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Rho Kinase-mediated Coronary Arteriolar Constriction to Endothelin-1: Mechanistic Implications for Cardiac Syndrome X

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Rho kinase (ROCK) has been implicated in mediating diverse biological functions of various cells. Accumulating evidence has suggested that abnormal activation of ROCK contributes to the development of cardiovascular disease. The elevation of endothelin-1, the most potent endogenous vasoconstrictor, is also known to be associated with numerous cardiovascular disorders. Recent reports suggest that the constriction of the coronary microvasculature to endothelin-1 is mediated by the activation of ROCK signaling. In this article, we provide an overview on the ROCK and endothelin-1 vascular biology in relation to the control of vasomotor activity and speculate on their contributions to local myocardial ischemia and cardiac syndrome X. Although the underlying cellular and molecular mechanisms involved in exerting endothelin-1/ROCK regulation of coronary microvascular function remain elusive, the development of specific and selective ROCK inhibitors appears to have therapeutic potential in cardiovascular disease treatment.

Introduction

Rho-associated coiled-coil-forming protein kinase (Rho kinase or ROCK) is a serine/threonine kinase and one of the major downstream effectors of the small GTPase RhoA [1-3]. There is compelling evidence demonstrating that activation of RhoA/ROCK signaling regulates a plethora of cellular functions, including adhesion, motility, proliferation, contraction, actin cytoskeleton organization, inflammation, cytokinesis and gene expression, all of which are involved in the pathogenesis of cardiovascular diseases such as hypertension, restenosis, atherosclerosis, stroke and heart failure. There are several recent reviews focusing on the physiology and pathophysiology of ROCK signaling [4-6], but the discussion on how ROCK regulates vasomotor function is lacking. The involvement of ROCK in hypertension [7], atherosclerosis [8], coronary heart disease [9], stroke [10], pulmonary artery hypertension [11], and neurological disorders [12] has been well recognized. However, the current understanding of ROCK signaling mechanisms contributing to vascular tone, agonist-induced constriction and cardiovascular disease is almost exclusively based on the study of intact animals, conduit arteries or cultured vascular cells, and little is known about how ROCK regulates vascular function at the microcirculatory (i.e., resistance artery/arteriole) levels. Moreover, elevation of the circulatory level of endothelin-1 (ET-1), a potent vasoconstrictor, in association with patients with coronary events is well documented, but its pathophysiological role in vasomotor control remains elusive. In this article, we provide an overview of ROCK signaling in the vasculature in relation to ET-1 activation and coronary microvascular disorders associated with cardiac syndrome X. The ROCK as a therapeutic target and the use of its inhibitors for the treatment of coronary ischemia are discussed.

Molecular Biology of ROCK

ROCK and Smooth Muscle Contraction. Myosin light chain (MLC) phosphorylation is indispensable for the activation of contractile elements in smooth muscle contraction. The traditional signaling pathway for smooth muscle contraction considers that myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP) work coordinately to regulate MLC phosphorylation. Either activation of MLCK or reduction of MLCP activity will increase MLC phosphorylation, leading to smooth muscle contraction. Activation of ROCK was initially found to enhance smooth muscle contraction in a calcium-independent manner by inhibiting MLCP through phosphorylation of its myosin binding subunit. This pathway has been considered as a major mechanism for calcium sensitization in smooth muscle cells [2,13-15]. ROCK can also stoichiometrically phosphorylate MLC at serine-19 [16], the same molecular target for MLCK, and thereby facilitate actin activation [17] and stress fiber assembling [18]. It is speculated that MLC phosphorylation by ROCK

also contributes to the regulation of smooth muscle contraction in addition to MLCK activation. However, this direct phosphorylation pathway has not yet been demonstrated to be a physiologically significant mechanism in smooth muscle contraction [14].

