Expression of Rho Kinase in Cardiovascular Diseases

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FULL PAPERS

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ABSTRACT

Rho/rho-kinase (ROCK) is a serine-threonine protein kinase, which is one of the first immediate downstream targets of RhoA and expressed ubiquitously. ROCK is involved in many cellular functions, such as, cell growth, migration, apoptosis via actin cytoskeleton organization, and gene expression. They regulate cell contraction through serine-threonine phosphorylation of adducin, ezrin-radixin-moesin proteins, LIM kinase, myosin light chain phosphatase, and Sodium-Hydrogen ion (Na/H) exchanger.

Recent studies have shown that ROCK may play a pivotal role in cardiovascular diseases such as vasospastic angina, ischemic stroke, heart failure and metabolic syndrome via its involvement in regulation of vascular tone, endothelial dysfunction, inflammation, and remodeling. Indeed, inhibition of ROCK by statins or other selective inhibitors leads to upregulation and activation of endothelial nitric oxide synthase (eNOS) and reduction of vascular inflammation and atherosclerosis. In this thesis, we hypothesized that ROCK activity is increased in a selected population of patients with acute coronary syndrome (ACS) and congestive heart failure (CHF) and that ROCK activity is able to predict long-term clinical outcomes in these two populations.

In the first part of this thesis, 176 ACS patients and 51 control subjects were studied. All The patients were enrolled between December 2007 and May 2009 and followed up till 15th March 2010 (mean: 15.4±7.6 months, from 0.5 month to 27.5 months). The main outcome measures were all cause mortality, readmission with ACS or congestive heart

failure (CHF) at 2 years from presentation. Altogether, there were 23 deaths (13.1%), 33 readmissions with ACS (18.8%) and 13 admissions with CHF (7.4%) within 2 years.

We also recruited a group of 178 patients with CHF. All the patients were enrolled between December 2007 and January 2009 and followed up until 1st February 2010 (mean: 14.4±7.2 months, from 0.5 month to 26 months) or until the occurrence of cardiac death. Forty-five patients died (25.3%) within 2 years follow up.

In both ACS and CHF study cohorts, all the clinical parameters were recorded and analyzed.

The main findings are:

ROCK activity was increased in ST elevation myocardial infarction (STEMI), non-STEMI (NSTEMI) and unstable angina (UA) groups when comparing with disease controls and healthy controls. On multivariate analysis, heart failure symptom on presentation, LDL-C level, and number of diseased coronary vessels were independent predictors of ROCK activity in ACS patients.

Furthermore, ACS patients with a high N-terminal pro-B-type natriuretic peptide (NT-proBNP) and a high ROCK activity on admission had a five-fold risk to experience a cardiovascular event, when compared to those with low NT-proBNP and low ROCK activity. In addition, patients with high NT-proBNP and high ROCK activity were also

more likely to die or experience a cardiovascular event at two years when comparing to those with high NT-proBNP and low ROCK activity.

The ROCK activity in CHF patients was significantly higher than that of the disease control and normal control groups. New York Heart Association (NYHA) class, low left ventricular ejection fraction (LVEF) and high creatinine were independent predictors of the baseline ROCK activity in CHF. In terms of long-term heart failure mortality, ROCK activity was not an independent predictor. However, combining ROCK activity and NT-proBNP provided an incremental value in predicting long-term heart failure mortality over NT-proBNP alone.

Thus, increased ROCK activity is likely involved in cardiovascular diseases and further studies would be helpful to elucidate the potential role of ROCK activity inhibition in cardiovascular diseases.

摘要

Rho/rho-kinase (ROCK) 是第一个被发现的 RhoA 的直接的下游基因。ROCK 参与许多细胞功能，它通过调节肌动蛋白细胞骨架功能，参与细胞生长、迁移和凋亡，并且参与基因表达。ROCK 通过丝氨酸苏氨酸磷酸化的内收蛋白，膜-细胞骨架连接蛋白，LIM 激酶，肌球蛋白轻链磷酸酶，钠-氢离子交换器调节细胞收缩。

最近的研究显示，ROCK 可能在心血管疾病中扮演重要角色，它通过调节血管紧张程度，内皮功能，炎症发生和血管重建，参与了痉挛性心绞痛，缺血性中风和心衰和代谢综合征。事实上，他汀类药物和某些选择性抑制剂可抑制 ROCK 功能，导致上调并且激活 eNOS，并且可降低血管炎症和动脉粥样硬化。在这篇论文里，我们主要目的是研究 ROCK 活性在冠心病和充血性心衰里面的表达是否升高，ROCK 活性是否可以作为一个远期预测指标来预测心血管事件的发生。

本论文的第一部分研究的疾病是冠心病。该部分研究纳入 176 例冠心病患者为病例组，对照组有 51 例。所有冠心病患者的入组时间在 2007 年 12 月和 2009 年 5 月之间，在 2010 年 3 月 15 日为随访截止时间（平均随访时间为 15.4±7.6 个月，从 0.5 个月到 27.5 个月不等）。以所有原因造成的死亡、因为冠心病或者心衰而需再次入院为主要的检测结果。通过 2 年跟踪随访，其中有 23 例死亡（13.1%）、33 例因冠心病再入院（18.8%）和 13 例因心衰再入院（7.4%）。

同时，本研究还纳入 178 例充血性心衰患者。所有患者的入组之间从 2007 年 12 月至 2009 年 1 月，随访截止时间为 2010 年 2 月 1 日（其平均随访时间为 14.4±7.2 个月，从 0.5 个月至 26 个月不等）。主要检测结果为心源性死亡。45 例病人在近 2 年的追踪时间内死亡，死亡率为 25.3%。

在两项研究中，记录并分析所有病人的临床指标。

以下是本研究的主要发现：

在 ST 段抬高性冠心病组、非 ST 段抬高性冠心病组和非稳定性心绞痛组，ROCK 活性都比正常对照组或者疾病对照组高。多变量分析结果显示，入院时心衰、LDL 水平和冠脉阻塞支数为影响 ROCK 活性在冠心病患者中高低的独立的影响因素。

入院时，同时具有高 NT-proBNP 水平和高 ROCK 活性的关心病患者，其远期心血管发生事件的发生率是低 NT-proBNP 水平和低 ROCK 活性的患者的 5 倍。另外，高 NT-proBNP 水平和高 ROCK 活性的冠心病患者的心血管事件发生率比高 NT-proBNP 水平和低 ROCK 活性的患者要高。

另外，在充血性心衰患者中，ROCK 活性显著的比健康对照组和疾病对照组高。美国纽约心脏病学会(NYHA)心功能分级、低心脏射血分数和高肌酸酐是 ROCK 活性的独立的影响因素。

虽然在充血性心衰患者中，ROCK 活性对远期死亡率不是一个独立的预测因子，但是结合 NT-proBNP 和 ROCK 活性可以很好的预测远期死亡率。其预测效果比单用 NT-proBNP 要好。

总之，改研究显示 ROCK 活性在在心血管疾病中显著地升高，进一步的研究将着眼于 ROCK 抑制剂在心血管疾病中的应用。

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LIST OF ABBREVIATIONS

ACE	angiotensin-converting enzyme
AF	atrial fibrillation
ANF	atrial natriuretic factor
Ang II	angiotensin II
BMI	body mass index
BW	body weight
BH	body height
CCM	cerebral cavernous malformations
CCS	canadian cardiovascular society
CHF	congestive heart failure
CIHD	coronary ischemic heart disease
CK-MB	creatine kinase-MB
CPK	creatine phosphokinase
CRP	c-reactive protein
cTnT	cardiac troponin T
CVA	cardio vascular accidents
DM	diabetes mellitus
DBP	diastolic blood pressure
EC	endothelial cells
EC-1	endothelin-1
ECG	electrocardiogram

eGFR	estimated Glomerular Filtration Rate
GRACE	Global Registry of Acute Coronary Events
HR	heart rate
HDL-C	high-density lipoprotein cholesterol
HMG-CoA	3 hydroxy-3-methylglutaryl coenzyme A
I/R	ischemia/reperfusion
LDL-C	low-density lipoprotein cholesterol
LVEF	left ventricular ejection fraction
MURC	muscle-restricted coiled-coil protein
NT-proBNP	N-terminal pro-B-type natriuretic peptide
NO	nitric oxide
NSTEMI	non-ST-segment elevation myocardial infarction
PCI	percutaneous coronary intervention
PAI-1	plasminogen activator inhibitor-1
ROCK	rho kinase
RFP	restrictive filling pattern
SBP	systolic blood pressure
SLE	systemic lupus erythematosus
SRF	serum response factor
STEMI	ST-segment elevation myocardial infarction
SMC	smooth muscle cells
TEMED	tetramethylethylenediamine

TG	triglycerides
TC	total cholesterol
VEGF	vascular endothelial growth factor-A
VSMC	vascular smooth muscle cells
WBC	white blood cell
5-HT	5-hydroxytryptamine

