Chapter 2
Pathophysiology and Molecular Mechanisms of Coronary Artery Spasm

Kimio Satoh and Hiroaki Shimokawa

Abstract Rho-kinase plays a central role in the pathogenesis of coronary artery spasm caused by vascular smooth muscle cell (VSMC) hypercontraction. Rho-kinase belongs to the family of serine/threonine kinases and is an important downstream effector of the small GTP-binding protein RhoA. Two isoforms of Rho-kinase, ROCK1 and ROCK2, have different functions with ROCK1 for circulating inflammatory cells and ROCK2 for vascular smooth muscle cells. The RhoA/Rho-kinase pathway plays an important role in many cellular functions, including contraction, motility, proliferation, and apoptosis, leading to the development of cardiovascular diseases. In addition to vasospasm, important roles of Rho-kinase in vivo have been demonstrated in the pathogenesis of 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 animals and humans. Thus, the Rho-kinase pathway is an important new therapeutic target in vasospasm and other cardiovascular diseases.

Keywords Vasospasm · Rho-kinase · Cardiovascular disease · Oxidative stress · Small G proteins

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2.1 Development of Animal Models of Coronary Artery Spasm and Identification of Important Pathogenetic Roles of Rho-Kinase

Rho-kinase activation plays a central role in the pathogenesis of coronary artery spasm caused by vascular smooth muscle cell (VSMC) hypercontraction. In an animal model in pigs in vivo, we examined whether atherosclerotic coronary lesion, induced by a combination of balloon endothelium removal and high-cholesterol feeding, exhibits hyperresponsiveness to vasoconstrictor agents [1]. Importantly, intracoronary administration of serotonin induced coronary artery spasm at the atherosclerotic lesion, and there was a close topological correlation between the spastic site and atherosclerotic lesion (Fig. 2.1a, b) [1]. This is the first experimental evidence for the close relationship between coronary artery spasm and coronary atherosclerosis [1]. Next, we further examined whether chronic adventitial inflammation could cause vasospastic activity of the coronary artery without endothelium removal in pigs. Two weeks after the adventitial application of interleukin-1β (IL-1β), coronary angiography showed the development of mild stenotic lesion, where intracoronary administration of serotonin repeatedly caused coronary spasm (Fig. 2.1c) [2]. Histological examination showed adventitial accumulation of inflammatory cells, mild neointimal formation, and a marked reduction in vascular cross-sectional area (Fig. 2.1d) [2]. These results provided the first experimental evidence for the role of adventitial inflammation in the pathogenesis of coronary artery spasm. Delayed cerebral ischemia due to cerebral vasospasm remains a major cause of morbidity in patients with subarachnoid hemorrhage (SAH). It has been demonstrated that Rho-kinase is substantially involved in the pathogenesis of cerebral vasospasm after SAH [3]. Coronary artery spasm plays an important role in variant angina, myocardial infarction, and sudden cardiac death [4]. It was demonstrated that elevated serum level of cortisol, one of the important stress hormones, causes coronary hyperreactivity through activation of Rho-kinase in pigs in vivo [5]. The activity and the expression of ROCKs are enhanced at the inflammatory/arteriosclerotic coronary lesions [6]. Accumulating evidence indicates that Rho-kinase plays a crucial role in the pathogenesis of coronary artery spasm. Intracoronary administration of fasudil [7] and of hydroxyfasudil [8] inhibited coronary spasm in pigs in vivo [2]. We have demonstrated that fasudil is effective in preventing coronary spasm and resultant myocardial ischemia in patients with vasospastic angina [9]. 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 [10]. The clinical trials for 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 [11]. We also have recently demonstrated that Rho-kinase activity in circulating neutrophils is an useful biomarker for the diagnosis and disease activity assessment in patients with VSA [12].