ROCK Isoforms and Biological Function. Two isoforms of ROCK, ROCK1 and ROCK2, have been identified and are involved in various physiological and pathophysiological signaling pathways. ROCK1 is also known as p160-ROCK and ROKβ, and ROCK2 is also known as Rho-kinase and ROKα. Human ROCK1 and ROCK2 genes are located on chromosome 18 (18q11.1) and chromosome 2 (2p24), respectively [1,19]. ROCK1 and ROCK2 contain 1354 and 1388 amino acids, respectively, and each isoform contains a kinase domain, a regulatory domain, and a pleckrin-homology domain. The two isoforms are highly homologous, sharing 65% homology in amino acid sequence and 92% homology in their kinase domains. Animal studies have shown that ROCK1 is expressed preferentially in the lung, liver, spleen, kidney and testis, whereas ROCK2 is highly expressed in the brain and the heart [20-22]. At the molecular level, ROCK requires dimerization before activation [23-25] and a hydrophobic motif is essential for kinase domain dimerization and substrate phosphorylation [25]. ROCK translocates to the cell membrane upon stimulation, and is active only when it is bound to the membrane [26-28]. Alternatively, ROCK activation can be achieved by removal of its inhibitory domain [29,30] or by phosphorylation [31,32]. Recent studies indicate that phosphorylation of ROCK2 by Polo-like kinase-1 is involved in cytokinesis and cancer development [31,32]. There are some studies supporting the idea that the kinase activity of ROCK can be modulated through an auto-phosphorylation process after Rho binding [1,20,33-35]. ROCK phosphorylation status can be affected by TNF-α [36], osteopontin [35], thrombin [26] and ROCK inhibitors [25,32,33]. Although the activity of many kinases can be regulated by phosphorylation, how phosphorylated ROCK plays a role in the regulation of cardiovascular function is not completely understood.

Using genetic approaches, deletion of ROCK1 (i.e. ROCK1-/-) resulted in failure of eyelid and ventral body wall closure, leading to a neonatal phenotype with open eyes at birth and omphalocele [21]. Most ROCK1-/- mice died soon after birth because of cannibalization of the omphalocele by the mother. A few ROCK1-/- mice survived to adulthood with a phenotype that appeared normal and no apparent compensatory changes in ROCK2 levels [21]. By comparison, a ROCK2 knockout affected viability since more than 90% of the embryos died in utero during the late stage of pregnancy [22]. The surviving ROCK2-/- mice were born as runts and although fertile, they subsequently showed growth retardation, placental dysfunction and thrombus formation. [22]. Taken together, these studies show that there is no compensatory increase in one isoform in response to loss of the other isoform[22,37-40]. Interestingly, both ROCK1 and ROCK2 knockouts affect eyelid formation and function [21,38] with reduced intraocular pressure and corneal neovascularization [41].

In the cardiovascular system, ROCK1 appears to mediate leukocyte/macrophage recruitment and neointima formation following vascular injury [42]. Interestingly, ROCK1-deficient macrophages exhibit impaired ability to take up lipids and to develop into foam cells when challenged with modified low-density lipoprotein [40]. Transplant of bone marrow from ROCK1-deficient mice into low-density lipoprotein receptor knockout mice protected the animal from the development of atherosclerosis [40]. These studies highlight the significant role of ROCK1 in atherogenesis. Furthermore, mice deficient in ROCK1, but not ROCK2, are protected from the development of perivascular fibrosis [39,43,44] and cardiomyocyte apoptosis [29] in the hypertrophied heart secondary to pressure overload. Studies on the role of ROCK2 in the cardiovascular system are sparse. In the pulmonary circulation, ROCK2 protein expression and activity are correlated to the development of arteriolar hypertrophy in the rat model of pulmonary arterial hypertension [45]. A more recent study has shown that cardiac hypertrophy induced by systemic administration of angiotensin II is significantly attenuated in mice subjected to cardiomyocyte-specific deletion of ROCK2, suggesting the prominent role of ROCK2 in the adaptation of cardiomyocytes to pressure overload and/or to angiotensin II activation [46]. In a cellular study, using an siRNA approach, selective suppression of ROCK2 expression significantly attenuated vascular smooth muscle cell contraction by modulating myosin phosphatase activity [47]. These studies suggest a prominent and specific role for ROCK1 and ROCK2 in cardiovascular function and disease development. However, the role of ROCK in the regulation of vasomotor function at the level of the microcirculation remains unclear.