CHAPTER 1

INTRODUCTION

CHAPTER 1. INTRODUCTION

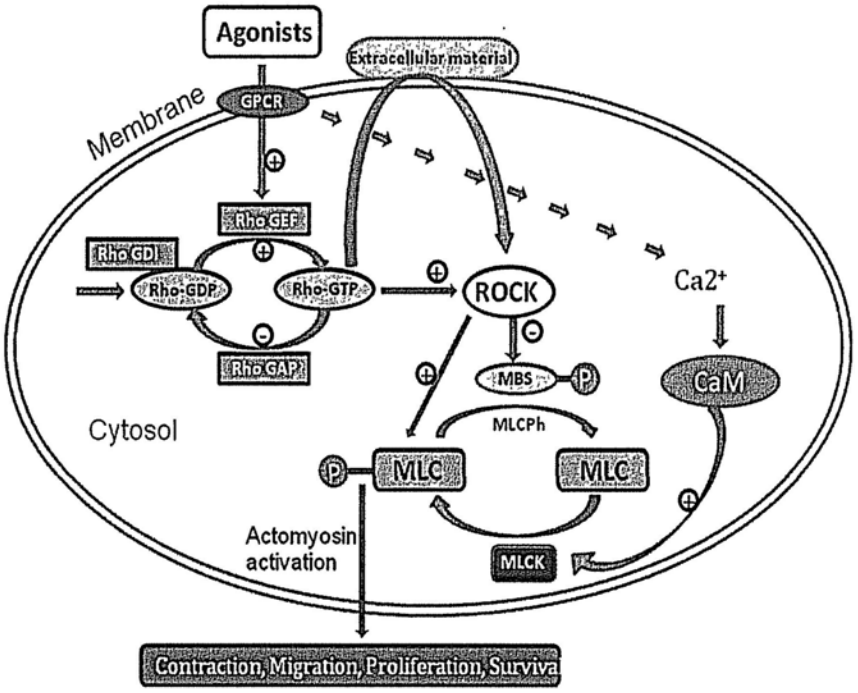
The Rho kinases (ROCKs) were initially discovered as downstream targets of the small GTP-binding protein Rho. Because ROCKs mediate various important cellular functions such as cell shape, motility, secretion, proliferation, and gene expression, it is likely that this pathway will intersect with other signaling pathways known to contribute to cardiovascular disease. Indeed, ROCKs have already been implicated in the regulation of vascular tone, proliferation, inflammation, and oxidative stress. However, it is not entirely clear how ROCKs are regulated and whether ROCK1 and ROCK2 mediate different cellular functions. Clinically, inhibition of ROCK pathway is believed to contribute to some of the cardiovascular benefits of statin therapy that are independent of lipid lowering, i.e., pleiotropic effects. To inhibit what extent of ROCK activity in patients on statin therapy is unknown, though it might have important clinical implications. Indeed, ROCK inhibitors are currently under development by several pharmaceutical companies because evidence from animal studies suggested the potential involvement of ROCK in systemic and pulmonary hypertension, vascular inflammation as well as atherosclerosis.

1.1 Introduction of Rho/Rho kinase (ROCK)

Families of small G-proteins such as Rho, Ras, Rab, Sarl/Arf and Ran are substantially involved in intracellular signaling.[1] The Rho family members, including Rho, Rac and Cdc42, regulate both cytoskeletal reorganization and gene expression. The effector domains of RhoA, RhoB and RhoC (collectively referred to here as Rho) have the same

amino acid sequence, and these G proteins seem to have similar intracellular targets. As with other Rho GTPases, Rho acts as a molecular switch, cycling between an active GTP-bound state and an inactive GDP-bound state.[2] The exchange between the active and the inactive states is regulated by several regulatory proteins such as guanine dissociation inhibitor (GDI), guanine-nucleotide-exchange factor (GEF) and GTPase activating protein (GAP). In unstimulated cells, Rho resides predominantly in the cytosol in its inactive GDP-bound form, and Rho GDI binds to Rho- GDP and extracts it from the membrane to the cytosol. When cells are stimulated with certain agonists, Rho-GDP is converted to Rho-GTP through the action of Rho GEF. Then, Rho-GTP is targeted to the cell membrane, where it interacts with its specific targets (Figure 1.1).

Figure 1.1 The Rho GDP –Rho GTP signaling pathway from membrane to the cytosol.



Rho GAP inactivates Rho by dephosphorylating GTP to GDP. The best-characterized downstream effector of Rho is Rho-kinase (ROCK), which mediates various cellular functions.[2] ROCK was identified in the mid-1990s as one of the downstream effectors of Rho.[1, 2] There are two isoforms of ROCK (ROCK1 and ROCK2).[1, 2] The genes expressing human ROCK1 and ROCK2 are located on chromosome 18 (18q11.1) and chromosome 2 (2p24), respectively.[3, 4] ROCK1 and ROCK2 are highly homologous, sharing 65% homology in amino acid sequence and 92% homology in their kinase domains. Both isoforms are ubiquitously expressed in human. ROCK2 is highly expressed in the brain and the heart, whereas ROCK1 is expressed preferentially in the lung, liver, spleen, kidney and testis.[5]

1.2 Downstream targets of ROCK

The substrates of ROCK have been identified, including the myosin-binding subunit (MBS) of myosin light-chain phosphatase (MLCPh), the ERM (ezrin, radixin, moesin) family, adducin, intermediate filaments (e.g. vimentin and desmin), the Na^+–H^+ exchanger and LIM-kinase.[1] In addition to ROCK, several other proteins have been identified as effectors of Rho, including protein kinase N, rhophilin, rhotekin, citron, p140mDia and citron kinase.[1] Although studies showed the effectors' involvement of actin cytoskeleton organization[6] and neuronal differentiation,[7] their roles remain to be examined.

The ROCKs phosphorylate various targets and mediate a broad range of cellular responses in response to GTP-bound RhoA activation by lysophosphatidic acid (LPA) or sphingosine-1 phosphate (S1P) (figure 1.1). Functions such as actin cytoskeleton assembly and cell contractility are controlled by ROCK phosphorylation of various downstream target proteins, including myosin binding subunit (MBS) on myosin light chain phosphatase (MLCP), myosin light chain (MLC), Ezrin/radixin/moesin (ERM) proteins, protein LIM kinase, adducin and troponin. ROCKs can undergo autophosphorylation, suggesting that the function of ROCKs may be autoregulated.[8]

MBS on MLCP is an important downstream target protein of ROCKs. ROCKs can phosphorylate MBS at Thr697, Ser854, and Thr855.[9] Phosphorylation of Thr697 or Thr855 attenuates MLCP activity and, in some instances, the dissociation of MLCP from myosin.[10, 11] Phosphorylation of MBS on MLCP leads to the phosphorylation of MLC and subsequent contraction of VSMCs.[12] ROCKs can also directly phosphorylate Ser19 of MLC (the same residue that is phosphorylated by MLC kinase).[13] Thus, Rho-kinase could regulate the phosphorylation of MLC and contraction via the two processes: inactivation of myosin phosphatase and direct MLC phosphorylation. However, recent studies have shown that it is the Rho-kinase-mediated inactivation of myosin phosphatase, rather than the direct phosphorylation of MLC by Rho-kinase, that could be responsible for Ca^{2+}-sensitization of smooth muscle.[14,15] In addition, this pathway is involved in cytokinesis, cell morphology, cell migration and invasion via the phosphorylation of various proteins.[2]

Furthermore, ROCKs phosphorylation of ERM proteins, which serve as crosslinkers between actin filaments and membrane proteins at the cell surface leads to disruption of the head-to-tail association of ERM proteins and actin cytoskeletal reorganization.[16] However, it is not known whether phosphorylation of MBS on MLCP or ERM proteins is specific to ROCK isoforms.

Adducin is another downstream target of ROCKs.[17, 18] Adducin is a membrane skeletal protein that associates with and promotes the association of spectrin with F-actin. Adducin is localized at cell-cell contact sites and is thought to participate in the assembly of the spectrinactin network by capping the fast-growing ends of actin filaments and recruiting spectrin to the filament ends.[17] The phosphorylation of α-adducin by ROCKs enhances the binding activity of α -adducin to F-actin, thereby increasing VSMC contractile response.

Troponin is a muscle protein that, together with tropomyosin, forms a regulatory protein complex controlling the interaction of actin and myosin; when combined with calcium ions, it permits muscular contraction. Recently, ROCK activation has been shown to alter cardiac myofilaments response to calcium by a mechanism involving troponin phosphorylation.[19] ROCK-mediated troponin phosphorylation induces depression of the tension generation and the ATPase rate of cardiac myofilaments. However, the

physiological or pathophysiological role of ROCK-dependent troponin phosphorylation remains to be determined.

1.3 Upstream targets of ROCK

MicroRNAs (miRs) participate in many cardiac pathophysiological processes, including ischemia/reperfusion(I/R)-induced cardiac injury. For example, miR-494 targets both proapoptotic (PTEN, ROCK1, and CAMKIIδ) and anti-apoptotic (FGFR2 and LIF) proteins.[20] The ultimate consequence is activation of the Akt pathway, which leading to cardioprotective effects against I/R-induced injury. Currently, it is well accepted that inhibition of ROCKs has a major impact on improving myocardial survival after an I/R episode. Overexpression of miR-494 reduced the level of ROCK1 which might work in concert to activate Akt signaling, a critical survival pathway in the myocardium. However, it may be experimentally complicated to ROCK1 for elucidating mechanisms underlying the protective effects of miR-494 against I/R. Further studies are needed to ascertain the causal relationship between miRs-modulated targets and their functional consequences in association with ROCK.

1.4 ROCK at cellular level

1.4.1 ROCK and vascular smooth muscle cells

The Rho/ROCK pathway is a major regulator of vascular smooth muscle cell (VSMC) contraction as well as in controlling migration, proliferation, differentiation, apoptosis,

survival and gene transcription.[21] The major mediator of smooth muscle contraction is phosphorylation/dephosphorylation of myosin light chain (MLC).[22] MLC is phosphorylated by the Ca^{2+}-calmodulin–activated MLC kinase and dephosphorylated by the Ca^{2+}-independent MLC phosphatase. On the other way, stimulation of tyrosine kinase and G-protein–coupled receptors leads to activation of Rho, the direct upstream activator of ROCKs, via recruitment and activation of RhoGEF.[23] ROCKs are important effectors of Rho in regulating the actin cytoskeleton. This is due predominantly to the phosphorylation and inhibition of MLCP by ROCK, which increases MLC phosphorylation and cellular contraction, by facilitating interaction of myosin with F-actin (Figure 1). Thus, ROCKs regulate cell polarity and migration predominantly through enhancing actomyosin contraction and focal adhesions. This ROCK-mediated contraction can occur independently of intracellular Ca^{2+} changes and is known as Ca^{2+} sensitization.[24] It has recently been shown that in normotensive arteries ROCK mediated calcium sensitization is involved in maintenance of basal myogenic tone and, to a lesser extent, in pressure-dependent development.[25] Pressure-dependent activation of this enzyme is enhanced in hypertension with greater contribution to the maintenance of myogenic tone.[25]

