinase on eNOS expression are exerted through inhibition of eNOS mRNA stability but not that of eNOS transcription. It is envisaged that, in diseased and dysfunctional endothelium, overactive of Rho/Rho-kinase leads to decrease in endothelial-derived NO bioavailability that gives rise to activation of VSMC Rho/Rho-kinase pathway leading to vascular hypercontraction,[117] excessive vascular inflammation (figure 2). Though it has been demonstrated that a decrease in NO bioavailability is often accompanied by increased ROS production in endothelial dysfunction disease,[118,119] whether Rho/Rho-kinase can directly produce ROS still needs more evidences (Figure 2).

Effect on RTK

Crosstalk between GPCRs and RTKs is mediated by phosphatidylinositol 3-kinase (PI3K), p70S6 kinase, and glycogen synthase kinase-3 (GSK-3).[120-122] Rho kinase has been proved to interact and phosphorylate insulin receptor substrate-1 (IRS-1) and thus modulate insulin signaling. However, both in vitro and in vivo studies have yielded conflicting results about the effects of RhoA/Rho kinase/IRS pathway on insulin signaling (RTK). The majority of the reported studies favor a detrimental role of Rho kinase activation in insulin signaling. These studies suggest that Rho kinase phosphorylates IRS-1 at Ser307 and impairs activation of PI-3K in rat VSMCs, H9c2 rat cardiac myoblasts and C2C12 mouse myoblasts.[123,124] On the contrary, Furukawa's group reported that Rho kinase mediated phosphorylation of IRS-1 at Ser632/635 positively regulates insulin action by facilitating tyrosine phosphorylation of IRS-1 in 3T3-L1 adipocytes and L6 myotubes.[125] Although it was consistently reported that Rho kinase activation increases serine phosphorylation levels of IRS-1 and IRS-2, the effects on insulin-stimulated tyrosine phosphorylation of IRS-1 and IRS-2 and on PI-3K activation appear to be complex and may be context dependent. Whether treatment with Rho kinase inhibitors improves or impairs insulin signaling is also context dependent. Both effects have experiments based evidences,[125,126] but the impairing effect mechanism would explain the recently demonstrated risk of statin therapy for development of diabetes (Figure 1).

Effect on Proliferation

Transcriptional activation by GATA-4, a well-known regulator of hypertrophic gene expression in cardiomyocytes, is potentiated by activated RhoA. The mechanism is that the effect of RhoA relies on p38 MAPK-dependent phosphorylation of GATA-4.[127] Rho kinase activation could also be involved, as a recent study reported that expression of dnROCK prevents GATA-4 responsive promoter-driven luciferase expression in cardiomyocytes.[128] Treatment with Y-27632 was found to decrease PE induced increases in GATA-4 DNA binding, further suggesting RhoA/Rho-kinase signaling in the regulation of GATA-4. That may be the major pathway of Rho/Rho-kinase to induce cell hyperplasia and hypertrophy.[129,130]

Effect on Apoptosis

There are few data concerning involvement of Rho/Rho-kinase in apoptosis and cell survival. The available literature is also conflicting, probable suggesting that cell fate in response to Rho/Rho-kinase is cell type-specific. Most of the published data prefer that RhoA can serve a protective function.[131-133] Thus, inhibiting RhoA/Rho kinase signaling by treatment with C3 exoenzyme which ribosylates RhoA or with Y-27632 and HA-1077 has been shown to lead to apoptosis in human umbilical vein endothelial cells,[131] neuroblastoma, and Jurkat cells,[133] further prove the protective function of RHOA. In contrast to the prosurvival effects of RhoA signaling in these cell types, over expression of wild type or activated RhoA has been shown to induce apoptosis in NIH3T3 cells.[134] RhoA is also proved to mediate the apoptosis in response to heterologously expressed and constitutively activated Ga_{12} and Ga_{13} in COS-7, Chinese hamster ovary, and HEK293 cells.[135] RhoA mediated apoptosis is shown to be Bcl-2-sensitive, which is known to affect cell survival by regulating the permeability of mitochondria. Del Re's team has proved that RhoA/Rho-kinase induces p53 mediated up-regulation of Bax expression and activation, which shifts the balance between proapoptotic, Bax, and antiapoptotic, Bcl-2 family proteins. This process can directly regulate the mitochondria and determine cell fate.[136] Further study suggests Bax translocates to and permeabilizes the mitochondria, allowing the release of

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apoptogenic mediators and subsequent caspase-9 and caspase-3 activation, resulting in cardiomyocyte apoptosis.[137]

