The interaction between cardiac myocytes and cardiac fibroblasts is a key event during Ang II–induced cardiac hypertrophy.[2,5,24,29,30] In particular, several studies have shown that cardiac myocyte hypertrophy was stimulated by growth factors and cytokines secreted from cardiac fibroblasts.[2–4,31,32] To prove that CyPA could be one of these factors that in a paracrine fashion ultimately induces cardiac myocyte hypertrophy, we first showed that CyPA is released from cardiac fibroblasts after Ang II treatment, and then we proved that extracellular CyPA stimulates cardiac hypertrophy. However, we cannot exclude the involvement of CyPA produced by vascular smooth muscle cells or cardiac myocytes in the enhancement of hypertrophy and fibrosis.

The precise mechanism by which CyPA directly enhances cardiac hypertrophy remains to be elucidated, in part because the CyPA receptor has not been identified. Nonetheless, the present study suggests that inhibition of CyPA may be a useful therapeutic strategy to attenuate cardiac hypertrophy in patients who experience high oxidative stresses, such as smoking, hypertension, and hyperlipidemia.

Acknowledgments

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Disclosures

None.

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Supplemental Material

Cyclophilin A promotes cardiac hypertrophy in apolipoprotein E-deficient mice

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Supplementary methods

Generation of mice

All animal experiments were conducted in accordance with the experimental protocols that were approved by the Institutional Animal Care and Use Committee at the University of Rochester. *Ppia*$^{-/-}$ mice were purchased from Jackson Laboratory (Bar Harbor, Maine) and were backcrossed to C57BL/6J mice for 10 generations. The *Apoe*$^{-/-}$ mice on a C57BL/6J background were obtained from the Jackson Laboratory (Bar Harbor, Maine, USA). Double knockout *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice were generated by crossing *Ppia*$^{-/-}$ mice with *Apoe*$^{-/-}$ mice. The F1 generation was backcrossed with *Apoe*$^{-/-}$ mice to fix the *Apoe*$^{-/-}$ genotype, and littermates were crossed. All mice were genotyped by PCR on tail clip samples, and all experiments were performed with generations F4–F6 using littermate *Apoe*$^{-/-}$*Ppia*$^{+/+}$ as wild-type controls. Animals were housed under a 12-hour light and 12-hour dark regimen and placed on a normal chow diet.

Blood pressure measurement and echocardiography

Blood pressures were obtained from the mice using a noninvasive tail-cuff system (BP-2000 Blood Pressure Analysis System; Visitech Systems, Apex, North Carolina, USA) as described previously.[1] Unanesthetized mice were restrained on a temperature-controlled mouse board, and echocardiography was performed on mice using a Vevo 770 ultrasound system (Visual Sonics, Toronto, Canada). An echocardiographer blind to animal genotype captured 2-dimensional long axis views of the left ventricle. An M-mode cursor was positioned perpendicular to the interventricular septum and the posterior wall of the left

ventricle at the level of the papillary muscles. The following measurements were obtained for systole and diastole using 5 cardiac cycles: interventricular septal thickness, left ventricular posterior wall thickness, left ventricular internal diameter, heart rate, and left ventricular mass.

Histological Analysis

After hemodynamic measurements, animals were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (5 mg/kg). For morphological analysis, aortas were perfused with normal saline and fixed with 10% phosphate-buffered formalin at physiological pressure for 5 minutes. The whole hearts were harvested, fixed for 24 hours, embedded in paraffin, and cross-sections (5 μm) were prepared. Paraffin sections were stained with Elastica-Masson staining or used for immunostaining.

Immunohistochemistry

Formaldehyde-fixed paraffin sections were incubated with primary antibody overnight at 4°C. The primary antibody used was PECAM-1 (1:100 dilution; BD Pharmingen). As a negative control, species- and isotype-matched IgG were used in place of the primary antibody. Slides were viewed with a microscope (BX41, Olympus) and with digital camera (Spot Insight 2, Diagnostic Instruments, Inc.).

Preparation of conditioned medium

Conditioned medium from AngII-stimulated cardiac fibroblasts or control medium from

DMEM-incubated cells was collected and filtered to remove cell debris. The medium was concentrated 100-fold with a Centricon Plus-20 filter (Millipore Corporation, Bedford, MA) to yield concentrated conditioned medium [1].

RNA isolation and semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

RNA was isolated from rat neonatal cardiac myocytes using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Semi-quantitative RT-PCR was performed with a C1000^{TM} Thermal Cycler (Bio-Rad) by first synthesizing cDNA using oligo(dT) primers with the Reverse Transcription PCR kit (Promega) followed by standard PCR using Go-Taq (Promega) according to the manufacturer's instructions. Rat atrial natriuretic peptide (ANP), brain nariuretic peptide (BNP), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were measured using the following primer pairs: ANP: 5'-ATCTGATGGATTTCAAGAACC-3' (Forward) and 5'-CTCTGAGACGGGTTGACTTC-3' (Reverse);
BNP: 5'-ACAATCCACGATGCAGAAGCT-3'
(Forward) and 5'-GGGCCTTGGTCCTTTGAGA-3' (Reverse);
GAPDH: 5'- GACATGCCGCCTGGAGAAAC-3' (Forward) and 5'-AGCCCAGGATGCCCTTTAGT-3' (Reverse).

Expression and Purification of His-tagged CyPA from High Five^{TM} cells

The recombinant baculovirus encoding N-terminal 6 x His tagged rat CyPA protein was

constructed using the Invitrogen Bac-to-Bac® Baculovirus expression system (Invitrogen, Carlsbad, CA, USA) as described below. Briefly, a 508bp cDNA fragment containing the full-length CDS of rat *PPIA* was obtained by excising from BamHI/EcoRI sites of plasmid pGEX-2TK-CyPA. After gel purification, it was inserted in the *Bam*HI/*Eco*RI sites of pFastBac™HT B vector. The resulting plasmid was termed pFastBac His-CyPA. After transformation and amplification in *E. coli*, purified pFastBac His-CyPA was sequenced and was verified that CyPA sequence was fused at its N-terminal to 6 x His tag. This plasmid was further transformed into DH10Bac™ *E.coli* for transposition into the bacmid. After package, amplification, purification from Sf9 cells, the resulting baculovirus was titred and was further used to infect High Five™ cells. Finally, His-CyPA was purified from High Five™ cells by using Ni-NTA Agarose (Qiagen, Germandtown, MD, USA) according to the manufacturer's instructions.