ROCK Expression and Microvascular Regulation

Vascular Expression. The term microcirculation is used to describe a group of small vessels embedded within a tissue responsible for the distribution and regulation of blood flow to/within the tissues. Based on size and function, the microcirculation consists of arterioles, capillaries, and venules. Arterioles (about 10-100 μm in diameter) play a key role in blood flow regulation by changing flow resistance (i.e., diameter) through relaxation and contraction of smooth muscle, and in blood pressure regulation if a systemic change in arteriolar tone is achieved. ROCKs are expressed in both arteries and arterioles in different organ systems. For example, ROCK2 expression in the brain is much higher in cerebral arterioles than in surrounding neurons [48]. Similar results have been shown in pulmonary [49,50] and retinal arterioles [51] with both ROCK1 and ROCK2 isoforms expressed in the arteriolar wall. Immunohistochemical studies of large conduit vessels such as femoral [52] and carotid arteries

[42] have shown that ROCK2 is mainly distributed in the medial layer. In ROCK2 deficient mice, where the disrupted ROCK2 gene is replaced by a β-Gal gene knock-in, the reporter gene product LacZ is strongly expressed in the medial layer of the umbilical artery [22], indicating a vascular smooth muscle-dominant ROCK2 expression pattern.

It is well documented that both ROCK1 and ROCK2 are strongly expressed in the heart [20,22,53,54], but their expression in the coronary vasculature is less studied. Confocal immunofluorescence results suggest that ROCK1 expression is stronger in rat coronary capillary endothelial cells than in their adjacent cardiomyocytes and is indiscriminately distributed to the luminal and abluminal sides of the capillary membrane [55]. The cell membranes of pericytes also strongly express ROCK1 [55]. Recently, we have reported in the porcine heart that ROCK1 and ROCK2 expression are about 2.6- and 4.7-fold higher, respectively, in coronary arterioles than in their adjacent cardiomyocytes and that ROCK2 expression in arterioles is 2-fold higher than ROCK1, with preferential medial-layer distribution [56]. This is consistent with the finding that ROCK2 plays a predominant role in the regulation of vascular smooth muscle contraction [47].

Vasomotor Regulation. The involvement of ROCK in pre-arteriolar and arteriolar regulation of vasomotor activity has been studied in cerebral [57-59], renal [60,61], retinal [62-64], and mesenteric [65,66] tissues. ROCK may be important in the regulation of renal afferent arteriolar resistance by activating the myogenic response [60,61]. Pharmacological studies also have shown that ROCK-mediated vasoconstriction is more apparent in the renal afferent, but not efferent, arterioles [61]. However, the underlying mechanism responsible for this heterogeneous vasomotor regulation in renal microvasculature is not currently understood. The influence of ROCK on cerebral vascular reactivity appears to be enhanced in animals exposed to cigarette smoke [59] or with type II diabetes [57]. This enhancement might be the result of endothelial dysfunction induced by those risk factors [57,59]. ROCK also participates in the vasomotor regulation of retinal arterioles from various species, including humans [62], and may be involved in the endothelial dysfunction and elevated oxidative stress elicited by C-reactive protein [63,64]. These studies provide direct evidence that ROCK plays an important role in regulating arteriolar function in health and disease. Activation of RhoA/ROCK signaling is also closely associated with hypertensive disease by modulating arteriolar tone and reactivity [7]. Interestingly, inhibition of ROCK appears to preserve endothelium-dependent dilation of coronary arterioles [67], reduce myocardial infarct size and exert cardioprotective effects on coronary ischemia-reperfusion injury [68]. It is worth noting that the potent vasoconstrictor ET-1 released during ischemia-reperfusion has been implicated as a detrimental peptide determining the outcome of myocardial injury [69,70]. It is likely that activation of ROCK signaling by ET-1 may contribute to the pathophysiology of coronary dysfunction and disease development as discussed below.