Inhibitors of the Rho/Rho-kinase Signaling

The pyridine derivative Y-27632 is commonly used as a Rho kinase inhibitor. Y-27632 is a cell permeable, ATP-competitive inhibitor, which inhibits the activities of ROCK-I and ROCK-II.[138] Y-27632 is a reasonably selective ROCK-I and ROCK-II inhibitor, and provides a useful pharmacological tool to study the role of Rho kinase in patho/physiological events, both in vitro and in vivo when applied at the appropriate concentrations. Several analogues of Y-27632, both Y-30141 and Y-30694, have been synthesized, that have similarly high inhibitory constants for Rho kinase both Y-30141 and Y-30694.[139]

Fasudil, or HA-1077 has a similar affinity for Rho kinase as Y-27632, as indicated by its inhibition constant for Rho kinase activity.[140] Fasudil is only 3-fold more selective for Rho kinase compared to PKA , which is frequently used in animal models, and currently is the only Rho kinase inhibitor available for clinical use. In China and Japan, fasudil is approved for the prevention of vasospasm in patients with subarachnoid hemorrhage.[141] Interestingly, another ROCK inhibitor, SAR407899, has been shown to be eightfold more active than fasudil. In animal models, the antihypertensive effect of the novel ROCK inhibitor has been shown to be superior to that of fasudil and Y-27632.[142] Thus, SAR407899 might represent a novel potent ROCK inhibitor for the treatment of these diseases mentioned above. Finally, an isoform-selective ROCK2 inhibitor, SLx-2119 (Surface Logix), appears to be 100-fold more selective towards ROCK2 than ROCK1, and could have more favorable safety profile than dual ROCK inhibitors.[143]

Azaindole 1, a derivative of a class of azaindoles, is a potent, highly selective and orally active ROCK inhibitor.[144] Two aminofurazan-based inhibitors, GSK269962A and SB-7720770-B, have been identified as a novel class of potent inhibitors of ROCK activity.[145]

Positive and Negative Feedback between Rho/Rho Kinase and Some Certain Signal Pathways

Rho/Rho-kinase can be activated by almost all of the important known signal pathways in animals, including GPCR, RTK, JAK/STAT, ROS pathways, and other receptor-independent pathway like depolarization induced by high k^+, stretch, high glucose. Yet they are neither one direction pathway nor just crosstalk-some of them comprise signal loops and can stimulate each other in the style of positive feedback. The signal loop induces unstable condition, which is rare in the usual physiological regulations. Taking Rho/Rho-kinase and JAK/STAT pathway for example, activation of Rho/Rho-kinase can activate JAK/STAT cascade which then subsequently upgrades activation of RhoA. Through this circulation, the inflammatory mediators (leukotriene) and other cytokines (interleukin) maintain at a high level inducing pathological and pathophysiological changes. The same manner also happens to RTK pathway. The positive feedback might explain some inflammatory disease i.e. refractory asthma, RA, MS which cannot be controlled thoroughly by routine medicine. This phenomenon reminds us blocking the signal loop may provide a new way for the therapy of these inflammatory diseases.

The negative feedback exists between NO/cGMP/cGK and RhoA/Rho-kinase signalings. Increased NO production in endothelium will lead to an inhibition of the Rho/Rho-kinase cascade in VSMCs; thus, protecting against the enhanced contractile and proliferative effects of Rho/Rho-kinase signaling. On the contrary, stimulated Rho/Rho kinase activity in endothelium appears to diminish NO production; thus, even amplifying the increase in contractility and vascular remodeling induced by VSMCs Rho/Rho kinase signaling.

To the obvious questions, whether Ca^{2+} sensitization works in positive feedback under physiological conditions, the answer here is definitely negative. The reasonable explanation is that the Ca^{2+} sensitization can work in this way only under overactive status. Actually, endothelium-dependent vasodilation in healthy subjects tend to worsen with fasudil therapy compared with placebo. This finding might be explained by the fact that inhibition of Rho kinase in healthy individuals could lead to a negative-feedback loop with increased transcription of Rho. This would in turn lead to a compensatory increase in the downstream effects of Rho, including suppression of eNOS production. Also, Rho kinase inhibition in healthy individuals might lead to an excess of NO production, resulting in the formation of peroxynitrite, and this could lead to eNOS uncoupling and worsening

endothelial function.[146] These results suggest that some basal Rho kinase activity is probably required for the maintenance of vascular homeostasis and emphasizes the importance of selective Rho kinase inhibitors for use in the clinic.

CONCLUSION

Rho/Rho-kinase can maintain inflammatory mediators and cytokines at a high level under overactive status through the positive feedback with some certain signal pathways e.g. RTK, JAK/STAT. It is worth noting that, though Rho/Rho-kinase pathway exert pivot role in some inflammatory diseases above, inhibiting the way cannot fully reverse these pathophysiological process. That means some other unknown pathways might substitute Rho/Rho-kinase, which may be our potential new target for therapy and future study direction.

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