Mouse adult ventricular cardiac fibroblasts Isolation

Wild-type C57B6/j mice aged 12-16 weeks were anesthetized with 0.5 ml heparin (100 U/ml) and 0.5 ml of a ketamine/midazolam in saline combination via intraperitoneal injection. Once anesthetized, the heart was removed, immediately suspended on a Langerdorf apparatus by cannulation of the aortic root and perfused at constant rate of 4 ml/min at 37°C starting with 4 min of perfusion buffer (5 mM $NaHCO_3$, 30 mM taurine, 10 mM BDM, 5mM Glucose, pH 7.4). Subsequently, enzymatic digestion was achieved by the infusion of Calcium-free digestion buffer (120mg Collagenase type II in 50 ml perfusion buffer) for 3 min followed by 10 minutes of perfusion with calcium containing digestion

buffer (digestion buffer + 40 nM $CaCl_2$). The heart was then removed and placed in a dish filled with 2 mls of stopping buffer (10% FBS, 12.5 μM $CaCl_2$ in perfusion buffer). Following removal of the atria, the ventricles were teased apart and pipetted into small pieces. To remove undigested tissue, the cell suspension was filtered through a 200 μm mesh and allowed to settle by gravity for 10 min at 37°C. The pellet containing cardiac myocytes was discarded and the supernatant, containing mostly cardiac fibroblasts, was centrifuged at 1000 rpm for 5min, and resuspended in 5 ml of plating medium (DMEM, 10% FBS, 1x penicillin/streptomycin). The cardiac fibroblasts were plated on a 10 cm dish with DMEM low glucose+10% Newborn Calf Serum (NCS). After a 2 hours period of incubation at 37°C, allowing cardiac fibroblasts to attach, the plate was washed with PBS and the cells were cultured for 7 days in 10 ml of culture medium (DMEM, 10% FBS, 1X pencillin/streptomycin). After the first passage, more than 95% of the cells were cardiac fibroblasts as previously demonstrated by positive immunostaining for vimentin. Mouse cardiac fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS at 37°C in a humidified atmosphere of 5% CO_2/95% air as described.[2] Passage 2 to 3 cardiac fibroblasts at 70-80% confluence were used for experiments.

Neonatal rat cardiac myocytes isolation

Neonatal cardiac myocytes were obtained by enzymatic dissociation of cardiac ventricles from 2-3 day old Sprague-Dawley rat neonates. The ventricular tissue parts were subjected to multiple rounds of enzymatic digestion by collagenase II (Worthington). Cells were then collected by centrifugation at 800 rpm for 5 min at 4 °C. Non-myocytes were removed

via two rounds (45 min each) of pre-plating on culture dishes. The enriched cardiac myocytes were plated on 12-well plates coated with 0.2% gelatin in DMEM with 5% BCS, 5% horse serum, Insulin-Transferin-Selenium (ITS) and pencillin/streptomycin. The following day after cells adhered to the dish, cells were serum-starved for 24 hours in DMEM, ITS, pencillin/streptomycin and 10μM cytosine 1-β-D-arabinofuranoside to inhibit the growth of contaminating non-myocytes. More than 90% of cells were cardiac myocytes as verified by positive immunostaining for α-actinin.

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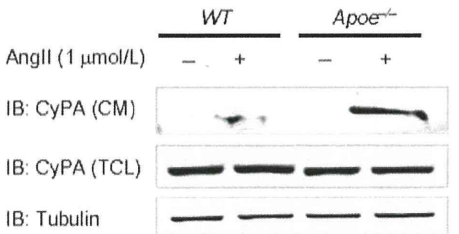
A



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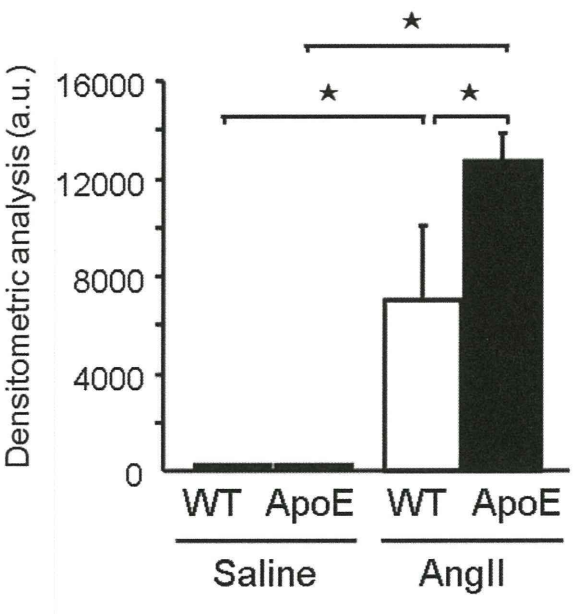
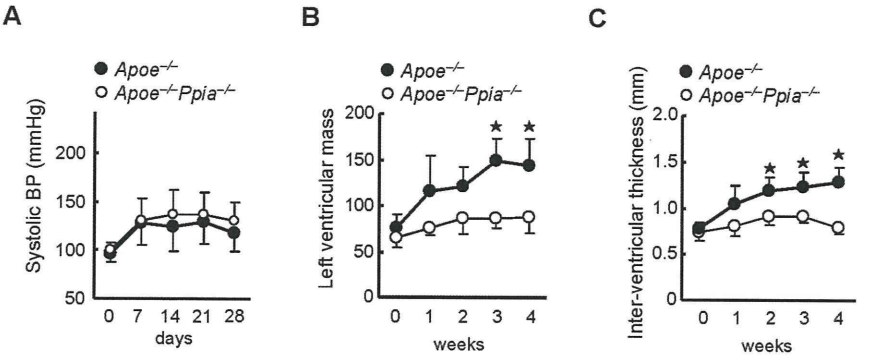
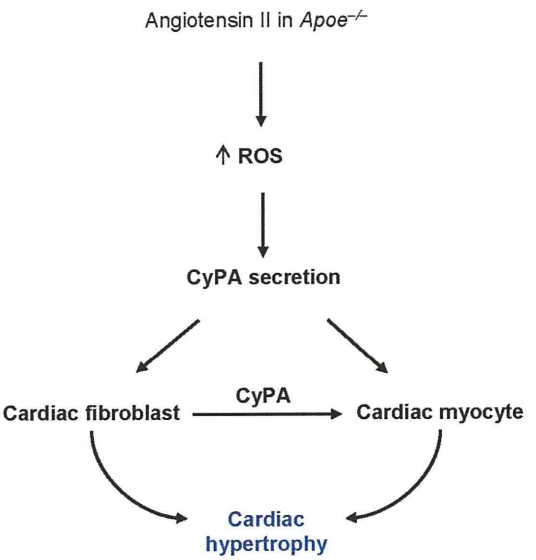


Supplemental Figure I. **(A)** CyPA secretion after 24 hrs of stimulation with AngII was greater in *Apoe*$^{-/-}$ compared to WT cardiac fibroblasts. (**B**) Densitometric analysis of CyPA secretion. $n = 3$ in each group. Data are mean ± SD. *$P<0.05$.