1.4.2 ROCK and endothelial cells

In endothelial cells, the activation of endothelial nitric oxide synthase (eNOS) is fund to the maintenance of normal vascular function. Endothelium- derived NO plays an important role in the regulation of vascular tone, inhibition of platelet aggregation,

suppression of smooth muscle cell proliferation, and prevention of leukocyte recruitment to the vessel wall. Increased bioavailability of NO is, in part, dependent on increased expression and activity of eNOS as well as on decreased inactivation of NO by reactive oxygen species. Rho/ROCK activation plays a role in oxidized LDL-induced endothelial cell contractility [26] and in the modulation of endothelial fibrinolytic activity.[27] The Rho/ROCK signaling pathway is involved in the regulation of endothelial barrier function, inflammation and trans-endothelial leukocyte migration, platelet activation, thrombosis, gene expression and oxidative stress.[28] Studies have found that ROCK activation decreases the expression of eNOS by reducing eNOS mRNA stability.[29] Direct inhibition of Rho by C3 transferase, inhibition by ROCK inhibitors or overexpression of dominant-negative ROCK prevent downregulation of eNOS expression and eNOS mRNA stability.[29] ROCKs also negatively regulate eNOS function via a tonic inhibitory effect on PI3-kinase/Akt pathway[30] and possibly by stimulation of arginase activity, which leading the damage of vessel function.[31]

In addition to lipid-lowering effects, statins exert 'pleiotropic' effects such as inhibition of vascular inflammation and atherosclerosis through Rho GTPases (Rho, Rac1 and Ras) on the vascular wall.[32] Statins upregulate and activate eNOS expression through inhibition of Rho geranylgeranylation.[32] Clinically, statins inhibit Rho geranylgeranylation at lipid lowering doses [33] and statin-induced improvement in endothelial-dependent vasomotion is mediated, in part, by inhibition of ROCK activity.[34]

1.4.3 ROCK and adventitia

Rho signaling is reportedly essential for vascular endothelial growth factor-A (VEGF) dependent in vivo angiogenesis and in vitro capillary formation.[35] In a porcine model of coronary arteriosclerosis, treatment with fasudil, a selective ROCK inhibitor, markedly reduces macrophage accumulation in the adventitia and migration into the media.[36] Indeed, the importance of adventitial accumulation of inflammatory cells has been suggested for the pathogenesis of arteriosclerosis[37] and acute coronary syndrome in general and for that of coronary lesion formation after coronary intervention[38] in particular. Furthermore, ROCK inhibition has been shown to suppress in-stent neointimal formation in porcine coronary arteries by reducing vascular inflammation, enhanced apoptosis and decreased collagen deposition.[39] Long-term treatment with fasudil or adenovirus-mediated transfer of dominant-negative ROCK have been shown to induce regression of both constrictive remodeling and coronary vasospastic activity in a porcine model with adventitial inflammation.[40]

Because the established chemical inhibitors of ROCK (Y-27632, H-1152, Wf-536, fasudil, and hydroxyfasudil) cannot distinguish between ROCK1 and ROCK2 and exhibit some nonspecific inhibition of other protein kinases; therefore, it is not clear how ROCK1 and ROCK2 differ in their regulation and function. Bryan B.A., et al. used small interfering RNA-mediated gene knockdown and knockout mouse models to indicate that VEGF-driven angiogenesis is largely mediated through ROCK2.[41] These data demonstrated that Rho/ROCK signaling is an important mediator in a number of angiogenic processes, including EC migration, survival, and cell permeability, and

suggest that Rho/ROCK inhibition may prove useful for the treatment of angiogenesis-related disorders. Future experiments utilizing gene targeting of ROCK1 and ROCK2 are necessary to provide more direct evidence for the role of these proteins in regulating biological processes in the endothelium. Given the recent advances in cancer treatment with antiangiogenic drugs and the effective clinical use of hydroxyl-fasudil in Japan (with relatively low side effects) for cardiovascular disease and cerebral vasospasm, a better understanding of the differences between ROCK1 and ROCK2 may lead to the development of more specificand effective therapeutics.

1.4.4 ROCK and cardiomyocytes

Mitochondrial-RNA of both ROCK1 and ROCK2 are expressed in the developing heart.[42] ROCK inhibition can block migration of pre-cardiac mesoderm and cardiac tube fusion in cultured chick and mouse embryos.[42] In cultured mouse embryos, inhibition of ROCK decreases cell proliferation but does not lead to programmed cell death, suggesting that ROCK regulates cardiomyocyte division but not apoptosis during heart development.[43] This effect is mediated through the regulation of cell-cycle protein expression, cyclin D3, CDK6 and p27Kip1 in cardiomyocytes.[43]

ROCK signaling is also involved in cell proliferation and migration during endocardial cushion development, myocardial hypertrophy and cardiac fibrosis.[44] Recent studies in ROCK1-deficient mice indicate that ROCK1 is required for the development of cardiac fibrosis, not hypertrophy.[45] Hattori et al reported that ROCK is substantially involved in

the pathogenesis of left-ventricular remodeling after myocardial infarction associated with upregulation of pro-inflammatory cytokines, indicating a potential therapeutic target for preventing post-infarct heart failure.[46]

1.4.5 ROCK and the Central Nervous System

In the central nervous system (CNS), Rho GTPases are essential regulators of neuronal growth cone motility, axonal migration, and dendritic spine morphogenesis. In addition, numerous recent studies have identified Rho GTPases as central players in the molecular pathways that determine neuronal survival and death. Interestingly, individual Rho family members have been shown to play either a pro-death or pro-survival role in the nervous system depending on both the type of neuron and the particular neurodegenerative insult involved.[47]

Rho/ROCK pathway is involved in neurite outgrowth and up-regulation of eNOS with ROCK inhibition has potential neuroprotective effect.[32] Thus, ROCK regulation may be potential important targets for axonal repair strategies in CNS injury, ischemic stroke and Alzheimer's disease.[48] In patient with stroke, Fasudil has been shown to increase cerebral blood flow in both ischemic and non-ischemic areas, reduces cerebral infarct size and improves neurological deficits.[32] In a study by Yamashita K., et al., demonstrated an increase in neural (axonal) ROCK expression and activity in ischemic brain tissue, which was reduced by fasudil and inhibited OGD-induced PC12 cell death

and glutamate-induced neurotoxicity.[49] In another study, Yamaguchi et al., demonstrated that ROCK regulated the TNF-α-induced IL-6 release suggesting that ROCK inhibition may alleviate cerebral vasospasms.[50] Tumor necrosis factor (TNF)-α is widely recognized as a prototypic proinflammatory cytokine. In the central nervous system (CNS), TNF-α is released from neurons, astrocytes and microglia.[51] Therefore, Rho-kinase inhibitor may be considered to a new clinical candidate for the treatment of CNS disorders in addition to cerebral vasospasms.

1.5 ROCK in the mechanisms underlying cardiovascular diseases

1.5.1 Inflammation and Atherosclerosis

Atherosclerosis is characterized by progressive inflammation, accumulation of lipids and fibrosis in the arterial wall[52] and mRNA expression of ROCK has been shown to be enhanced in atherosclerotic lesions in both animal[53] and human studies.[54] Within fibroblasts and inflammatory cells, ROCK upregulates pro-inflammatory molecules including activator protein-1, NF-κB,[55] NAD(P)H,[44] IL-6,[56] monocyte chemoattractant protein (MCP)-1,[57] macrophage migration inhibitory factor (MIF),[46] and interferon (IFN)-γ,[46] all of which are involved in the pathogenesis of atherosclerosis. The expression of ROCK itself is accelerated by inflammatory stimuli such as nicotine,[58] angiotensin II and IL-1β, which are mediated through PKC/NF-κB pathway,[59] and is negatively modulated by estrogen. Interestingly, ROCK is positively involved in its own expression.[59] Since Rho-kinase itself mediates the intracellular signaling initiated by

those inflammatory agonists,[60] it is also conceivable that Rho-kinase and inflammatory agonists form a vicious cycle in which they activate each other and promote the process of arteriosclerosis.

Cumulating evidence indicate that ROCK-mediated pathway is involved at all stages of the inflammatory process. Activated ROCK down-regulates eNOS,[29] whereas ROCK inhibition by hydroxylfasudil rapidly increases endothelial eNOS activity.[61] Nitric oxide itself antagonizes the vasoconstrictor effect of ROCK through activation of myosin phosphatase.[62] ROCK activation leads to endothelial hyperpermeability and hence enhances atherosclerosis.[63] Furthermore, ROCK1 plays a key role in macrophage chemotaxis, cholesterol uptake and foam cell formation, all of which are hallmark events in the pathogenesis of atherosclerosis.[64] ROCK1 also mediates neointimal proliferation via recruitment of circulating leukocytes and infiltration of inflammatory cells into the vessel wall.[65] In a low-density lipoprotein (LCL) receptor knockout mice model, activation of the transcription factor NF-κB via Rho/ROCK pathway was enhanced after a high-fat cholate-free diet while inhibition of ROCK significantly was associated with suppression of early atherosclerotic plaque development.[66]

The anti-inflammatory properties of statins (hydroxy-3-methylglutaryl coenzyme-A reductase inhibitors) may be mediated, at least in part, by inhibition of Rho proteins isoprenylation which prevent membrane attachment of Rho proteins and the subsequent activation of downstream effectors such as ROCK.[67] Nohira et al. were the first to

demonstrate statins inhibited ROCKs activity and improved endothelial function in patients with stable atherosclerotisis.[68] Intriguingly, higher dose statin (40mg/d) monotherapy appears to have greater inhibitory effects on ROCK activity and potentially more improvement in endothelial function than lower-dose statin plus ezetimibe (10/10mg/d).[34] It might because ezetimibe, either alone or in comnination with statin, is less effective in improving endothelial function than high dose statin montheropy.[69]

1.5.2 Thrombosis

ROCK up-regulates thrombogenic molecules (eg, platelet-activating factor [plasminogen activator inhibitor (PAI)]-1[70] and tissue factor[39]) and fibrogenic molecules (e.g. transforming growth factor-β1[46] and Bcl-2[39]). Thrombin induced tissue factor expression is regulated positively by Rho/Rho-kinase and p38 MAP kinase. Inhibition of Rho/ROCK can prevent endothelial tissue factor induction through and activation of Akt.[71] Thrombin is also an independent potent stimulator of endothelial permeability via ROCK pathway.[72]

1.5.3 Coronary and Cerebral Vasospasm

There is increasing evidence indicating that ROCK is substantially involved in the pathogenesis of coronary and cerebral vasospasm. Coronary spasm is caused by hyper-contraction of coronary smooth muscle triggered by an increase of intracellular Ca^{2+} in the presence of an increased Ca^{2+} sensitivity.[73] It has been shown that enhanced