ROCK Mediates ET-1-induced Vasoconstriction

ET-1 is a vasoactive peptide that has been reported to elicit robust and sustained constriction of coronary arterioles in vivo [71-73] and in vitro [74-77], and has thus been implicated in modulating coronary microvascular tone [78]. Hitherto the mechanisms contributing to the ET-1-induced constriction in the coronary microcirculation have been limited to the ET-1 receptor activation and the contribution of Ca2+. Our recent study utilizing an in-vitro approach of isolated porcine coronary arterioles suggests that the ROCK signaling pathway plays a key role in regulating coronary arteriolar tone and ET-1 induced constriction [56]. The vasomotor influence of ET-1 along with its physiological/pathophysiological implications in relation to ROCK in coronary arterioles are delineated below.

ET-1 Synthesis and Receptor Activation. ET-1 is a 21-amino-acid peptide produced primarily by the vascular endothelial cells [79]. However, evidence has shown that ET-1 can also be synthesized from vascular smooth muscle cells [80], cardiomyocytes [81-83] and cardiac fibroblasts [84]. Cells initially produce the ET-1 precursor, preproendothelin-1, which is subsequently processed to yield a biologically inactive intermediate, big ET-1 [85]. This precursor peptide is proteolytically cleaved by endothelin-converting enzyme (ECE) to generate ET-1 [85,86], which is preferentially released from the abluminal side of the endothelium in the vasculature. Interestingly, recent evidence has shown that ECE and ET-1 are highly expressed in the neointimal smooth muscle layer of human coronary arteries from patients at early stages following percutaneous coronary intervention [87], suggesting the close relation of ET expression with vascular injury. In addition, ECE and ET-1 expression were detected in both the endothelial and smooth muscle layers of coronary arteries obtained from hearts with cardiomyopathy [88]. In addition to the vasculature, the ET-1 synthesis in cardiomyocytes is also increased in the failing heart [81,83,89]. Taken together, these studies support the possible contribution of vascular (i.e., endothelial and smooth muscle cells) and myocardial ET-1 to coronary vascular regulation in the disease state.

ET-1 exerts its function via binding to two distinct receptor subtypes, i.e., ETA and ETB. Activation of either ETA or ETB receptors on vascular smooth muscle leads to sustained vasoconstriction [79], whereas activation of endothelial ETB receptors promotes vasodilation [90,91]. Receptor binding and molecular studies have identified strong expression of ETA receptors in the vascular smooth muscle layer along with a minimal amount of ETB receptors in human small coronary arteries [92,93] and arterioles [74]. Clinical and experimental studies have shown that intraco-

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ronary administration of an ETA receptor antagonist in human subjects with angiographically normal coronary arteries [78], as well as in dogs and pigs, results in coronary vasodilation and increased coronary blood flow [76,94], suggesting a role for ET-1-mediated activation of ETA receptors in regulating coronary arteriolar tone. On the other hand, a tonic vasodilator influence of ETB receptors in the porcine coronary microcirculation has been suggested using a non-selective ETA/ETB antagonist [94]. These in-vivo receptor studies have been substantiated by in-vitro evidence that ET-1-induced constriction of isolated epicardial and endocardial coronary arterioles are blocked by ETA receptor blockade [74-77], whereas ETB receptor blockade enhances constriction of coronary arterioles [75,77]. In the isolated human coronary arterioles, ETB receptor blockade did not alter the vasoconstrictor response to ET-1 [74], but it is important to note that the endothelial function might have been diminished in these vessels since they were obtained from patients with various cardiovascular risk factors undergoing coronary bypass surgery. Collectively, these studies highlight the predominant role of ETA receptors in the vasoconstriction of coronary arterioles in response to ET-1.