Supplemental Figure II. **(A)** No significant difference in systolic blood pressure (BP) between *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice during the AngII infusion. Maximal left ventricular mass (**B**) and diastolic inter-ventricular septum (IVS) thickness (**C**) was significantly reduced in *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice ($n = 5$) compared with *Apoe*$^{-/-}$ mice ($n = 6$) 4 weeks after AngII infusion. Results are mean ± SD.



Supplemental Figure III. Schematic representation of the effect of CyPA on cardiac fibroblasts and myocytes. CyPA is secreted in response to oxidative stress and induces cardiac hypertrophy by increasing hypertrophy of cardiac myocytes and hyperplasia of cardiac fibroblasts.

	WT (n=8)	*CyPA-/- (n=9)*	*p*
Blood pressure (mmHg)			
Before treatment	117.6 ± 17.7	107.5 ± 10.3	NS
After Ang II infusion	139.8 ± 22.0	134.5 ± 27.6	NS
Body weight (mg)			
Before treatment	27.5 ± 1.7	24.1 ± 3.1	NS
After Ang II infusion	26.8 ± 1.1	21.3 ± 3.9	NS
EF (%)			
Before treatment	80.2 ± 3.4	79.9 ± 8.7	NS
After Ang II infusion	79.1 ± 1.4	77.9 ± 10.4	NS
FS (%)			
Before treatment	47.5 ± 3.3	48.0 ± 9.1	NS
After Ang II infusion	47.5 ± 4.0	46.4 ± 7.3	NS
LV diameter (diastolic)			
Before treatment	2.99 ± 0.34	2.81 ± 0.29	NS
After Ang II infusion	3.31 ± 0.24	3.21 ± 0.17	NS
LV mass			
Before treatment	92.2 ± 21.1	72.0 ± 15.9	NS
After Ang II infusion	113.7 ± 25.3†	77.9 ± 10.4	$P < 0.05$

† $p < 0.01$ vs. before treatment. NS, no significant difference. Results are mean ± SD.

Supplemental Table I. Blood pressure and echocardiography data of *Ppia*$^{-/-}$ and *WT* before and after Ang II treatment.

	Apoe-/- (n=13)	*Apoe-/-Ppia-/- (n=11)*	*p*
Body weight (mg)			
Before treatment	16.9 ± 4.1	16.2 ± 1.3	NS
After Ang II infusion	21.1 ± 2.7 †	21.4 ± 1.6 †	NS
IVS (diastolic)			
Before treatment	0.78 ± 0.07	0.74 ± 0.09	NS
After Ang II infusion	1.29 ± 0.16	0.79 ± 0.06	$P < 0.01$
EF (%)			
Before treatment	81.3 ± 9.7	85.2 ± 4.0	NS
After Ang II infusion	91.1 ± 6.4	82.1 ± 9.6	NS
LV mass			
Before treatment	75.4 ± 15.8	65.1 ± 11.0	NS
After Ang II infusion	143.9 ± 30.1 †	88.5 ± 17.6	$P < 0.01$

† $p < 0.01$ vs. before treatment. NS, no significant difference. Results are mean ± SD.

Supplemental Table II. Body weight and echocardiography data of *Apoe*$^{-/-}$ and *Apoe*$^{-/-}$*Ppia*$^{-/-}$ mice after Ang II treatment.

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Review

Rho-kinase: important new therapeutic target in cardiovascular diseases

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Satoh K, Fukumoto Y, Shimokawa H. Rho-kinase: important new therapeutic target in cardiovascular diseases. *Am J Physiol Heart Circ Physiol* 301: H287–H296, 2011. First published May 27, 2011; doi:10.1152/ajpheart.00327.2011.— Rho-kinase (ROCKs) belongs to the family of serine/threonine kinases and is an important downstream effector of the small GTP-binding protein RhoA. There are two isoforms of Rho-kinase, ROCK1 and ROCK2, and they have different functions with ROCK1 for circulating inflammatory cells and ROCK2 for vascular smooth muscle cells. It has been demonstrated that the RhoA/Rho-kinase pathway plays an important role in various fundamental cellular functions, including contraction, motility, proliferation, and apoptosis, leading to the development of cardiovascular disease. The important role of Rho-kinase in vivo has been demonstrated in the pathogenesis of vasospasm, arteriosclerosis, ischemia-reperfusion injury, hypertension, pulmonary hypertension, stroke, and heart failure. Furthermore, the beneficial effects of fasudil, a selective Rho-kinase inhibitor, have been demonstrated for the treatment of several cardiovascular diseases in humans. Thus the Rho-kinase pathway is an important new therapeutic target in cardiovascular medicine.

cyclophilin A; oxidative stress; inflammation

THE RHO FAMILY OF small G proteins comprises 20 members of ubiquitously expressed proteins in mammals, including RhoA, Rac1, and Cdc42 (25, 65, 122). Among them, RhoA is the best-characterized protein that acts as a molecular switch that cycles between an inactive GDP-bound and an active GTP-bound conformation, interacting with downstream targets to elicit a variety of cellular responses (Fig. 1) (23). The activity of RhoA is controlled by the guanine nucleotide exchange factors that catalyze the exchange of GDP for GTP (102). In contrast, GTPase-activating proteins stimulate the intrinsic GTPase activity and inactivate RhoA (12). It has been demonstrated that guanine nucleotide dissociation inhibitors block spontaneous RhoA activation (Fig. 1) (81).