Rho/ROCK activity reduces endothelial NO activity resulting in increased Ca^{2+} sensitivity. ROCKs modulate sensitivity of contractile apparatus to intracellular Ca^{2+} by increasing MLC phosphorylation either directly via phoshprylation or inhibition of myosin binding subunit of MLCP.[60] Studies have shown that Rho/ROCK activity is enhanced in the rat arteries with hypertension and vasospasm.[1, 74] Shimokawa and colleagues[1] developed the swine model of coronary spasm and have shown that ROCK activity is enhanced in smooth muscles of the coronary artery involved in spasm, as well as in human arteries.[54]

Intracoronary administration of fasudil[75] and hydroxyfasudil[76] markedly inhibit coronary spasm induced by IL-1β in a porcine model via suppression of enhanced MLC phosphorylations at the spastic coronary segments.[76] The effect of fasudil has also been demonstrated in other animal coronary spasm models including a rabbit myocardial ischemic model induced by intravenous administration of endothelin-1,[77] a dog model of pacing-induced myocardial ischemia in the presence of coronary stenosis,[78] and a rat model of vasopressin-induced chronic myocardial ischemia.[79] Similar results were found in a cerebral vasospasm dog model using Y27632.[80]

ROCKs are negative upstream regulators of eNOS expression and activation eNOS in endothelial cells.[29] In the coronary artery, decrease in endothelial NO activity causes increase in Rho/ROCK activity.[1, 24] Rho/ROCK inhibition leads to stabilization of eNOS

mRNA and increases expression of eNOS.[81] In cerebral vasospasm, increased contractility of vascular muscle, which may, at least in part, be the result of increased rhoA/rho-kinase activity, is now thought to contribute to the severe arterial narrowing observed in cerebral vasospasm after subarachnoid hemorrhage.[82]

Recently, inhibition of ROCKs activity by Fasudil has been shown to relieve coronary spasm induced by acetylcholine in humans.[83] Fasudil has also been shown to inhibit coronary vasospasm in patients with unstable angina pectoris[84] and for the treatment of cerebral vasospasm after SAH.[85] The vasodilatory effect of fasudil is more potent than that of nitroglycerin[86] and has been shown to further dilate segments of vasospastic coronary artery which has already been pre-treated with nitroglycerin.[87] These findings support the potential of fasudil as a novel therapeutic agent for coronary and cerebral vasospasm.

1.5.4 Ischemia and reperfusion injury

Rho-kinase activation is involved in the pathogenesis of myocardial ischemia and reperfusion injury. Recently, Rho-ROCK-flippase singnaling pathway was proved involved in maintaining asymmetrical membrane phospholipid distribution in cardiomyocytes after myocardial infarction.[88] Pre-treatment with fasudil before reperfusion has been shown to prevent endothelial dysfunction and suppresses the development of myocardial infarction in dogs in vivo.[89] Up-regulated expression of Rho

in ischemic myocardium and subsequent activation of ROCKs occurs specifically during early reperfusion.[90] The mechanisms by which fasudil acts against cerebral infarction are thought to involve an increase in regional cerebral blood flow and a decrease in the inflammatory response.[32] Increasing regional cerebral blood flow via inhibition of ROCK is believed to be achieved through vascular dilatation,[32] hemodilution and decrease in blood viscosity.[91] The latest findings indicate that the vascular dilatation induced by fasudil is achieved not only via an inhibition of smooth muscle contraction, but also through an up-regulation of eNOS.[32] The decrease in inflammatory responses induced by ROCK blockade is achieved by an inhibition of neutrophil migration.[92] These observations suggest that ROCK may play an important role in mediating the inflammatory response to ischemic and reperfusion injury.[30] Y-27632 has also been shown to reduce myocardial infarct size in rats via the same mechanism confirming that ROCK activation may be deleterious through suppression of the reperfusion injury salvage kinase pathway.[90] Furthermore, Y-27632 has been shown to enhance post-ischemia cardiac function, reduce myocardial apoptosis and decrease accumulation of neutrophils in the heart following ischemic and reperfusion injury in mice.[93] These findings suggest that ROCK activation occurs during early reperfusion and inhibition of ROCK at this critical period may limit infarct size.

1.6 ROCK and cardiovascular diseases

1.6.1 ROCK and hypertension

Systemic hypertension is characterized by a high arterial pressure level resulting from increased vascular resistance attributable to both enhanced contractility and arterial wall remodeling. The ROCK inhibitors Y-27632 and fasudil normalize arterial pressure in animal models of hypertension indicating the importance of the ROCK signaling pathway in the vascular hyper-reactivity associated with hypertension.[74] Direct measurements of the amount of active GTP-bound RhoA in arteries from several animal models of hypertension have suggested that an increased RhoA activity is responsible for enhanced ROCK activation in this pathological context.[94] In addition, long-term blockade of ROCK suppresses vascular lesion formation such as medial hypertrophy and perivascular fibrosis in small coronary artery from spontaneously hypertensive rats.[95] Similar observations have been made in the rat model of hypertension induced by chronic inhibition of NO synthesis.[96] In both models, the activity of RhoA/ROCK pathway is found increasing. Because the inhibition of AII type 1 receptor prevents the upregulation of RhoA/ROCK activity, it has been suggested that an increasein AII activity participates in the activation ROCK in hypertensive rats.[96] This is in agreement with another report showing that in vivo, long-term infusion of AII increases the activity of RhoA and ROCK increases medial thickness and promotes perivascular fibrosis in coronary arteries.[44] Both AII-induced coronary hypertrophy and fibrosis are inhibited by ROCK inhibitors. This effect of ROCK inhibition is associated with a marked reduction of AII-induced superoxide anion production,88 AII-induced monocyte chemoattractant protein-1, and PAI-1.[57, 70] Although Ang II seems to substantially participate in the activation of ROCK in hypertensive vascular disease, a potential role of the increased

arterial pressure cannot be excluded. In hypertension, mechanical strain on the vessel wall is increased and it has been shown that mechanical stress stimulates vascular smooth muscle cell proliferation.[97] Indeed, stretch-induced ERK activation and vascular smooth muscle cell growth are inhibited by ROCK inhibition.[98, 99] An additional and important role of the ROCK pathway that can account for its involvement in hypertension is the alteration of the expression of genes important in the regulation of arterial tone and structure such as PAI-1 and eNOS. Excessive RhoA/ROCK activity could thus participate in endothelial dysfunction and the decreased NO production associated with arterial diseases. Together, these recent data point to a substantial role of ROCKs in hypertension and show that different upstream signals can converge toward ROCKs in hypertensive vascular diseases.

1.6.2 ROCK and pulmonary hypertension

Pulmonary hypertension is abnormally high blood pressure in the arteries of the lungs. The pathogenesis of pulmonary hypertension is a multifactorial process that comprises sustained vasoconstriction and structural remodeling of pulmonary arteries leading to reduction of the lumen area of the pulmonary microvasculature and fixed elevation of pulmonary resistance. Reduced endothelium-derived NO production in pulmonary arterial vessels has been implicated in the pathophysiology of hypertension.[100] It has recently been shown in vitro in human pulmonary endothelial cells that hypoxia-induced decrease in eNOS expression is mediated by ROCK.104 In addition, several studies indicate that activation of the RhoA/ROCK pathway contributes to both vasoconstriction

and vascular remodeling associated with pulmonary hypertension.[101, 102] The ROCK inhibitor Y-27632 attenuates acute hypoxia-induced vasoconstriction and reduces the development of chronic hypoxia-induced pulmonary hypertension and vascular remodeling.[103] The ROCK-dependent calcium sensitization in small pulmonary arteries has been shown to be enhanced by chronic hypoxia in rats in association with a 2-fold increase in ROCK expression.[104] The ROCK pathway is also substantially involved in monocrotaline-induced pulmonary hypertension in rats and long-term inhibition of ROCK by orally given or inhaled fasudil, prevents or causes a marked improvement of monocrotaline-induced pulmonary hypertension through multiple mechanisms including inhibition of smooth muscle cell proliferation and increased apoptosis, reduced macrophage infiltration and improvement of endotheliumdependent relaxation.[102, 105, 106] Although the mechanisms leading to the increased RhoA/ROCK activity are not identified, these data indicate that activation of the RhoA/ROCK pathway is involved in the pathogenesis of pulmonary hypertension.

1.6.3 ROCK and vascular aneurysms

Vascular aneurysm is an abnormal widening or ballooning of a portion of an artery related to weakness in the wall of the blood vessel. Atherosclerosis and hypertension accelerate the development of abdominal aorta aneurysms via enhancement of Angiotension II pathway.[107] Agonism of a G protein–coupled receptor by Angiotension II activates ROCK and other signaling pathways and leads in activation of proteolysis and apoptosis. Increased proteolysis and smooth muscle cell apoptosis are important

mechanisms associated with vascular aneurysm. [107] Both the incidence and the severity of Angiotension II-induced aortic aneurysms in apoE-KO mice could be reduced by the fasudil, a Rho-kinase inhibitor.[108] This advantage is through the inhibition of ROCK-mediated extracellular matrix proteolysis and apoptosis.[108] In contrast to this observation, inhibition of ROCK leads the reduced neointima formation by enhancing SMC apoptosis and probably by suppressing early SMC migration, which is through Bax upregulation.[109, 110] It suggested that inhibition of ROCK enhanced neointimal SMC apoptosis but did not affect intimal SMC replication and apoptosis. Thus, the role of apoptosis in the pathogenesis of aneurysms may be different from that of other vascular diseases in which links between ROCK and apoptosis may be different.