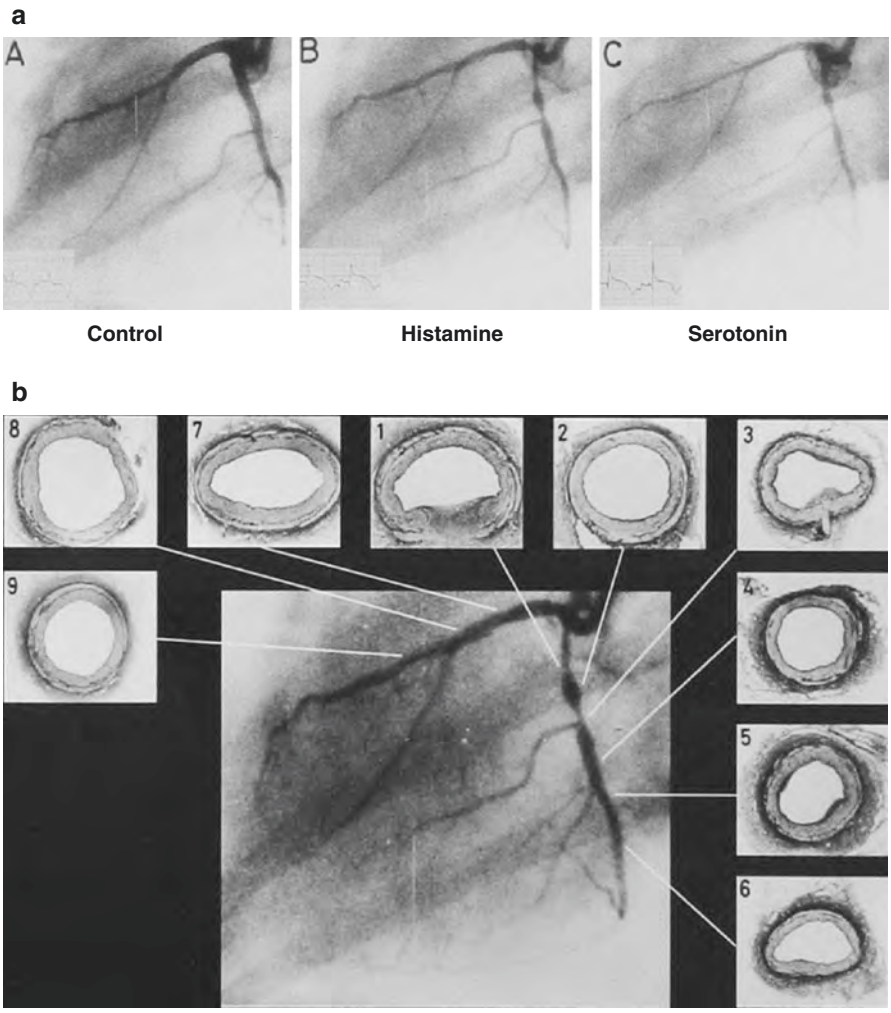

Fig. 2.1 Coronary artery spasm induced in two porcine models in vivo. (**a**, **b**) Coronary artery spasm was induced in atherosclerotic miniature pigs induced by balloon endothelial injury and high-cholesterol feeding (**a**), where topological correlation was noted between the spastic sites and the early atherosclerotic lesions (**b**). (**c**, **d**) Coronary artery spasm was induced in pigs with adventitial inflammation (**c**), where intimal thickening and negative remodeling were noted (**d**). (Reproduced from Shimokawa et al. [1, 2])