In 1996, Rho-kinase (Rho-kinase-α/ROCK 2 and Rho-kinase-β/ROCK 1) was identified as the effector of Rho (Fig. 2) and has been extensively studied, especially on its functions in the cardiovascular system (6, 53). Phosphorylation of myosin light chain (MLC) is a key event in the regulation of vascular smooth muscle cell (VSMC) contraction. MLC is phosphorylated by Ca^{2+}-calmodulin-activated MLC kinase and dephosphorylated by MLC phosphatase. Agonists bind to G protein-coupled receptors and induce contraction by increasing both cytosolic Ca^{2+} concentration and Rho-kinase activity through mediating guanine nucleotide exchange factor. The substrates of Rho-kinase have been identified, including MLC, myosin-binding subunit or myosin phosphatase target subunit 1, ezrin/radixin/moesin family, adducin, phosphatase and tensin homolog on chromosome 10, and LIM-kinases (Fig. 1). Rho-kinase enhances MLC phosphorylation through the inhibition of myosin-binding subunit of myosin phosphatase and mediates agonists-induced VSMC contraction (Fig. 1).

The interaction between endothelial cells (ECs) and VSMCs plays an important role in regulating vascular integrity and vascular homeostasis. ECs release vasoactive factors, such as prostacyclin, nitric oxide (NO), and endothelium-derived hyperpolarizing factor, participating in the regulation of vascular tone and arterial resistance (110, 118, 135). It has been demonstrated that both endothelial NO production and NO-mediated signaling in VSMCs are targets and effectors of the RhoA/Rho-kinase pathway. In ECs, the RhoA/Rho-kinase pathway negatively regulates NO production. In contrast, VSMCs are among the most plastic of all cells in their ability to respond to different stimuli. Growth factors secreted from VSMCs play an important role in mediating various cellular responses in vascular remodeling (10, 11, 30). Recent evidence suggests that many other stimuli that modulate VSMC functions, including reactive oxygen species (ROS), promote VSMC growth by inducing auto/paracrine growth mechanisms (127). Among those auto/paracrine factors, cyclophilin A (CyPA) has been identified as a ROS-related protein that is secreted from VSMCs by RhoA/Rho-kinase activation (95, 120) (Figs. 3 and 4). We have recently demonstrated that the extracellular CyPA decreases endothelial NO synthase (eNOS) expression (78), suggesting the indirect role of RhoA/Rho-kinase for the negative regulation of endothelial NO production. The initial investigations in our laboratory on the therapeutic importance of Rho-kinase were previously summarized (117). Since then, significant progress has been made in our knowledge on the therapeutic importance of Rho-kinase in cardiovascular medicine. In this article, we will briefly review the recent progress in the translational research on the

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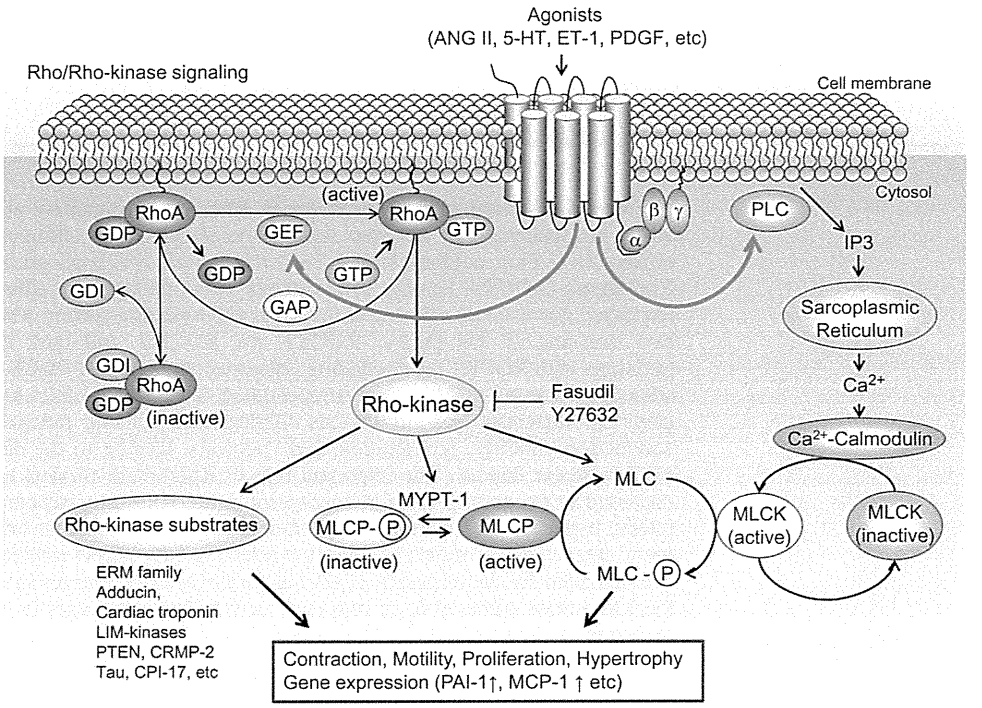

Fig. 1. The RhoA/Rho-kinase signaling pathway. Rho GTPases are small GTP-binding proteins that act as molecular switches and regulate many intracellular signaling pathways. RhoA cycles between an inactive GDP-bound and an active GTP-bound conformation, interacting with downstream targets, including Rho-kinase. The activity of RhoA is controlled by the guanine nucleotide exchange factors (GEFs) that catalyze exchange of GDP for GTP. GTPase-activating proteins (GAPs) stimulate the intrinsic GTPase activity and inactivate RhoA. Guanine nucleotide dissociation inhibitors (GDIs) block spontaneous RhoA activation. Various substrates of Rho-kinase have been identified, including myosin phosphatase target subunit 1 (MYPT-1), myosin light chain (MLC), ezrin/radixin/moesin (ERM) family, adducin, phosphatase and tensin homolog on chromosome 10 (PTEN), and LIM-kinases, etc. 5-HT, 5-hydroxytryptamine (serotonin); ET-1, endothelin-1; CRMP-2, collapsin response mediator protein 2; CPI-17, PKC-potentiated inhibitory protein 17; IP_3, inositol 1,4,5-trisphosphate; PAI-1, plasminogen activator inhibitor-1; MCP-1, monocyte chemoattractant protein-1.

therapeutic importance of the Rho-kinase pathway in cardiovascular medicine.