1.6.4 ROCK and cardiac hypertrophy and ventricular remodeling

Cardiac hypertrophy is a physiological adaptive response of the heart to pressure or volume overload. With the time prolonged, the heart is switched from a compensated to decompensated state and this initial adaptive response becomes maladaptive and finally leading to heart failure. It is complex of the molecular response to pressure overload and this process may include modulation of various intracellular signal pathways, such as expression of cardiac fetal genes (myosin light chain and atrial natriuretic factor (ANF)), activation of many protein kinases (mitogen-activated protein kinase and phosphatidylinositol 3 kinase) and increase of protein synthesis. Furthermore, pressure overload causes the secretion and production of vasoactive peptides, such as endothelin

1 and Ang II, both playing pivotal roles in the induction of these hypertrophic responses.[111, 112]

Recently, many cellular and molecular biology studies have proved an involvement of RhoA/ROCK signaling pathway in many aspects of cardiovascular functions such as cardiac hypertrophy and ventricular remodeling after myocardial infarction.[113] Sauzeau et al. reported that human urotensin II-induced VSMC proliferation was inhibited by a ROCK inhibitor, suggesting that RhoA and ROCK mediate the stimulation of VSMC growth.[114] In the adult rat myocardium, pressure overload induces a rapid activation of ROCK, suggesting that it could play a critical role in the coordination of initial mechanisms and adaptive changes triggered by mechanical stress in cardiac myocytes.[115] The ventricular hypertrophy and function is significantly ameliorated by ROCK inhibition in Dahl salt-sensitive hypertensive rats.[116, 117] It has been suggested that the cardioprotective effect of ROCK inhibition involved up regulation of the down regulated eNOS and the reduction of oxidative stress through the inhibition of NAD(P)H oxidase and lectin-like oxidized low-density lipoprotein receptor-1 expression.[116] Several neurohormonal factors, such as Ang II, are believed to participate in ventricular hypertrophy and to the transition to heart failure. Long-term inhibition of ROCK by fasudil treatment reduces the Ang II-induced cardiomyocyte hypertrophy in wildtype[44] as well as in apoE-KO mice.[118] In addition, ROCK inhibition improves cardiac function by preventing Ang II-induced decrease in ventricular contractility, cardiac output, and cardiac stoke volume.[118] In vitro, the assembly of contractile proteins into organized

sarcomeric units is one of the prominent features of the neurohormonal factor induced cardiac myocyte hypertrophic response.[119] In neoneatal ventricular myocytes, ROCK activation is one of the key events mediating α_1-adrenoceptor activationinduced myofibrillar organization and ANF expression.[119] Similarly, it has been shown that ROCK inhibitor significantly suppressed ET-1 induced hypertrophic response via the increase of ANF production, cell size, protein synthesis, and myofibrillar reorganization.[120] Activation of ERK1/2 and of the cardiac transcription factor GATA-4 is identified as downstream nuclear mediators of ROCKs during myocardial cell hypertrophy.[121]

However, the inhibitors of ROCK cannot distinguish between ROCK1 and ROCK2, the two isoforms of ROCK family, and could also have non-selective effects.[74] Recent genetic studies by Wei L's laboratory and others support the concept that ROCK1 and ROCK2 have distinct non-redundant functions in cardiac hypertrophy and remodeling.[122] ROCK1 deletion did not impair compensatory hypertrophic response, but significantly reduced cardiomyocyte apoptosis and fibrosis in response to pressure overload induced by transverse aortic constriction.[123] In addition, ROCK1 deletion did not affect the development of cardiac hypertrophy in Gαq transgenic mice, but prevented chamber dilation and contractile dysfunction at young ages (12 weeks).[124] These results indicate that ROCK1 does not play a significant role in compensatory hypertrophic responses, and raise the possibility that ROCK1 plays a critical role in the maladaptive response which contributes to the transition from compensatory cardiac hypertrophy to

heart failure. A latest study provided the long-term beneficial effects of ROCK1 deficiency in hypertrophic decompensation and suggested that ROCK1 may be an attractive therapeutic target to limit heart failure progression.[125]

1.6.5 ROCK and atrial fibrillation

Atrial fibrillation (AF) is one of the most common arrhythmias which causes substantially excess cardiovascular morbidity and mortality.[126] Clinically, increased vulnerability to AF is associated with underlying heart disease, such as valvular heart disease, HF, coronary artery disease, and hypertension, particularly when left ventricular hypertrophy is present.[126] The Rho family GTPase RhoA controls the formation of actin structures, and the RhoA-actin signaling pathway regulates serum response factor (SRF) transcriptional activity. SRF regulates seruminducible and muscle-specific gene expression by binding to the serum response element. RhoA regulates cardiac sinus and atrioventricular nodal function and its overexpression results in bradycardia and development of ventricular contractile failure with chamber enlargement and interstitial fibrosis.[127] On the other hand, inhibition of RhoA and Rac1 activities by overexpression of Rho GDP dissociation inhibitor resulted in an AV block with atrial enlargement and ventricular hypertrophy.[128] These results suggest that fine-tuning of Rho GTPase signaling is required for maintaining cardiac rhythm, conduction, and structure. Recently, muscle-restricted coiled-coil protein (MURC) was proved playing a critical role in the development of cardiac dysfunction and conduction disturbance with increased vulnerability to atrial arrhythmias, especially in the setting of CHF via modulating the

Rho/ROCK signaling pathway.[129] Sustained overexpression of MURC facilitates functional deterioration, including cardiac function and conduction disturbances.

In human, the beneficial effects of statins on atrial remodeling and AF are suggestive of Rac1, but because statins could also inhibit other isoprenoid-dependent pathways, such as the Ras and Rho/ROCK pathways, their inhibitory effects on atrial fibrosis and AF may not be due entirely to Rac1 inhibition. Indeed, deletion or inhibition of ROCK1 also leads to decreased angiotensin II-induced CTGF expression and cardiac fibrosis.[45, 122] Thus, the potential benefits of statin therapy in AF may extend beyond their inhibitory effects on Rac1. Nevertheless, these findings do provide some of the mechanistic basis for the clinical benefits of angiotensin receptor blockers or statins in patients with AF.

1.6.6 ROCK and stroke

Cerebral cavernous malformations (CCM) are vascular lesions causing seizures and stroke. Mutations causing inactivation of one of three genes, ccm1, -2, or -3, are sufficient to induce vascular endothelial cell defects resulting in CCM. The loss of expression of the CCM1, -2, or -3 proteins causes a marked increase in expression of the GTPase RhoA. Increased RhoA activation was associated with ROCK-dependent phosphorylation of myosin light chain 2. Functionally, loss of CCM1, -2, or -3 inhibited endothelial cell vessel-like tube formation and extracellular matrix invasion, each of which is rescued by chemical inhibition or short hairpin RNA knockdown of ROCK. Such as fasudil could prove beneficial in acute situations of pathological vascular leak

(e.g., sepsis) as well as CCM.[130] It was recently shown that loss of endothelial cell expression of CCM2 resulted in activation of the GTPase RhoA.[131] Crose et al. demonstrated the defective RhoA degradation resulting from loss of CCM2-mediated localization of Smurf1, a CCM2 binding partner, which controls RhoA degradation required for maintenance of normal endothelial cell physiology.[131] RhoA overabundance induced by loss of CCM2 was shown to increase cytoskeletal stability, inhibit vessel-like tube formation, and increase endothelial cell monolayer permeability.[131] Loss of CCM1, -2, or -3 expression results in a common phenotype associated with RhoA overexpression and activation. ROCK is defined as a critical RhoA effector whose increased activation dysregulates endothelial cell function. ROCK is activated by RhoA and phosphorylates several substrates, including myosin light chain, myosin light chain phosphatase, and LIM kinase for the regulation of actin cytoskeletal dynamics.[132] ROCK has also been shown to regulate vascular permeability and has been a drug discovery target for regulation of vascular bed diseases.[133]

1.7 ROCK and other diseases

1.7.1 ROCK and renal injury

Renal injury progresses to end-stage renal disease that requires renal replacement therapy. Recent studies demonstrate that ROCK inhibition by Y-27632 and fasudil dilates basal tone of afferent as well as efferent arterioles in the in vitro[134] and in vivo hydronephrotic kidney models,[135] although the vasodilator response of efferent arterioles is slightly less than that of afferent arterioles. Furthermore, both Y-27632 and fasudil

reverse the angiotensin II-induced vasoconstriction of afferent and efferent arterioles. Thus, the altered balance of renal pre- and post-glomerular microvascular tone may affect glomerular hemodynamics and subsequently could modify the development of renal disease, although it has not been evaluated whether the inhibition of ROCK alters the glomerular capillary pressure. These mechanisms of the ROCK inhibition may serve in part to exert renal protective action in chronic renal injury. In addition to a critical role of ROCK in the renal microvasculature, Rho proteins are important endogenous regulators of several types of renal tubular functions, including proliferation, migration, and apoptosis.[136] It has been demonstrated that Rho regulates the formation of stress fibers, focal adhesions, and peripheral bundles through reorganization of the actin cytoskeleton in a renal epithelial cell line, Madin-Darby canine kidney cells.[137] Furthermore, renal epithelial cells are able to transform mesenchymal-like cells (e.g., myofibroblasts) via the process of epithelial mesenchymal transdifferentiation.[138] These changes have been observed in the process of renal tubulointerstitial fibrosis. Mesangial cells are smooth muscle-like cells that reside in the renal glomerulus and produce extracellular matrix (ECM) protein or collagen to form mesangial matrix. Increased accumulation of ECM causes glomerulosclerosis, where TGF- β 1 has been implicated as a causative factor.[139] Although the effects of TGF-β1 on mesangial collagen I accumulation are mediated by several pathways, the Rho/ROCK mechanism constitutes an important role in mediating the cytoskeletal rearrangement of mesangial cells.[140] This transdifferentiation is characterized by the activation of smooth muscle α-actin expression, and ROCK inhibitors, Y-27632 and HA-1077, block this process in cultured renal mesangial cells.[141] Finally, in mesangial cells, mechanical stress, which is

considered to cause glomerular hypertension and glomerulosclerosis, enhances mitogen-activated protein kinase (MAP kinase) activity, stress fiber formation, and cellular proliferation.[142] In this disease process, RhoA plays an essential role, acting as a modulator of MAP kinase and the subsequent cellular impact. Collectively, these data strengthen a significant role of Rho/ ROCK pathway in the development of glomerulosclerotic renal disease. Podocytes are located in the renal glomerulus and are highly differentiated cells with a complex cellular morphology composed of F-actin.[143] Cytoskeleton Factin rearrangement is closely associated with podocyte shape changes and dysfunction in various renal disease.[144] A growing evidence demonstrates that in cultured renal podocytes, Endlich et al.[145] found that Y-27632 inhibited the reorganization of cytoskeleton induced by mechanical stress. Furthermore, the inhibition of ROCK activity is reported to prevent the transforming growth factor-β-induced increase in connective tissue growth factor accumulation in cultured human renal fibroblast cells.[146] Since Rho/ROCK regulates glomerular hemodynamics and has substantial effects on mesangial cell proliferation, matrix production, and contraction, ROCK inhibitors might be candidates as therapeutic tools for treating glomerulosclerotic diseases. The ROCK inhibitor, fasudil, was proved to attenuate glomerulosclerosis in salt-induced hypertensive rats partly by the inhibition of the transforming growth factor beta (TGF-β)/collagen cascade [147] and in subtotally nephrectomized spontaneously hypertensive rats (SHR, a model for hypertensive glomerulosclerosis) via the upregulation of $p27^{kip1}$, a cyclin-dependent kinase inhibitor.[148] They demonstrated that the Rho/ROCK pathway was activated in subtotal nephrectomized SHR and suggested an involvement of the Rho/ROCK pathway in the progression of hypertensive

glomerulosclerosis. The treatment with fasudil decreases urinary protein excretion, improved glomerular and tubulointerstitial injury score, and reduced the infiltration of ED-1 positive cells and proliferating cell nuclear antigen positive cells in the kidney of SHR with subtotal nephrectomy. In this study, fasudil did not significantly reduce the systemic blood pressure. Similarly, beneficial results of fasudil were reported in Dahl salt-sensitive rats.[149]