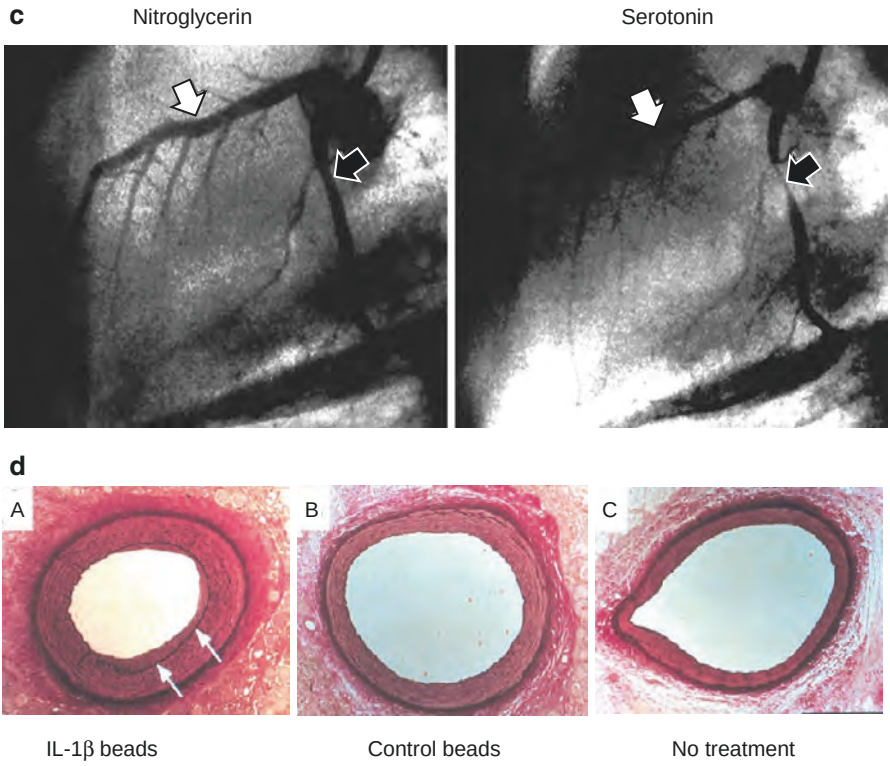

Fig. 2.1 (continued)

2.2 The Rho/Rho-Kinase System in Vascular Contraction

Rho-kinase belongs to the family of serine/threonine kinases and is an important downstream effector of the small GTP-binding protein RhoA. The Rho family of small G proteins comprises 20 members of ubiquitously expressed proteins in mammals, including RhoA, Rac1, and Cdc42 [13–15]. 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. 2.2) [16]. The activity of RhoA is controlled by the guanine nucleotide exchange factors (GEFs) that catalyze exchange of GDP for GTP [17]. In contrast, GTPase activating proteins (GAPs) stimulate the intrinsic GTPase activity and inactivate RhoA [18]. Additionally, it has been demonstrated that guanine nucleotide dissociation inhibitors (GDIs) block spontaneous RhoA activation (Fig. 2.2) [19].

In 1996, Rho-kinase (Rho-kinase α/ROCK 2/ROKα and Rho-kinase β/ROCK 1/ROKβ) was identified as the effector of Rho (Fig. 2.2) [20–22]. Phosphorylation of