Role of Rho-Kinase in the Regulation of Cardiovascular Function

Rho-kinase is a serine/threonine kinase with a molecular mass of ~160 kDa. Two isoforms of Rho-kinase encoded by two different genes have been identified (Fig. 2) (58, 69, 75). In humans, ROCK1 (Rho-kinase-β) and ROCK2 (Rho-kinase-α) genes are located separately on chromosome 18 and chromosome 2, respectively. They are ubiquitously expressed in invertebrates and vertebrates with ROCK1, especially in circulating inflammatory cells and ROCK2 in VSMCs. ROCKs consist of three major domains, including a kinase domain in its NH_2-terminal domain, a coiled-coil domain that includes Rho-binding domain in its middle portion and a putative pleckstrin homology (PH) domain in its COOH-terminal domain (25) (Fig. 2). ROCKs activity is enhanced by binding of GTP-bound active form of RhoA (69) (Fig. 1). Rho-kinase inhibitors, fasudil (7) and Y-27632 (131), have been developed, and they inhibit Rho-kinase activity in a competitive manner with ATP at the Rho-binding site (19). It has been demonstrated that hydroxyfasudil, a major active metabolite of fasudil, exerts a more specific inhibitory effect on Rho-kinase (37, 116).

Although the regulation of ROCK expression has not been fully elucidated, some studies have reported changes in ROCK expression. Functional differences between ROCK1 and ROCK2 have been reported; ROCK1 is specifically cleaved by caspase-3, whereas ROCK2 is cleaved by granzyme B (15, 104). The small G protein RhoE specially binds to the NH_2-terminal region of ROCK1 at the kinase domain (Fig. 2), whereas the myosin phosphatase target subunit 1 binds specially to ROCK2 (56, 139). RhoE binding to ROCK1 inhibits its activity and prevents RhoA binding to Rho-binding domain (85). Both ROCK1 and ROCK2 mRNAs and proteins are upregulated by angiotensin II (ANG II) via ANG II type 1 receptor stimulation and by interleukin-1β (IL-1β) (38). A number of Rho-kinase substrates have been identified (64) (Fig. 1), and Rho-kinase-mediated substrate phosphorylation causes actin filament formation, organization, and cytoskeleton rearrangement (Fig. 1) (86). The NH_2-terminal regions, upstream of the kinase domains of ROCKs, may play a role in determining substrate specificity of the two Rho-kinase isoforms (Fig. 2) (86).

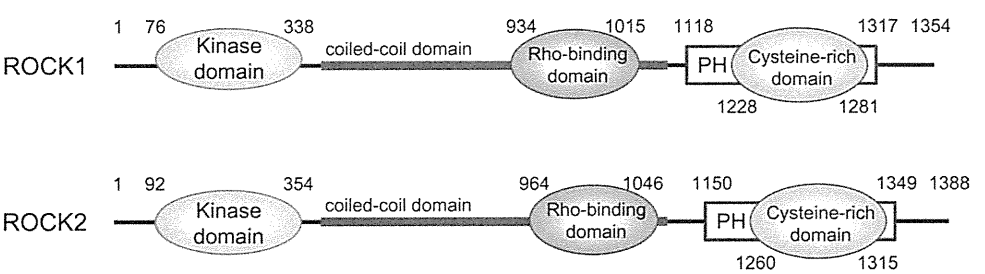

Fig. 2. Molecular structures of Rho-kinase isoforms. There are 2 isoforms of Rho-kinase, ROCK1 and ROCK2, which consist of 3 major domains, including a kinase domain in its NH_2-terminal domain, a coiled-coil domain with Rho-binding domain in its middle portion and a putative pleckstrin homology (PH) domain in its COOH-terminal domain. ROCK1 and ROCK2 are highly homologous with an overall amino acid sequence identity of 65%.

The majority of Rho-kinase substrates have been identified in vitro. Thus ROCK1- and ROCK2-deficient mice have been generated to further elucidate the functions of the ROCK isoforms (108, 130). Importantly, ROCK1-deficient mice showed the eyelids opened at birth (108), whereas ROCK2-deficient mice showed placental dysfunction and fetal death (61, 79, 130, 146). Thus the role of ROCK2, the main isoform in the cardiovascular system, remains to be fully elucidated in vivo. To address this point, we have recently developed VSMC-specific ROCK2-deficient mice and found the crucial role of ROCK2 in the development of hypoxia-induced pulmonary hypertension (107). These mutant mice revealed normal growth and body weight under physiological conditions. However, chronic hypoxia significantly increased ROCK2 expression and ROCK activity in lung tissues from littermates, and the development of right ventricular systolic pressure and right ventricular hypertrophy induced by chronic hypoxia in vivo was evident in littermates but was suppressed in the VSMC-specific ROCK2-deficient mice. In vitro, the growth and migration of VSMCs were significantly reduced in ROCK2-deficient VSMCs compared with control VSMCs.

Rho-Kinase and Vascular Function

Rho-kinase has been implicated in the pathogenesis of cardiovascular disease, in part by promoting VSMC proliferation (4, 8, 82). Changes in the vascular redox state are a common pathway involved in the pathogenesis of atherosclerosis, aortic aneurysms, and vascular stenosis. Vascular ROS formation can be stimulated by mechanical stretch, pressure, shear stress, environmental factors (e.g., hypoxia), and growth factors (e.g., ANG II) (32). Importantly, Rho-kinase is substantially involved in the vascular effects of various vasoactive factors, including ANG II (28, 33, 37, 123), thrombin (103, 134), platelet-derived growth factor (54), extracellular nucleotides (99), and urotensin (100) (Fig. 1). It has been previously shown that statins enhance eNOS mRNA by cholesterol-independent mechanisms involving the inhibition of Rho geranylgeranylation (124). We have also demonstrated that statins and Rho-kinase inhibitors completely block the secretion of CyPA from VSMCs (93, 120). Rho-kinase plays an important role in mediating various cellular functions, not only VSMC contraction (109, 111) but also actin cytoskeleton organization (5, 34), adhesion, and cytokinesis (117). Thus Rho-kinase plays a crucial role for the development of cardiovascular disease through ROS production, inflammation, EC damage, and VSMC contraction and proliferation. Rho-kinase inhibitors have excellent vasodilator activity and can induce vasodilation when vasoconstrictor tone is increased by a variety of mechanisms, including the activation of G-coupled receptors-enhanced calcium entry, ventilatory hypoxia, NOS inhibition, and other mechanisms (14, 20, 21, 137).