ET-1 and Coronary Flow Regulation. Coronary blood flow is tightly coupled to the metabolic activity of the heart. Accumulating evidence supports the concept that the ET-1 level in the heart contributes to metabolic regulation of coronary arteriolar diameter and coronary blood flow. Administration of ET-1 receptor antagonists causes coronary vasodilation and increases coronary blood flow [76,78,94], suggesting the tonic regulation of coronary flow resistance by ET-1. A series of recent studies proposed that cardiomyocytes play a key role in not only the production of vasodilators but also the vasoconstrictor ET-1 [95-98]. This concept is based on evidence showing that α-adrenergic activation of cardiomyocytes causes ET-1-dependent vasoconstriction of coronary arterioles *in vivo* and *in vitro* [95,97]. Administration of an ETA receptor antagonist or an ECE inhibitor prevented the constriction of coronary arterioles in response to the α-adrenergic agonist phenylephrine [95]. These results suggest that the cardiomyocytes promote production and/or release of ET-1 since earlier in-vitro studies have shown that α-adrenergic agonists do not cause constriction of coronary arterioles in vitro [97,99-101]. Recent studies have expanded this concept and demonstrated that phenylephrine stimulation causes cardiomyocytes to release an unidentified factor, which then induces ET-1 release from coronary arterioles [96,98]. Collectively, evidence from these integrative approaches surmise that regulation of coronary microvascular tone depends on tight control of the production and/or release of vasodilators and vasoconstrictors by the cardiomyocytes. It has been proposed that the α-adrenergic-induced production of ET-1 in the heart can contribute to increased basal coronary resistance to prevent excess perfusion in the subepicardium and may promote subendocardial perfusion [96].

Interestingly, clinical studies have reported that abnormal ET-1 levels in the plasma are associated with microvascular angina [102-105] (discussed further in the following section). The elevated plasma levels of ET-1 in patients with essential hypertension [106] and the improved exercise-induced forearm blood flow in hypertensive subjects by ETA receptor blockade [107] imply altered regulation of metabolic flow control via ET-1 activation in the disease state. Moreover, a recent study has reported that ET-1 causes greater constriction of coronary arterioles in parallel with the elevated ETA receptor protein expression in patients with diabetes [108]. These observations highlight the importance of ET-1 activation in cardiovascular disease that may influence coronary microvascular tone under resting and increased metabolic states, which can potentially compromise myocardial perfusion and lead to impaired cardiac function.

ET-1 and ROCK Activation. In-vivo studies have shown that administration of the Ca2+ chelator ethylenediaminetetraacetic acid [71] or Ca2+ channel blockers [109] reverses the sustained constriction of canine coronary arterioles in response to ET-1, suggesting the involvement of extracellular Ca2+ mobilization through voltage-gated Ca2+ channels. However, the specific signaling pathways downstream from Ca2+ for vasomotor regulation remain to be determined. ROCK has been shown to be a possible signaling molecule modulating contractile myofilament sensitivity to Ca2+, thus regulating the force of smooth muscle contraction [110]. However, it is unclear whether ET-1 also utilizes this signaling molecule, in addition to Ca2+ mobilization, in the coronary arterioles to exert its contractile action. We recently found that specific pharmacological blockade of ROCK with H-1152 and Y-27632 significantly reversed ET-1-induced constriction as well as inhibited myogenic basal tone of porcine coronary arterioles [56]. These results indicate the pivotal role of the ROCK pathway in evoking coronary arteriolar constriction and maintaining resting vascular tone. Since accumulating evidence suggests that ROCK activation is closely associated with numerous vascular diseases [5], it is speculated that enhanced ET-1 release during coronary disease development may contribute not only to the increased basal tone (i.e., reduction in resting diameter) and enhanced vasoconstriction but also to the vascular pathology involved in structural changes (i.e., remodeling). Moreover, ET-1 induced constriction of human coronary arterioles has recently been shown to be sensitive to protein kinase C inhibitors [74]. Future studies are necessary to understand the possible link between ROCK, protein kinase C and cytosolic Ca2+ in mediating coronary arteriolar constriction to ET-1.

Cardiac Syndrome X, ET-1, and ROCK

Cardiac Syndrome X (CSX). CSX is a condition defined as the presence of angina-like chest pain and a positive response to

stress testing, but exhibiting normal or near-normal coronary arteries based on coronary angiography [111,112]. Accumulating evidence has emerged showing that coronary microvascular dysfunction is likely involved in CSX. However, rigorous definitions of the syndrome are elusive and the diagnostic criteria rely on "normal" coronary angiography. It is basically a diagnosis of exclusion because there is a lack of a standard way to precisely measure coronary microcirculatory function. This syndrome is predominant among females with onset of symptoms commonly between 40 and 50 years old, the age range of the menopausal transition [112]. Evidence obtained using the latest technology such as magnetic resonance, single-photon emission computed tomography and transthoracic color and pulsed-wave Doppler to study coronary blood flow supports the idea that coronary microvascular deficiency is a common factor among CSX patients [113-115]. In the following sections we will focus on the development of cardiac ischemia as a consequence of coronary microvascular dysregulation in association with elevated systemic/cardiac ET-1, endothelial dysfunction and ROCK activation as a possible mechanism for CSX.