Furthermore, Ang II is proved contributing to renal impairment in chronic renal disease, even though systemic blood pressure is unchanged.[150] The latter observation is consistent with previous reports that Ang II may play an important role in the sclerotic changes within the glomerulus independent of changes brought about by increased glomerular pressure.[151] In this regard, it has been reported that Ang II [61] and mechanical stretch [98] activate ROCK in vascular smooth muscle cells. Moreover, enhanced ROCK activity is involved in the pathogenesis of neointimal formation.[36] Therefore, it can be extrapolated that the role of ROCK is exaggerated in pathophysiological conditions, such as chronic renal injury. In subtotal nephrectomy, alterations in glomerular capillary pressure[152] may also play a role in the enhancement of Rho/ROCK activity through the mechanical stretch.[98] It is possible therefore that the ROCK inhibition confers beneficial action on renal injury through blood pressure dependent and -independent mechanisms. Nishikimi et al. study showed that activation of the Rho/ROCK pathway is related to the pathogenesis of nephrosclerosis, and that long-term fasudil treatment has renoprotective effects in severe hypertension.[153] The mechanism of the renoprotective effect of fasudil

may be due to reduction of the TGF-β/collagen and TGF-β/PAI-1 cascades, control of inflammation, and decreased oxidative stress.[153] More recently Ishikawa et al. also reported that the ROCK inhibitor has renoprotective effects partly via the inhibition of extracellular matrix gene expression, monocyte/macrophage infiltration, oxidative stress, and the upregulation of endothelial nitric oxide synthase gene expression in malignant hypertensive rats independent of blood pressure-lowering activity.[154]

1.7.2 ROCK and diabetes

Diabetes can cause many serious complications such as cardiovascular diseases, cerebrovascular diseases, nephropathy and retinopathy. Long-term complications of diabetes result in increased physical disability or mortality.

Plasminogen activator inhibitor-1 (PAI-1) is a key regulator of fibrinolysis by inhibiting plasminogen activators. High plasma PAI-1 level correlates with the development of diabetes in patients with metabolic syndrome and future cardiovascular events.[155] Inhibition of PAI-1 may be beneficial not only in preventing cardiovascular complications of diabetes but the development of diabetes as well. It was reported that exposure of human endothelial cells to hyperglycemia increased ROCK activity in a concentration-dependent manner and PAI-1 mRNA expression.[156] This increase could be inhibited by PKC inhibitor and antioxidants. The increase of PAI-1 mRNA and protein levels induced by hyperglycemia could be inhibited by ROCK inhibitors

hydroxyfasudil and Y27632 or by a dominant-negative mutant of ROCK. ROCK inhibitors suppressed hyperglycemia-stimulated PAI-1 promoter activity but without affecting mRNA stability. Hyperglycemia failed to stimulate ROCK activity and PAI-1 expression in heterozygous ROCK1 knockout murine endothelial cells despite the presence of the highly homologous ROCK2. These results suggest that hyperglycemia stimulates ROCK activity via PKC- and oxidative stress–dependent pathways, leading to increased PAI-1 gene transcription. ROCK1 plays a predominant role in hyperglycemia-induced increases in ROCK activity and PAI-1 expression.

Iwasaki et al.[157] undertook an additional study and found that PAI-1 expression in bovine aortic endothelial cells was significantly increased under high glucose (HG) condition. Stimulation with HG significantly increased RhoA activation and NF-κB promoter activity. Pretreatment with Y-27632 significantly blocked HG-induced PAI-1 expression at a basal level and inhibited NF-κB activity. These indicate that HG-induced PAI-1 expression in endothelial cells is mediated by NF-κB activation through Rho/ROCK pathway. NF-κB is one of the transcriptional downstream molecules of Rho/ROCK signaling.

The activation of Rho/ROCK pathway plays an important role in the change of vascular contractility and reactivity induced by vasoactive agents. Didion et al. [158, 159] measured responses of cerebral arterioles and carotid arteries in vivo by using a cranial window

and in vitro by using tissue baths in db/db mice and in TallyHo mice. Their results showed the vasodilatation to acetylcholine (Ach) was markedly impaired and superoxide levels were increased. Phenylephrine (Phe)- and serotonin (Ser)-induced vascular contraction was increased. Responses to Phe and Ser were restored to normal in the presence of Y-27632. These findings provide the evidence that oxidative stress and enhanced activity of ROCK may contribute to altered vascular function in the genetic models of type 2 diabetes. A further study showed that RhoA and ROCK were activated in type 2 diabetic db/db mice vasculature and cultured aortic smooth muscle cells with HG.[160] Activated ROCK in diabetic condition directly phosphorylates MLC and inhibits MLCP, altogether resulting in a net increase of MLC in the phosphorylated state and enhanced vascular smooth muscle contraction, therefore amplifying vasoconstriction and contributing to the mesenteric artery contractile hyperreactivity in response to Phe in vitro. There is an enhanced role for Rho/ROCK pathway in alpha1-adrenergic vasoconstriction in metabolic syndrome. Recently, investigators found that aortas isolated from STZ-diabetic rats presented hyper-contracture to angiotensin II (AT-II) mainly dependent on the up-regulation of ROCK1 expression/activity.[161] Pre-treatment with HA-1077, an inhibitor of ROCK, reduced AT-II efficacy in vitro. Losartan treatment in vivo, which limited increases in ROCK1 expression and activity in STZ-diabetic rats, corrected hyper-contracture to AT-II. In the study, AT-II contraction efficacy were more intense than Phe, this may be the reason that RhoA/ROCK1 activation is also related to G12/13 protein-linked AT1[162] except mainly depending on HG, namely, the aortas contractile hyperreactivity to angiotensin II is due to amplification of Rho/ROCK pathway in diabetic condition. The migration of vascular

smooth muscle cells (SMCs) has been known as one of the major pathogenic changes in diabetic macroangiopathy. The study[163] demonstrated that the protein level of platelet-derived growth factor receptor-β (PDGFR-β) in cultured human aortic SMCs was increased under HG condition concomitant with the increased protein level and activity of Rho. Anti-PDGF neutralizing antibody and PDGFR-β inhibitor suppressed the increased protein level and activity of Rho under HG condition. The increased protein levels of Rho were observed in aortae of diabetic rats, which were abolished by the administration of Imatinib, the inhibitor of PDGFR. Furthermore, HG significantly increased the migration of SMCs, which was suppressed by Y-27632 and anti-PDGF neutralizing antibody. These observations indicate that the upregulation of the PDGFR-β / Rho/ROCK pathway increases the migration of SMCs under HG condition.

1.7.3 ROCK and autoimmunity

Accumulating murine and human studies supported that IL-17 and IL-21 play as a key role in the pathogenesis of several autoimmune disorders, such as systemic lupus erythematosus (SLE) and rheumatoid arthritis.[164, 165] In particular, studies have demonstrated that interferon regulatory factor 4 (IRF4), a member of the IRF family of transcription factors, plays a unique and integral role in the control of these 2 cytokines since it is absolutely required for the production of both IL-17 and IL-21.[166] Interestingly, the expression of IRF4 is upregulated upon T cell activation.[167] In addition to directly controlling IRF4, Def6 can also function as an activator for Rac, and lack of Def6 results in defective activation of Rac in mature $CD4^+$ T cells. Interestingly, extensive crosstalk

exists amongst different Rho GTPases.[168] In particular, Rac and RhoA can antagonize each other, since Rac activation inhibits RhoA activity and vice versa. Consistent with this notion, the only other protein sharing a high degree of homology with Def6,[169] was recently shown to result in increased RhoA activation in dendritic cells.[141] This finding led to the suggestion that this small family of proteins may control the antagonistic interaction between Rac and RhoA. One of the major mechanisms by which activated RhoA can exert its biologic effects is by binding to and activating ROCK. Consistent with their role in the regulation of cytoskeletal reorganization, ROCK has been shown to regulate the migration of $CD4^+$ T cells. Interestingly, increased ROCK activity has been observed in $CD4^+$ T cells from SLE patients, and ROCK inhibitors have been shown to ameliorate the cytoskeletal abnormalities exhibited by T cells from SLE patients.[167] In addition to its effects on the T cell cytoskeleton, ROCK may also play a role in the activation of $CD4^+$ T cells, as suggested by the finding that ROCK inhibitors can decrease the production of cytokines such as IL-2 and IFN-γ by naive T cells.[170] Intriguingly, however, these inhibitors did not alter the production of these cytokines by primed $CD4^+$ T cells. WT $CD4^+$ T cells activated ROCK2 when exposed to Th17, but not Th0, which leading the production of IL-17 and IL-21 as a result of its ability to phosphorylate IRF4.[171] In contrast to the WT T cells, Def6-deficient $CD4^+$ T cells aberrantly activated ROCK2 under neutral conditions.[171] Aberrant ROCK2 activation was also detected in $CD4^+$ T cells from another spontaneous mouse model of autoimmunity, the lupus-prone MRL/lpr mouse. Administration of ROCK inhibitors to either Def6-deficient or MRL/lpr mice decreased the production of IL-17 and IL-21 and ameliorated the autoimmune symptoms that spontaneously develop in these mice. These