Rho-Kinase, Inflammation, and Oxidative Stress

Rho-kinase augments inflammation by inducing proinflammatory molecules, including IL-6 (83), monocyte chemoattractant protein-1 (28), macrophage migration inhibitory factor (MIF) (35, 36), and sphingosine-1-phosphate (136). In ECs, Rho-kinase downregulates eNOS (125) and substantially activates proinflammatory pathways, including an enhanced expression of adhesion molecules. The expression of Rho-kinase is accelerated by inflammatory stimuli, such as ANG II and IL-1β (38), and by a remnant lipoproteins in human coronary VSMCs (80). Rho-kinase also upregulates NAD(P)H oxidases (nox1, nox4, $gp91^{phox}$, and $p22^{phox}$) and augments ANG II-induced ROS production (37). Several growth factors are known to be secreted from VSMCs in response to oxidative stress. We have recently demonstrated that ROS activate a pathway containing vesicles that results in the secretion of CyPA (45, 120). Secreted extracellular CyPA stimulates ERK1/2, Akt, and JAK in VSMCs that contribute to ROS production and compose a vicious cycle for ROS augmentation (94, 97). CyPA is secreted from VSMCs via a highly regulated pathway that involves vesicle transport and plasma membrane binding (Fig. 3) (120). Rho GTPases, including RhoA, are key regulators in signaling pathways linked to actin cytoskeletal

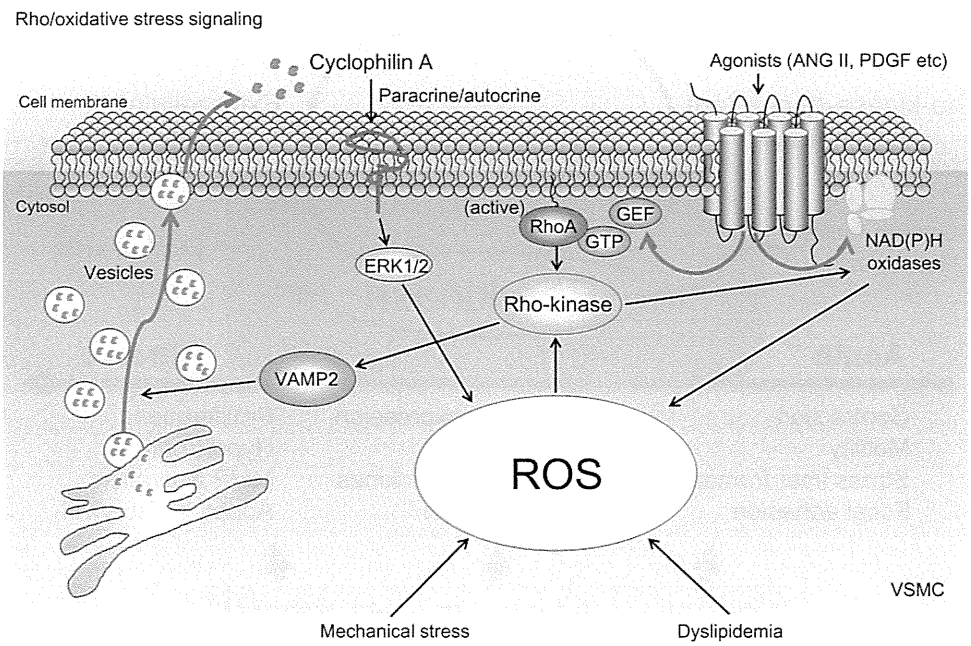

Fig. 3. Rho-kinase and reactive oxygen species (ROS) production. Intracellular signaling pathways for Rho-kinase activation, ROS production, and cyclophilin A (CyPA) secretion are closely linked through vesicle-associated membrane protein 2 (VAMP2) vesicle formation. Secreted extracellular CyPA activates ERK1/2, Akt, and JAK, promoting ROS production and Rho-kinase activation again. VSMC, vascular smooth muscle cell.

rearrangement (66). RhoA plays a central role in vesicular trafficking pathways by controlling the organization of actin cytoskeleton. It has been reported that active participation of Rho GTPases is required for secretion. We showed that the expression of dominant-negative mutants of RhoA inhibited ROS-induced CyPA secretion, suggesting that RhoA-dependent signaling events regulate CyPA secretion (120). Myosin II is involved in the secretory mechanisms as a motor for vesicle transport (77). ROCKs, downstream effectors of RhoA, mediate myosin II activation via phosphorylation and inactivation of myosin II light chain phosphatase (53). We have also recently demonstrated that Rho-kinase inhibitor reduced ROS-induced CyPA secretion (95, 120) (Fig. 4). These results suggest that myosin II-mediated vesicle transport is required for CyPA secretion from VSMCs. CyPA is transported to the plasma membrane and colocalized with vesicle-associated membrane protein 2 in response to ROS stimulation (Fig. 3).

We demonstrated that extracellular CyPA stimulates proinflammatory signals in ECs, including the expression of E-selectin and vascular cell adhesion molecule-1 (44). In addition to the effects on vascular cells, CyPA has been shown to be a direct chemoattractant for inflammatory cells (16, 52), promoting matrix metalloproteinases (MMPs) activation (138, 144). All of these roles of CyPA derive from the activation of Rho-kinase in the cardiovascular system (Fig. 4). Recently, we have demonstrated that the extracellular CyPA activates Rho-kinase in human pulmonary VSMCs from patients with pulmonary hypertension (91). Thus CyPA may be a key mediator of Rho-kinase that generates a vicious cycle for ROS augmentation, affecting ECs, VSMCs, and inflammatory cell functions (Fig. 4) (94, 97).

Rho-Kinase and Arteriosclerosis/Restenosis

As mentioned above, Rho-kinase plays a crucial role in the ROS augmentation and vascular inflammation. ROS have been implicated in the pathogenesis of neointima formation in part by promoting VSMC growth (8, 84) and by stimulating proinflammatory events (40, 59, 62, 87). Accumulating evidence indicates that Rho-kinase inhibitors have broad pharmacological properties (111, 115, 117). The beneficial effects of the long-term inhibition of Rho-kinase for the treatment of cardiovascular disease have been demonstrated in various animal models, such as coronary vasospasm, arteriosclerosis, restenosis, ischemia-reperfusion injury, hypertension, pulmonary hypertension, stroke, and cardiac hypertrophy/heart failure (111, 115, 117). Gene transfer of dominant-negative Rho-kinase reduced the neointimal formation in pigs (24). Long-term treatment with a Rho-kinase inhibitor suppressed neointima formation after vascular injury in vivo (101, 105), monocyte chemoattractant protein-1-induced vascular lesion formation (72), constrictive remodeling (113, 114), in-stent restenosis (70) and the development of cardiac allograft vasculopathy (36).