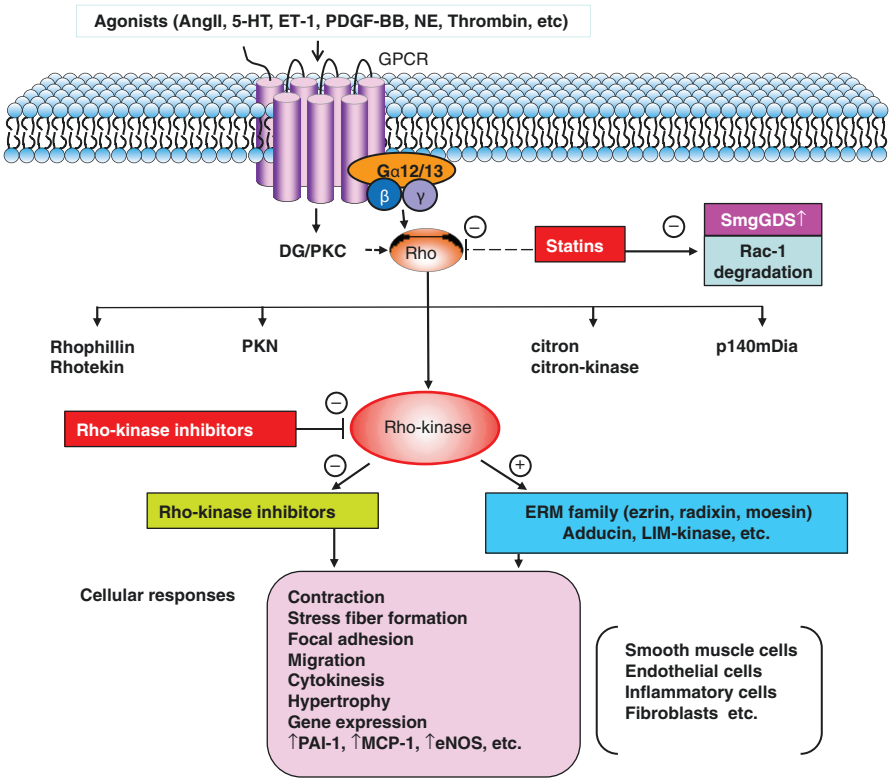

Fig. 2.2 The important roles of Rho/Rho-kinase pathway in the pathogenesis of cardiovascular diseases. The Rho/Rho-kinase pathway plays important roles in the pathogenesis of vasospastic disorders as well as atherosclerotic cardiovascular diseases in general. (Reproduced from Shimokawa et al. [27])

myosin light chain (MLC) is a key event in the regulation of VSMC contraction (Fig. 2.3). MLC is phosphorylated by Ca^{2+}-calmodulin-activated MLC kinase (MLCK) and dephosphorylated by MLC phosphatase (MLCP) (Fig. 2.3). Agonists bind to G-protein-coupled receptors and induce contraction by increasing both cytosolic Ca^{2+} concentration and Rho-kinase activity through mediating GEF. The substrates of Rho-kinase have been identified, including MLC, myosin-binding subunit (MBS) or myosin phosphatase target subunit (MYPT-1), ERM family, adducin, PTEN, and LIM-kinases (Figs. 2.2 and 2.3). Rho-kinase enhances MLC phosphorylation through inhibition of MBS of myosin phosphatase and mediate agonists-induced VSMC contraction (Fig. 2.3).

The interaction between endothelial cells (ECs) and VSMCs plays an important role in regulating vascular integrity and vascular homeostasis [23, 24]. ECs release vasoactive factors, such as prostacyclin, nitric oxide (NO) and endothelium-derived hyperpolarizing factor (EDHF), participating in the regulation of vascular tone and arterial resistance [1, 25–27]. It has been demonstrated that both endothelial NO