ET-1 and Myocardial Ischemia. As discussed above, ET-1 is the most potent endogenous vasoconstrictor to date that has been identified. Coronary arterioles appear to be the most sensitive vasculature in response to ET-1 stimulation (via abluminal site), with a threshold in the picomolar range [56,116]. This is in contrast with skeletal muscle [117,118], cerebral [119-122] and mesenteric [65,66,123] arterioles, which have thresholds at subnanomolar to nanomolar concentrations. In addition, the EC50 value for coronary arterioles is 10- to 100-fold lower than that of other vascular beds. It is worth noting that the extent of ET-1-induced constriction in the coronary circulation is inversely related to vessel size [71,124]. Therefore, it is reasonable to speculate that increased ET-1 level in systemic circulation might cause vasoconstriction primarily in the coronary microcirculation, and thus reduce local blood flow leading to myocardial ischemia. Several clinical studies support this view when examining the correlation between ET-1 level and CSX [102-105,125]. Elevated levels of ET-1 have been shown to be closely associated with reduced coronary vasomotor responses in patients with chest pain and normal coronary arteriograms [102]. Moreover, an increased circulating level of ET-1 has been reported to be associated with adverse clinical outcomes among myocardial infarction patients, including reduced survival rate [126-129]. The level of ET-1 released in acute myocardial infarction has been suggested to be an independent predictor of myocardial no-reflow, left ventricular function, and long-term mortality [126]. The apparent close association between elevated ET-1 and myocardial ischemia supports the pathophysiological role of ET-1 in CSX.

Source of ET-1. The normal circulatory level of ET-1 in the peripheral blood is about 1-3 pM (2-8 pg/ml) [103-105,125,130], and studies have demonstrated that plasma ET-1 levels rise in CSX patients. If one closely examines the elevated level of ET-1 in CSX patients, it is noted that the ~2-fold increase in plasma ET-1 is rather trivial [103-105,125] in terms of the extent sufficient to elicit a significant change in coronary arteriolar tone. Interestingly, the arterial and coronary sinus ET-1 levels do not change significantly in CSX patients after pacing, despite a positive EKG change and lactate elevation being noted [102]. It appears that the myocardial ischemia is dissociated from the plasma level of ET-1. The population based Rancho Bernardo Study indicates that the ET-1 level is independently associated with coronary heart disease in female patients with median ET-1 values of 3.3 and 3.1 pg/ml for diseased and healthy groups, respectively [131]. This small amount of ET-1 elevation, albeit statistically significant, makes it difficult to extrapolate its contribution to coronary arteriolar constriction leading to myocardial ischemia. It should be noted that there is no report on the incidence of angina when a 3- to 12-fold increase in arterial plasma ET-1 is achieved by systemic infusion of ET-1 in healthy human volunteers, despite the apparent inhibition of cardiac function (e.g., bradycardia, decreased stroke volume and cardiac output, and reduced left and right ventricular diastolic filling) [132-137]. However, patients with cardiovascular risk were not tested in those studies and it is unclear whether predisposing clinical factors are requisite to cause/enhance the ET-1 response. Moreover it should be noted that the elevated plasma ET-1 might not lead to vasoconstriction since a preferential coronary arteriolar dilation was observed when ET-1 was infused intracoronary at the level of 2-20 pmol/min [71]. Reduction of coronary blood flow (i.e., arteriolar constriction) was not observed until a high concentration of ET-1, 375 pmol/min, was infused [138]. In contrast, topical application (abluminal administration) of a low dose of ET-1 to the coronary arterioles consistently evoked vasoconstriction [56,71]. Therefore, a simple increase in circulatory ET-1 per se is unlikely to be sufficient or important for the initiation of an acute coronary event. Instead, the vasoconstrictor ET-1 is likely to be derived from a vascular and/or extravascular source.