Arteriosclerosis is a slowly progressing inflammatory process of the arterial wall that involves the intima, media, and adventitia (111, 117). Accumulating evidence indicates that Rho-kinase-mediated pathway is substantially involved in EC dysfunction (125, 134), VSMC contraction (46), VSMC proliferation and migration in the media (143), and accumulation of inflammatory cells in the adventitia (72). Those Rho-kinase-mediated cellular responses led to the development of vascular disease. In fact, the mRNA expression of ROCKs is enhanced in the inflammatory and arteriosclerotic arterial lesions in animals (46) and humans (48). In the context of atherosclerosis, Rho-kinase should be regarded as a proinflammatory and proatherogenic molecule. Thus Rho-kinase is an important new therapeutic target for the treatment of atherosclerosis.

Rho-Kinase and Coronary Vasospasm

It has been demonstrated that Rho-kinase is substantially involved in the pathogenesis of coronary vasospasm. Coronary vasospasm plays an important role in variant angina, myocardial infarction, and sudden death (121). It was demonstrated that the serum level of cortisol, one of the important stress hormones,

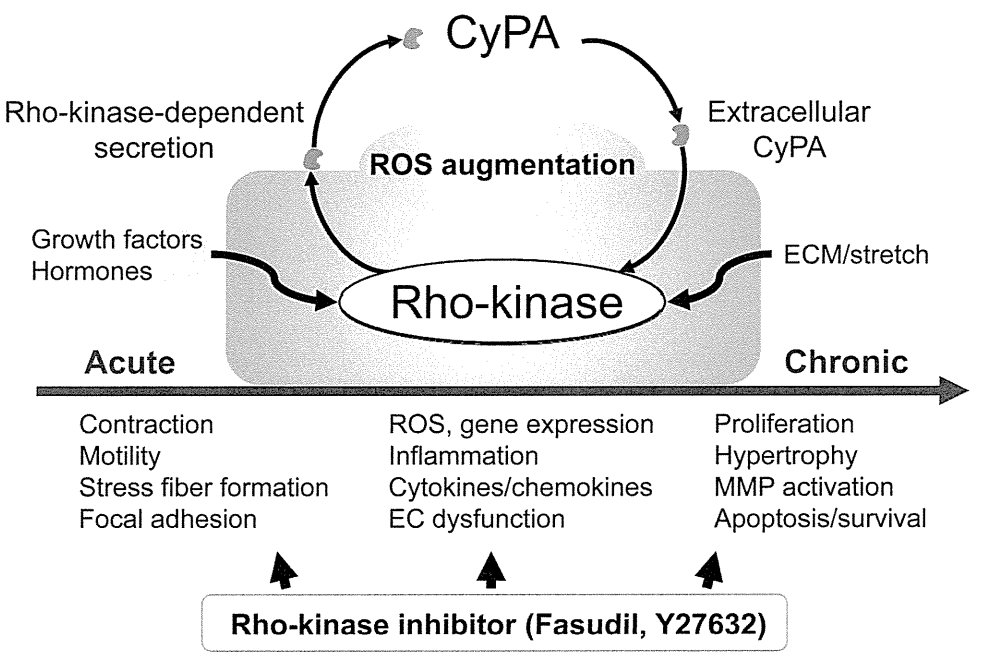

Fig. 4. Roles of the Rho-kinase/CyPA system. CyPA is secreted from VSMCs through a process requiring Rho-kinase activity and generates a vicious cycle for ROS augmentation. Extracellular CyPA induces Rho-kinase activation. CyPA and Rho-kinase augment ROS production and promote VSMC proliferation/migration, inflammation, matrix metalloproteinase (MMP) activation, endothelial dysfunction, endothelial nitric oxide synthase downregulation, and adhesion molecules expression. EC, endothelial cell.

causes coronary hyperreactivity through the activation of Rho-kinase in pigs in vivo (39). The activity and the expression of ROCKs are enhanced at the inflammatory/arteriosclerotic coronary lesions (47). Accumulating evidence indicates that Rho-kinase plays a crucial role in the pathogenesis of coronary vasospasm. Intracoronary administration of fasudil (49) and of hydroxyfasudil (116) inhibited coronary spasm in pigs in vivo (113). We have demonstrated that fasudil is effective in preventing coronary vasospasm and resultant myocardial ischemia in patients with vasospastic angina (68). Thus fasudil is useful for the treatment of ischemic coronary syndromes caused by coronary artery spasm. Fasudil is also effective in treating patients with microvascular angina (73). The clinical trials of the effects of fasudil in Japanese patients with stable effort angina demonstrated that the long-term oral treatment with the Rho-kinase inhibitor is effective in ameliorating exercise tolerance in those patients (112). We also have recently demonstrated that Rho-kinase activity in circulating neutrophils is a useful biomarker for the diagnosis and disease activity assessment in patients with vasospastic angina (51).

Rho-Kinase and Myocardial Ischemia-Reperfusion Injury

ROS production and Rho-kinase activation play a crucial role in myocardial damage after ischemia-reperfusion. We have demonstrated that pretreatment with fasudil before reperfusion prevents endothelial dysfunction and reduces the extent of myocardial infarction in dogs in vivo (142). The beneficial effect of fasudil has been also demonstrated in a rabbit model of myocardial ischemia induced by an intravenous administration of endothelin-1 (89), a canine model of pacing-induced myocardial ischemia (132), and a rat model of vasopressin-induced chronic myocardial ischemia (98).