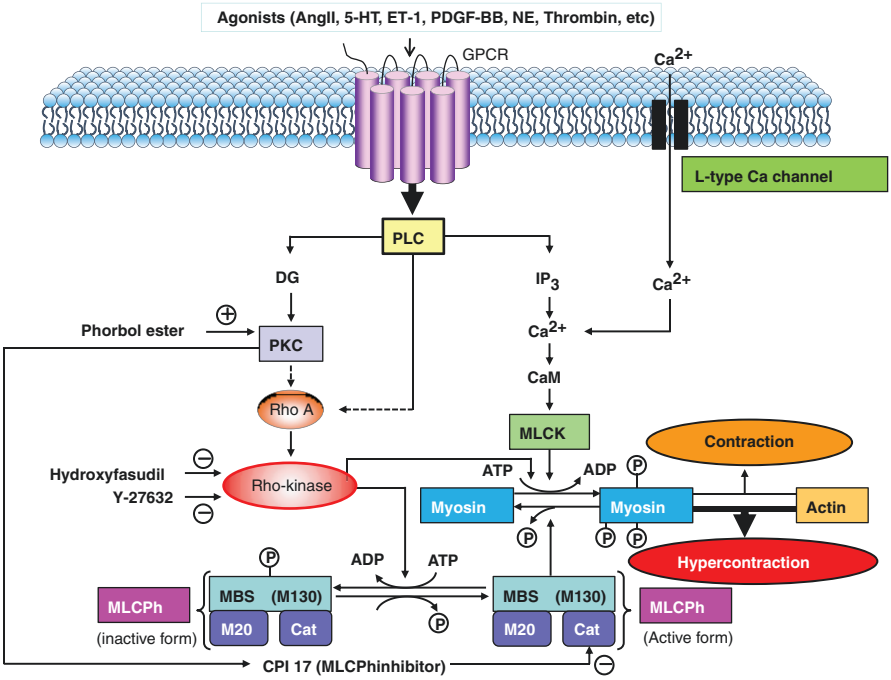

Fig. 2.3 Molecular mechanisms of vascular smooth muscle cells hypercontraction for coronary spasm. The central molecular mechanism of vascular smooth muscle cell hypercontraction for coronary spasm is Rho-kinase-mediated enhancement of myosin light chain phosphorylations through inhibition of myosin light chain phosphatase. (Reproduced from Shimokawa et al. [27])

production and NO-mediated signaling in VSMCs are targets and effectors of the RhoA/Rho-kinase pathway [23, 28]. In ECs, the RhoA/Rho-kinase pathway negatively regulates NO production [29]. In contrast, VSMCs are among the most plastic of all cells in their ability to respond to different stimuli [30–32]. The initial works in our laboratory on the therapeutic importance of Rho-kinase were previously summarized [23, 33]. Since then, a 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 therapeutic importance of the Rho-kinase pathway in cardiovascular medicine.

2.3 Substrates of Rho-Kinase

Rho-kinase is a serine/threonine kinase with a molecular weight of ~160 kDa. Two isoforms of Rho-kinase encoded by 2 different genes have been identified [34–36]. In humans, ROCK1 and ROCK2 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 3 major domains, including a kinase domain in its N-terminal domain, a coiled–coil domain that includes Rho-binding domain in its middle portion, and a putative pleckstrin homology (PH) domain in its C-terminal domain [13]. Rho-kinase activity is enhanced by binding of GTP-bound active form of RhoA [35] (Fig. 2.2). Rho-kinase inhibitors, fasudil [8] and Y-27632 [37], have been developed and they inhibit Rho-kinase activity in a competitive manner with ATP at the Rho-binding site [38]. It has been demonstrated that hydroxyfasudil, a major active metabolite of fasudil, exerts a more specific inhibitory effect on Rho-kinase [8, 39].

Although regulation of Rho-kinase expression has not been fully elucidated, some studies have reported changes in Rho-kinase expression. Functional differences between ROCK1 and ROCK2 have been reported; ROCK1 is specifically cleaved by caspase-3, whereas ROCK2 is cleaved by granzyme B [40, 41]. The small G-protein RhoE specially binds to the N-terminal region of ROCK1 at the kinase domain, whereas the MYPT1 binds specially ROCK2 [42, 43]. RhoE binding to ROCK1 inhibits its activity and prevents RhoA binding to the Rho-binding domain [44]. Both ROCK1 and ROCK2 mRNAs and proteins are upregulated by angiotensin II (AngII) via AT1 receptor stimulation and by interleukin-1β (IL-1β) [45]. A number of Rho-kinase substrates have been identified [46] (Fig. 2.2) and Rho-kinase-mediated substrate phosphorylation causes actin filament formation, organization, and cytoskeleton rearrangement (Fig. 2.2) [47]. The N-terminal regions, upstream of the kinase domains of Rho-kinase, may play a role in determining substrate specificity of the 2 isoforms [47].