Myocardial ET-1. It has been suggested that ET-1 is a local vasopressor hormone, rather than a circulating one [139]. Coronary arteriolar expression of ET-1 is strongly associated with the regional environment, especially in relation to a1-adrenergic stimulation of the myocardium [98]. There are several reasons to believe that the local (myocardial and vascular) release of ET-1 plays a critical role in the regulation of coronary vascular function. ET-1 is mainly tissue-bound [140] and its level in the myocardial interstitium is about 3- to 6-fold higher than that in the plasma [141,142]. Significant compartmentalization of ET-1 exists within the human myocardium [141,142] and it can function in an autocrine and/or paracrine manner. Studies have shown that ET-1 is primarily released from endothelial cells toward the basal side, i.e., vascular smooth muscle, rather than into the apical lumen [137]. Moreover, ETA receptors are expressed on vascular smooth muscle and show strong affinity to

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ET-1. Secreted ET-1 from the endothelium can quickly interact with the underlying smooth muscle cells instead of being released to the circulation unless the "spillover" phenomenon from local tissue compartments occurs [142] or endothelial integrity is compromised [143-146].

As discussed above, cardiomyocytes are known to synthesize and secrete ET-1 [81] and it is this released ET-1 that appears to cause/facilitate coronary vasoconstriction induced by α-adrenergic stimulation [98], although the release of ET-1 from the coronary vasculature cannot be excluded [96]. Moreover, the interstitial concentration of ET-1 in porcine myocardium as measured by a microdialysis probe is about 20 pM [142], which is within the range of threshold for coronary arteriolar constriction in the same species [56]. Since coronary arterioles appear to be able to sense and respond readily to a small local elevation of abluminal ET-1, the focal vascular spasm or increase in vascular tone by ET-1 may contribute to local ischemia and CSX in patients with apparently normal coronary angiography. It is notable that coronary arteriolar constriction to ET-1 is generally suppressed by the functional endothelium [116]. Therefore, the elevated circulatory level of ET-1 may have a significant impact on the coronary microcirculation by promoting vasoconstriction if the endothelial function is compromised under disease states [83,89]. Although it is less studied, the release of ET-1 from fibroblasts for vasomotor regulation cannot be excluded. As the understanding of the physiology of ET-1 is extended, the pathological role of ET-1 appears ever more complex. The detrimental action of ET-1 as mentioned above cannot discount the growing evidence that ET-1 may contribute to tissue repair, such as inhibition of cardiomyocyte apoptosis via ETA receptor-mediated calcineurin signaling [147-150]. Although it remains unclear whether ET-1 is cardioprotective in an actual disease state, with these new findings in mind the association between ET-1 and coronary microvascular function, as well as overall clinical outcomes, should be interpreted with caution. Further clinical and translational studies are needed to address these issues.

CSX and ROCK Inhibitors in Clinical Therapy. Numerous studies have suggested the involvement of cardiovascular risk factors including age, gender, cholesterol level, blood pressure, smoking status and diabetes in coronary dysfunction [151]. These factors are reportedly associated with ROCK signaling, and possibly associated with CSX through abnormal activation of ROCK in the coronary microcirculation. Although the technology for assessment of ROCK activity in the coronary microvasculature in vivo is not available at the present time, ROCK inhibitors [152-161] have been employed to prevent coronary microvascular spasm and angina in patients with a microvascular type of myocardial ischemia.