Rho-Kinase and Aortic Aneurysms

Aortic aneurysm is formed by chronic inflammation of the aortic wall, associated with decreased medial VSMCs and progressive destruction of structural components, particularly the elastic lamina (63). Key mechanisms include VSMC senescence (57), oxidative stress (31, 127), increased local production of proinflammatory cytokines (13), and increased activities of MMPs that degrade extracellular matrix (119, 145). Chronic ANG II infusion into apolipoprotein E-deficient mice promotes aortic aneurysm formation (17, 18). In animal models of aortic aneurysm, the genetic and pharmacological inhibition of ROS production (29, 128) and MMPs (67, 129) suppressed the development of aneurysms. A chronic inhibition of Rho-kinase by fasudil has been reported to reduce ANG II-induced aortic aneurysm formation (140). The activation of Rho-kinase promotes CyPA secretion from VSMCs and extracellular CyPA stimulates VSMC migration, proliferation, and MMP activation (45, 60) (Fig. 4). Extracellular CyPA is also a chemoattractant for inflammatory cells (45, 50, 120) and further activates vascular Rho-kinase (Fig. 4). Recently, we have demonstrated that CyPA augments ANG II-induced ROS production, MMP activation, and inflammatory cell recruitment into the aortic VSMCs, contributing to the aortic aneurysm formation (95). CyPA is highly expressed in the aorta of patients with aortic aneurysm (95). Our findings suggest that Rho-kinase/CyPA signaling pathway is a novel therapeutic target for aortic aneurysm. All these data are a proof of concept that both Rho-kinase and CyPA play a crucial role in VSMCs by augmenting ROS generation. ANG II induces Rho-kinase activation and promotes CyPA secretion (Fig. 3). Secreted extracellular CyPA augments Rho-kinase activity in a synergistic manner (91) (Fig. 4). Secreted CyPA, acting as a proinflammatory cytokine, then synergistically augments ANG II-mediated ROS production, contributing to the onset of vascular inflammatory cell migration and aortic aneurysm formation (128, 141).

Rho-Kinase, Cardiac Hypertrophy, and Heart Failure

ANG II plays a key role in many physiological and pathological processes in cardiac cells, including cardiac hypertrophy (71). Understanding the molecular mechanisms for ANG II-induced myocardial disorders is important to develop new therapies for cardiac dysfunction (88). One important mechanism now recognized to be involved in ANG II-induced cardiac hypertrophy is ROS production (3, 76); however, the precise mechanism by which ROS cause myocardial hypertrophy and dysfunction still remains to be fully elucidated (106). It has been demonstrated that cardiac troponin is a substrate of Rho-kinase (133). Rho-kinase phosphorylates troponin and inhibits tension generation in cardiac myocytes. We have recently demonstrated that Rho-kinase inhibition with fasudil suppresses the development of cardiac hypertrophy and diastolic heart failure in Dahl salt-sensitive rats (26). Furthermore, our recent study provides strong mechanistic evidence of synergy between CyPA and Rho-kinase to increase ROS generation (95). Since ROS stimulates myocardial hypertrophy, matrix remodeling, and cellular dysfunction (126), Rho-kinase and CyPA may work together to promote ROS production and ANG II-induced cardiac hypertrophy (Fig. 4). In fact, CyPA was required for ANG II-mediated cardiac hypertrophy by directly potentiating ROS production, stimulating proliferation and migration of cardiac fibroblasts, and promoting cardiac myocyte hypertrophy in mice (96). In patients with heart failure, intra-arterial infusion of fasudil caused a preferential increase in forearm blood flow compared with that in control subjects, suggesting an involvement of Rho-kinase in the increased peripheral vascular resistance in patients with heart failure (55).

Rho-Kinase and Hypertension

Short-term administration of Y-27632, another Rho-kinase inhibitor, preferentially reduces systemic blood pressure in a dose-dependent manner in rat models of systemic hypertension, suggesting an involvement of Rho-kinase in the pathogenesis of hypertension (131). The expression of Rho-kinase was significantly increased in spontaneously hypertensive rats (74). Rho-kinase may be also involved in the central mechanisms of sympathetic nerve activity (41, 42).

Rho-Kinase and Pulmonary Hypertension

Pulmonary hypertension is associated with hypoxic exposure, endothelial dysfunction, VSMC hypercontraction and proliferation, enhanced ROS production, and inflammatory cell migration, for which Rho-kinase may also be substantially involved. Indeed, a long-term treatment with fasudil suppresses the development of monocrotaline-induced pulmonary hyper-

tension in rats (1) and of hypoxia-induced pulmonary hypertension in mice (2). Recently, we were able to obtain direct evidence for Rho-kinase activation in patients with pulmonary arterial hypertension (PAH) (22). Because the secretion of CyPA is regulated by Rho-kinase (95, 120), we tested the hypothesis that CyPA contributes to Rho-kinase activation and pulmonary vascular remodeling in PAH patients and noted enhanced CyPA expression on the α-smooth muscle actin-positive cells in the lung from patients with PAH (91). Additionally, statins and Rho-kinase inhibitor reduced the secretion of CyPA from VSMCs (95, 120) and pravastatin ameliorated hypoxia-induced pulmonary hypertension in mice (90, 92). Thus it is possible that the inhibition of CyPA secretion by statins (90) or Rho-kinase inhibitors (1, 43) may contribute to the therapeutic effects of these drugs on pulmonary hypertension. It has been reported that an intravenous injection of a number of chemically different Rho-kinase inhibitors reduces systemic and pulmonary arterial pressures under resting baseline tone conditions (9, 14, 20, 21). These data suggest that Rho-kinase plays a physiological role in the maintenance of baseline vasoconstrictor tone in the pulmonary and systemic vascular beds. Furthermore, intravenous infusion of fasudil significantly reduced pulmonary vascular resistance in patients with PAH, indicating an involvement of Rho-kinase in the pathogenesis of PAH in humans (27). Therefore, fasudil will decrease pulmonary arterial pressure in any situation in which vasoconstrictor tone is increased in the pulmonary vascular bed. A most important point in clinical settings is the chronic effects of the drugs (Fig. 4). The effects of long-acting fasudil in patients with PAH are now under investigation.

Conclusion

The identification of Rho-kinase as a mediator of cardiovascular diseases associated with inflammation and oxidative stress provides insight into the development of new therapies. Accumulating evidence suggests that Rho-kinase is substantially involved in the pathogenesis of a variety of cardiovascular diseases and that Rho-kinase inhibitors are useful for the treatment of those cardiovascular diseases. Clinical studies with fasudil have suggested that the Rho-kinase inhibitor may be useful for the treatment of a wide range of cardiovascular diseases, as mentioned in this article. Importantly, Rho-kinase inhibitors and statins significantly reduce CyPA secretion from VSMCs in animals. Blocking the malignant cycle that augments ROS production through CyPA secretion may be partially involved in the beneficial effect of Rho-kinase inhibitors. In conclusion, accumulating experimental and clinical evidence indicates that Rho-kinase is an important new target for the treatment of cardiovascular disease.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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