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 [48, 49]. Importantly, ROCK1-deficient mice showed the eyelids opened at birth [49], whereas ROCK2-deficient mice placental dysfunction and fetal death [48, 50–52]. Thus, the role of ROCK2, the main isoform in the cardiovascular system, remained to be fully elucidated in vivo. In order 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 [30].

2.4 Rho-Kinase-Mediated Inflammation and Oxidative Stress

Rho-kinase augments inflammation by inducing pro-inflammatory molecules, including IL-6 [53], monocyte chemoattractant protein (MCP)-1 [54], macrophage migration inhibitory factor (MIF) [55, 56], and sphingosine-1-phosphate (S1P) [57]. In ECs, Rho-kinase downregulates eNOS [58] and substantially activates pro-inflammatory pathways including enhanced expression of adhesion molecules. The expression of Rho-kinase is accelerated by inflammatory stimuli, such as AngII and

IL-1β [45], and by remnant lipoproteins in human coronary VSMCs [59]. Rho-kinase also upregulates NAD(P)H oxidases and augments AngII-induced ROS production [39]. Several growth factors are known to be secreted from VSMC in response to oxidative stress. Rho GTPases including RhoA are key regulators in signaling pathways linked to actin cytoskeletal rearrangement [60]. RhoA plays a central role in vesicular trafficking pathways by controlling organization of actin cytoskeleton. It has been reported that active participation of Rho GTPases is required for secretion. Myosin II is involved in secretory mechanisms as a motor for vesicle transport [61]. Rho-kinase mediates myosin II activation via phosphorylation and inactivation of myosin II light chain phosphatase [20]. Thus, the Rho/Rho-kinase is important for the secretion of inflammatory cytokines and growth factors (Fig. 2.2).

2.5 Rho-Kinase in Vascular Function and Contraction

Rho-kinase has been implicated in the pathogenesis of cardiovascular disease, in part by promoting VSMC proliferation [62–64]. Changes in vascular redox state are a common pathway involved in the pathogenesis of vasospastic angina (VSA), atherosclerosis, aortic aneurysms, and vascular stenosis. Vascular ROS formation can be stimulated by mechanical stretch, pressure overload, shear stress, environmental factors (e.g. hypoxia), and growth factors (e.g. AngII) [65]. Importantly, Rho-kinase is substantially involved in the vascular effects of various vasoactive factors, including AngII [39, 54, 66, 67], thrombin [68, 69], platelet-derived growth factor [70], extracellular nucleotides [71], and urotensin [72] (Fig. 2.2). It has previously been shown that statins enhance eNOS mRNA by cholesterol-independent mechanisms involving inhibition of Rho geranylgeranylation [73]. Rho-kinase plays an important role in mediating various cellular functions, not only VSMC contraction [74, 75] but also actin cytoskeleton organization [76, 77], adhesion, and cytokinesis [33]. Thus, Rho-kinase plays a crucial role in the development of cardiovascular disease through ROS production, inflammation, EC damage, VSMC contraction and proliferation (Figs. 2.2 and 2.3).

2.6 Rho-Kinase in Arteriosclerosis

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 [64, 78] and by stimulating pro-inflammatory events [79–81]. Accumulating evidence indicates that Rho-kinase inhibitors have broad pharmacological properties [33, 75, 82]. The beneficial effects of long-term inhibition of Rho-kinase for the treatment of cardiovascular disease

have been demonstrated in various animal models, such as coronary artery spasm, arteriosclerosis, restenosis, ischemia/reperfusion injury, hypertension, pulmonary hypertension, stroke, and cardiac hypertrophy/heart failure [33, 75, 82]. Gene transfer of dominant-negative Rho-kinase reduced the neointimal formation in pigs [83]. Long-term treatment with a Rho-kinase inhibitor suppressed neointima formation after vascular injury in vivo [84, 85], MCP-1-induced vascular lesion formation [86], constrictive remodeling [87], in-stent restenosis [88], and the development of cardiac allograft vasculopathy [56] (Fig. 2.2).