Fasudil is the first ROCK inhibitor that has been approved for clinical application in Japan and China and is used for preventing secondary cerebral vasospasm and for protection from cerebral ischemia after stroke [162]. A phase II double-blind clinical trial in the U.S. has demonstrated the safety and efficacy of its use in patients with stable angina [154]. Fasudil treatment significantly elevates coronary oxygenation, reduces lactate production, and ameliorates pacing-induced myocardial ischemia in patients with effort angina without altering systemic hemodynamics [153]. Fasudil also increases the ischemic threshold of angina patients during exercise [154]. These clinical studies provide support that abnormal ROCK activation is likely responsible for the myocardial ischemia elicited by microvascular spasm. Interestingly, in some cases, Fasudil surpasses the effect of nitroglycerin in relieving intractable severe coronary spasm after coronary artery bypass surgery, which fails to respond to conventional vasodilators, including isosorbide dinitrate, diltiazem and nicorandil [156]. Fasudil is also used to treat other vascular diseases in clinical trials, including Raynaud's syndrome (Clinical Trial Identifier: NCT00498615), atherosclerosis (NCT00120718) and carotid stenosis (NCT00670202). Another ATP-competitive type of ROCK inhibitor (SAR407899), with higher specificity and potency than Fasudil, has been shown to lower blood pressure in various models of arterial hypertension in rodents [163] and is currently in a clinical trial for treating erectile dysfunction (NCT00914277). In addition to their inhibitory effect on vascular tone and ET-1-mediated vasoconstriction, ROCK inhibitors also have been shown to mitigate inflammatory insults [164,165], a biological action involved in almost all forms of cardiovascular disease. Although a significant interest has been generated in treating cardiovascular disease by ROCK inhibitors, it should be noted that most of these inhibitors are not at the optimal level of selectivity for different ROCK isoforms. Since ROCK isoforms play different roles in different biological processes, a selective ROCK isoform inhibitor may be desirable for specific disease treatments.

It is worth mentioning that statins, HMG-CoA reductase inhibitors for cholesterol reduction, are widely used in the clinic and have been shown in large-scale clinical studies to exert protective effects in various cardiovascular diseases [166,167] by improving endothelial function, attenuating vascular and myocardial remodeling, and decreasing oxidative stress and inflammation [168,169]. Blockade of HMG-CoA reductase also decreases generation of mevalonate and subsequent synthesis of isoprenoid intermediates, such as farnesyl pyrophosphate and geranylgeranyl pyrophosphate, which serve as sources for cholesterol production as well as lipid attachments for the post-translational modification of Rho [170,171]. Hence, statins are thought to exert the aforementioned pleiotropic effects beyond cholesterol lowering at least in part through inhibition of the RhoA/ROCK signaling pathway [169,172]. Recent studies have demonstrated that simvastatin dilates human [173] and porcine retinal arterioles [63] via an endothelium-dependent, nitric oxide-guanylyl cyclase pathway in part due to mevalonate-ROCK inhibition [63]. High-dose statin monotherapy has

been shown to reduce ROCK activity in leukocytes and to restore endothelium-dependent vasodilation in a recent clinical study [172]. The improvement of flow-mediated vasodilation and alleviation/elimination of stress-induced cardiac ischemia by statins in patients with CSX are also apparent [174,175]. The benefits from improved vascular endothelial function [172,174-176] and the ability to mitigate adverse responses associated with abnormal ROCK activation [172,177-179] support the therapeutic use of statins for CSX [174-176].

Conclusions

The RhoA/ROCK pathway plays an important role in mediating various cellular functions. Activation of different ROCK isoforms appears to exert different biological functions and to have varying roles in disease development. ROCK2 appears to be distributed predominantly in the medial layer of the vasculature and plays an important role in maintaining resting vascular tone. In the coronary microcirculation, ET-1 activates vascular smooth muscle ETA receptors to evoke vasoconstriction through ROCK2 activation involving Ca2+ mobilization and protein kinase C signaling. Elevation of the circulatory level of ET-1 is generally associated with coronary events and may contribute to myocardial ischemia. However, experimental studies suggest that local vascular/myocardial sources of ET-1 may be the culprit in eliciting coronary arteriolar constriction. Abnormal activation of the ET-1/ROCK2 signaling pathway may contribute to local myocardial ischemia and CSX. Although it may be disease-dependent, the factors triggering local release of ET-1 remain to be determined. Since abnormal RhoA/ROCK activation is closely associated with many cellular disorders, development of isoform-selective ROCK inhibitors will help future treatment of these disorders, including cardiovascular diseases.

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