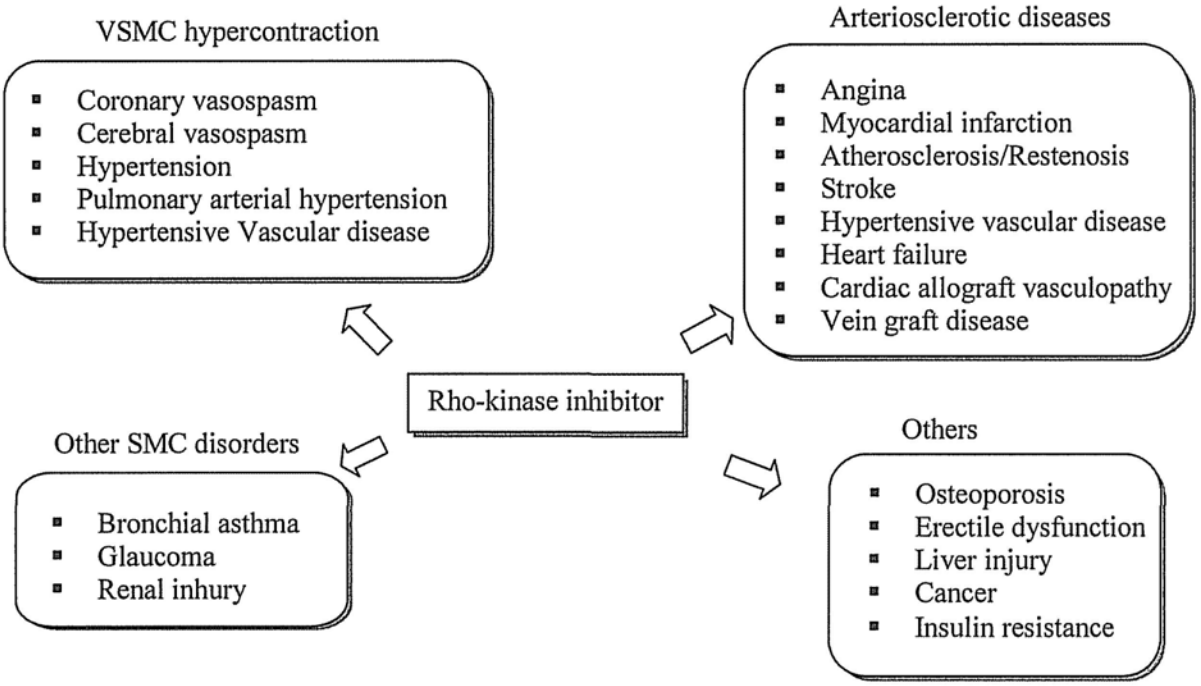
data demonstrated that ROCK2-mediated phosphorylation of IRF4 is a novel regulatory step controlling the production of IL-17 and IL-21 and that aberrant activation of ROCK2 in $CD4^+$ T cells may play a pathogenic role in autoimmunity.

1.8 ROCK inhibitors

Given the role of ROCKs in vascular function and inflammation, the development of selective and nonselective ROCK inhibitors has gained considerable interest. Current evidence supports ROCK inhibition as a potential treatment of various cardiovascular disorders caused by VSMC hyper-constriction, including coronary and cerebral vasospasm,[76, 80] hypertension and pulmonary hypertension.[106]

The benefit of ROCK inhibition may extend for the treatment of atheriosclerosis,[39, 40] ischemia-reperfusion injury,[89] stroke,[172] myocardial hypertrophy,[44] heart failure,[173] cardiac allograft vasculopathy[46] and vein graft disease.[174] Furthermore, they could be potentially used for the treatment of disorders associated with smooth muscle hyper-reactivity, such as asthma and glaucoma[1] and renal injury.[134] Finally, recent studies indicate that ROCK inhibitors could potentially be used to treat osteoporosis, renal disease, erectile dysfunction, cancers[1] and insulin resistance.[175] (Figure 1.2)

Figure 1.2 Current studies of ROCK inhibitors



1.8.1 Fasudil

Fasudil (HA-1077)[176] was the first ROCK inhibitor approved for clinical use in Japan in 1995 for the treatment of vasospasm after subarachnoid hemorrhage[85]. Fasudil selectively inhibits ROCK by competing with ATP for binding to the kinase.[177] Clinical development for this indication is in progress in the United States and Europe. The first report of a placebo-controlled double-blind trial in 1992 has demonstrated a significant reduction in angiographically revealed vasospasm by intravenous administration of fasudil.[178] Fasudil has significant vasodilatory activity[179] and is currently undergoing clinical trials for the treatment of stable effort angina pectoris[180]. Hydroxyfasudil (HA-

1100), a major active metabolite of fasudil after oral administration, has a more selective inhibitory effect on ROCK than does the parent drug.[44, 76] Another dimethylated analog of fasudil, H-1152P, is the most potent inhibitor of the three (HA-1077, H-1152P, HA-1100) ligands against ROCK.[181] It is a cell permeable, highly specific, potent and ATP-competitive inhibitor of ROCK and more selective than Y-27632. H-1152P has a potent intraocular pressure-lowering effect on an ocular hypertension model. These result suggested that H-1152P could be a candidate for the next generation of glaucoma therapy.[182]

Intracoronary administration of fasudil is effective at reducing coronary spasm and myocardial ischemia in patients with vasospastic angina and microvascular angina.[83] Long-term oral treatment with fasudil is also effective at ameliorating exercise tolerance in patients with stable-effort angina and adequate safety profiles.[60] Intra-arterial infusion of fasudil markedly enhances the vasodilator responses of forearm circulation in hypertensive patients.[183] Intravenous infusion of fasudil significantly reduces pulmonary vascular resistance in patients with severe pulmonary hypertension which was not ameliorated by oxygen inhalation, NO inhalation, or nifedipine.[184] Intra-arterial infusion of fasudil causes a preferential increase in forearm blood flow in patients with heart failure when compared with control subjects.[185] However, the potential usefulness of the oral administration of ROCK inhibitors for the treatment of unstable angina, MI, pulmonary hypertension, hypertensive vascular disease and/or cardiac hypertrophy remains to be examined in humans. Another clinical trial of the intravenous

administration of fasudil in the acute phase of stroke demonstrates that ROCK inhibitors exert beneficial effects on ischemic neuronal damage without causing serious adverse effects.[186]

Clinical trials of the anti-anginal effects of fasudil in patients with stable-effort angina in Japan[60] and the USA[187] have demonstrated that long-term oral treatment with this ROCK inhibitor is effective at ameliorating exercise tolerance in patients with adequate safety profiles. This Phase II dose-finding trial in patients with stable angina showed that ST-segment depression was improved with fasudil at both peak and trough compared with placebo. In addition, fasudil improved Seattle Angina Questionnaire scores. It was well tolerated and did not affect heart rate or blood pressure. No major adverse events were noted with fasudil treatment; most of the adverse events were mild and not considered to be related to study medication.[187] An effort has been made to develop more-specific and more-potent ROCK inhibitors.[188] Moreover, fasudil analogs have been designed on the basis of the complex structure of PKA and HA1077, and it was found that glycine derivatives of HA1077 are highly specific inhibitors of ROCK.[188] These inhibitors were applied to rabbit ocular hypertensive models, in which they reduced intraocular pressure – indicating that they might be useful for glaucoma, in addition to other ROCK-related diseases.[188] All these data from clinical studies suggest that ROCK is a valuable drug target for a broad range of cardiovascular diseases. More than 30, 000 patients have been treated with fasudil with only minimal and acceptable side effects.

1.8.2 Other ROCK inhibitors

A number of pyridine derivative compounds such as Y27632 have been developed with potent ROCK inhibitory effect.[74] Y27632 inhibits smooth muscle contractility and has been shown to normalize blood pressure in rat hypertensive models.[74] It was originally reported that Y27632 is a non-specific inhibitor of both ROCK1 and ROCK2 by competing with ATP for binding to their catalytic sites.[189] Y27632 is also a potent inhibitor of Rho-dependent PKC-related kinase 2.[177] This results in decreased phosphorylation of myosin, arterial smooth muscle relaxation and vasodilation of blood vessels. Y27632 has other inhibitory properties against RhoA-mediated cell transformation,[190] tumor cell invasion[191] and neutrophil chemotaxis.[192] These findings raised the possibility that inhibitors of ROCK may have additional potential therapeutic use in the treatment of cardiovascular and other diseases. More recently, a closely related molecule, Y32885, was found to inhibit protein kinase C-related kinase 1 (PRK1) (a distinct Rho-dependent protein kinase) at a concentration similar to which it inhibits ROCK isoforms.[193] PRK1 is present in various malignancies development and progression and it is a potential drug target for pharmacological intervention of RhoA-mediated signaling pathway.[194]

Recently, two aminofurazan-based inhibitors, GSK269962A and SB772077B, were characterized as members of a novel class of compounds.[195] These compounds highly inhibit both ROCK1 and ROCK2, and their potency is higher than that of Y27632 or

fasudil, especially on inhibiting ROCK1.[195] These compounds are potent vasodilators and that they lower blood pressure in spontaneously hypertensive rats and deoxycorticosterone acetate-salt-treated hypertensive rats.[195] Other highly ROCK2 selective inhibitors such as SR-715 and SR-899 have been developed.[196] Another ROCK2 inhibitor, SLx-2119 reduced connective tissue growth factor mRNA and remodeled the actin cytoskeleton in fibrosis-derived smooth muscle cells.[197] A weak ROCK inhibitor, Rockout (3-(4-pyridyl)indole), was discovered using automated microscopic screening for compounds that affected cell migration and mitotic progression.[198] Recently, the 3D crystal structure of ROCK and its binding site for fasudil has been determined, which should facilitate the development of more selective ROCK inhibitors .[199]

1.9 Summary and hypothesis

ROCK-dependent signaling pathway is recognized as an essential regulator of vascular functions and seems to play an important role in major arterial diseases such as hypertension, atherosclerosis, and pulmonary hypertension. Clearly, more studies are now required to understand how ROCK are activated, what are the downstream effectors, and how this complex signaling pathway regulates smooth cell functions in physiological conditions as well as in association with vascular diseases. However, the present knowledge of ROCK signaling suggests that pharmacological agents targeting the ROCK may have therapeutic benefits in arterial diseases. Data from large clinical trials are now needed to demonstrate the safety of long-term treatment with ROCK

inhibitors and to confirm the efficacy and the usefulness of these pharmacological agents in cardiovascular medicine.