Arteriosclerosis is a slowly progressing inflammatory process of the arterial wall that involves the intima, media, and adventitia [33, 75]. Accumulating evidence indicates that Rho-kinase-mediated pathway is substantially involved in EC dysfunction [58, 69], VSMC contraction [89], VSMC proliferation and migration in the media [90], and accumulation of inflammatory cells in the adventitia [86]. Those Rho-kinase-mediated cellular responses lead to the development of vascular disease. In fact, mRNA expression of ROCKs is enhanced at the inflammatory and arteriosclerotic arterial lesions in animals [89] and humans [91]. In the context of atherosclerosis, Rho-kinase should be regarded as a pro-inflammatory and pro-atherogenic molecule. Thus, Rho-kinase is an important new therapeutic target for the treatment of atherosclerosis (Fig. 2.2).

2.7 Rho-Kinase in Myocardial Ischemia and Heart Failure

ROS production and Rho-kinase activation play a crucial role in myocardial damage after ischemia/reperfusion. Consistently, we have demonstrated that pretreatment with fasudil before reperfusion prevents endothelial dysfunction and reduces myocardial infarction size in dogs in vivo [92]. The beneficial effect of fasudil has also been demonstrated in a rabbit model of myocardial ischemia induced by intravenous administration of endothelin-1 [93], a canine model of pacing-induced myocardial ischemia [94], and a rat model of vasopressin-induced chronic myocardial ischemia [95]. AngII plays a key role in many physiological and pathological processes in cardiac cells, including cardiac hypertrophy [96]. Understanding the molecular mechanisms for AngII-induced myocardial disorders is important to develop new therapies for cardiac dysfunction [97]. One important mechanism now recognized to be involved in AngII-induced cardiac hypertrophy is ROS production [98, 99], however, the precise mechanism by which ROS cause myocardial hypertrophy and dysfunction still remains to be fully elucidated [100]. It has been demonstrated that cardiac troponin is a substrate of Rho-kinase [101]. Rho-kinase phosphorylates troponin and inhibits tension generation in cardiomyocytes. We have demonstrated that Rho-kinase inhibition with fasudil suppresses the development of cardiac hypertrophy and diastolic heart failure in Dahl salt-sensitive rats [102]. In patients with heart failure, intra-arterial infusion of fasudil caused preferential increase in forearm blood flow as compared with control subjects, suggesting an involvement of

Rho-kinase in the increased peripheral vascular resistance in patients with heart failure [103].

2.8 Rho-Kinase in Hypertension and Pulmonary 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 [37]. The expression of Rho-kinase was significantly increased in spontaneously hypertensive rats (SHR) [104]. Local administration of a small amount of hydroxyfasudil into the nucleus tractus solitarii of the brain stem causes sustained decrease in heart rate and blood pressure in SHR but not in normotensive rats, suggesting that Rho-kinase is involved in the central mechanisms of sympathetic nerve activity [105]. Inhibition of Rho-kinase in the brain stem also augments baroreflex control of heart rate in rats [106]. Pulmonary hypertension (PH) 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, long-term treatment with fasudil suppresses the development of monocrotaline-induced PH in rats [107] and of hypoxia-induced PH in mice [108]. Recently, we were able to obtain direct evidence for Rho-kinase activation in patients with pulmonary arterial hypertension (PAH) [109]. 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 [110].

2.9 Conclusions

Accumulating evidence has indicated that Rho-kinase plays important roles in the pathogenesis of a wide range of cardiovascular diseases in general and coronary vasomotion abnormalities in particular. Additionally, Rho-kinase inhibitors are useful for the treatment of those cardiovascular diseases. In conclusion, accumulating experimental and clinical evidence indicates that Rho-kinase is an important new target for the treatment of VSA and cardiovascular diseases.

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