Although ROCK activity was widely studied in animal models and some clinical trials were studied recently, few are focused on cardiovascular diseases. Several publications[200] about the method of measuring ROCK activity were published recently indicating that this method is mature and stable. The hypothesis of my PhD study is to see if ROCK activity will increase in cardiovascular diseases. We also hope to find out whether ROCK activity can predict future event or increase the prediction value when combing with other golden biomarkers together. Here we focused on three cardiovascular diseases: acute coronary syndrome (ACS) and congestive heart failure (CHF).

Table 1.1 Current studies of ROCK inhibitors

	Diseases	**Target**	**Inhibitor**	**Mechanisms or findings**
Arteriosclero -tic diseases	Atherosclerosis / Restenosis	Rat	Statin	Prevent membrane attachment of Rho proteins and the subsequent activation of ROCKs[67]
		Human	Atorvastatin	High-dose atorvastatin rapidly inhibits the proatherogenic Rho/ROCK pathway, independent of cholesterol reduction[68]
			Lipophilic statin	Better inhibition of ROCKs activity than hydrophilic statin, [201]
			Simvastatin ezetimibe	Dose-related inhibition of ROCK. Higher dose statin have greater inhibitory effects on ROCK activity and potentially more improvement in endothelial function than lower statin dose[34]
	Angina	Human	Fasudil	Prevent ACh-induced coronary artery spasm and resultant myocardial ischemia in patients with vasospastic angina[83, 87]
	Stroke	Mice	Fasudil Y-27632	The neuroprotective effect of ROCK inhibition on ischemic stroke is mediated by increased eNOS expression and activity. [32]
	Heart failure	Mice Rats	Fasudil Statins	Long-term blockade of Rho-kinase with fasudil suppresses the development of LV remodeling and dysfunction after MI[113, 202]
		Human	Fasudil	Fasudil augmented the impaired ischemic vasodilation during reactive hyperemia after arterial occlusion of the forearm in the HF patients[185]

	Cardiac allograft vasculopathy	Mice	Fasudil	Fasudil suppresses the development of cardiac allograft vasculopathy via anti-inflammation. [46]
	Vein graft disease	Human	Fasudil	Useful to treat intractable and otherwise fatal coronary spasm resistant to intensive conventional vasodilator therapy after CABG[203]
			Statins	Prevent pulsatile stretch-induced proliferation of human saphenous vein smooth muscle cells [174]
VSMC hypercontrac -tion	Coronary vasospasm	Rabbit	Fasudil	Protective against vasopressin and endothelin-induced myocardial ischemic change in a coronary spasm animal model[77, 53]
		Porcine	Y-27632	
		Human	Fasudil	Effectiven to prevent anginal attacks in spastic angina[83, 84, 87, 203]
	Cerebral vasospasm	Dogs	Y27632	Cerebral vasospasm[80]
	Hypertension	Rat	Y-27632 fasudil	Decrease blood pressure in various hypertensive model rats, but did not in normotensive animals. [204-206] Rho-kinase inhibitors have been shown to inhibit hypertensive vascular lesion formation
		Human	Fasudil [183]	
	Pulmonary arterial hypertension	Rats	Fasudil	Fasudil acutely and effectively reduced the end-stage angioproliferative PAH in rats resembling severe human PAH histologically (presence of occlusion of precapillary vessels by proliferating endothelial cells) and

				hemodynamically (high RVSP and low cardiac output).[106, 207, 208]
		Mice	Fasudil	Treatment with fasudil is effective to inhibit the development of PAH induced by chronic hypoxia, in both eNOS-dependent and –independent mechanisms[102, 209]
		Human	Fasudil	Acute beneficial effects of intravenous fasudil in patients with severe PAH without adverse effects[184, 210, 211]
	Hypertensive vascular disease	Human	Fasudil	Fsudil induce vasodilation in hypertensive kidney transplant recipients.[212]
Other SMC disorders	Bronchial asthma	Pig	Y-27632 HA-1077	Y-27632 protects against acute allergen-induced bronchoconstriction, development of airway hyperresponsiveness after the early and late asthmatic reaction, and airway inflammation[213-215]
	Glaucoma	Cell	Y-27632	Rho-kinase, a potential cellular target involved in the regulation of aqueous humor outflow resistance.[216, 217]
	Renal injury	Rat kidney	Y-27632 Fasudil	Dilating basal tone of afferent as well as efferent arterioles by angiotensin II[134, 135, 149]
Others	Osteoporosis	Human cells	Statins	Reduce the risk of osteoporotic fractures[218-221]
	Erectile dysfunction	Rats	Y-27632	Rho-kinase antagonism stimulates rat penile erection independently of nitric oxide[222, 223]

	Cancer	Cells, Animal models	Fasudil Y-27632	Inhibit tumor cell motility and metastasis[224-226]
	Liver injury	Mice	Fasudil	Fasudil is a potent inhibitor of endotoxin-induced expression of TNF-alpha and CXC chemokines as well as leukocyte infiltration and hepatocellular apoptosis in the liver[227]
	Insulin resistance	Cells, VSMCs	Y-27632	ROCK is an important regulator of insulin signaling and glucose metabolism[76, 228]

CHAPTER 2

HYPOTHESES & OBJECTIVES

CHAPTER 2. HYPOTHESES AND OBJECTIVES

2.1 Introduction

Many conventional and promising new cardiac markers have evolved over the past decade and become not only an important diagnostic but also prognostic tool. Additionally, they may help identify patients who will derive the most benefit from therapeutic interventions. Furthermore, they have become a deciding factor for the need for aggressive management.

Since the discovery of ROCK in 1996, ROCK has been widely studied and more than 1400 articles were published until now. Most of them are focused on cardiovascular disease in the animal models or cell cultures. Recently, researches on human were increased much and ROCK inhibitor has been used on stroke already.

2.2 Hypothesis

1. ROCK activity is increased in patients with ACS or CHF
2. Baseline ROCK activity can predict long-term cardiovascular event in these two diseases or increase the predictive value of traditional biomarkers.

2.3 Objectives

1. To ascertain if ROCK activity really increased in ACS or CHF patients when compared to the normal and disease controls.
2. To investigate the predictors affecting the ROCK activity in ACS or CHF patients.
3. To find out if ROCK activity is an independent predictor for the long-term cardiovascular events.
4. To detect how ROCK activity can improve predictive value if it is not an independent predictor for long-term cardiovascular events.

2.4 Outcomes

1. ROCK activity in ACS study: all the patients were followed up till 1st April 2010 or until the occurrence of major events (death from any cause, readmission with ACS or admission with congestive heart failure).
2. ROCK activity in CHF study: ROCK activity in CHF study: all the patients were followed up until 1st February 2010 or until the occurrence of cardiac death.

CHAPTER 3

METHODOLOGY

CHAPTER 3. METHODOLOGY

3.1 Study protocol

Patients (according to ACC guideline, 2001) and controls (normal control and disease control) were enrolled from Prince of Wales Hospital. Blood were collected at baseline and 6 month following up and ROCK activity was tested at these two time points. 2 years following up outcomes were documented from ACS and CHF registry database.

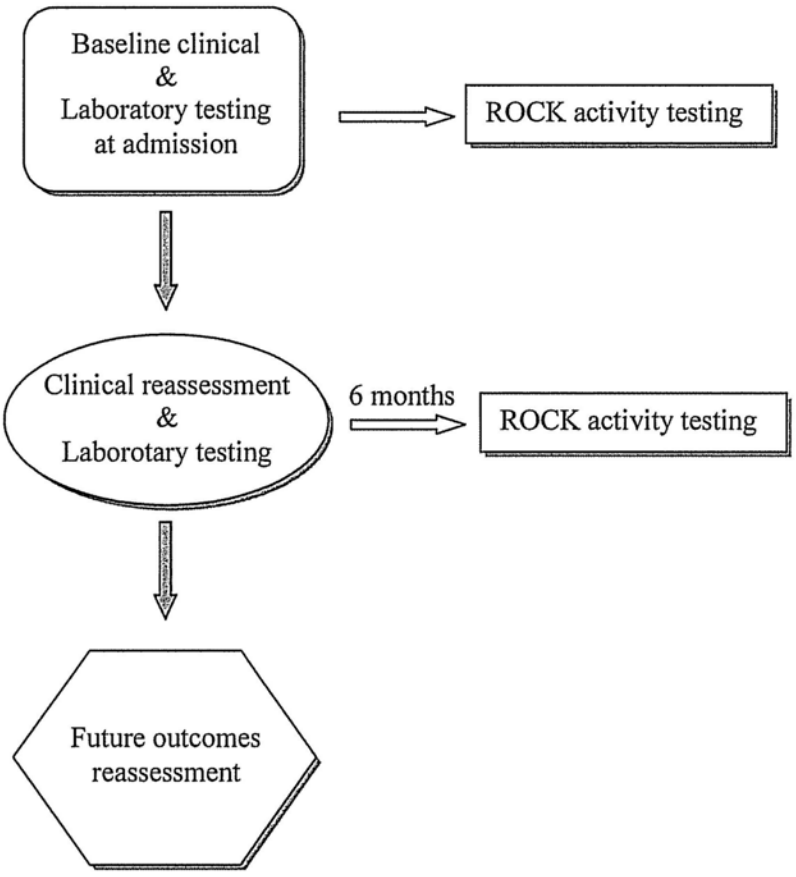


Figure 3.1 Flow chart of the study protocol

3.2 Study patients

(2001, ACC Clinical Data Standards – Reference Guide

http://www.acc.org/clinical/data_standards/ACS/acs_index.htm)

3.2.1 Patients with ACS

1. STEMI is defined as an ACS in which there is an evidence of a typical rise and gradual fall of myocardial necrosis biomarkers (e.g., troponin, CK-MB or CPK) and new (or presumably new if no prior ECG is available) ST segment elevation (new ST segment elevation at the J point in 2 or more contiguous leads with the cutoff points greater than or equal to 0.2mV in leads V1, V2, or V3, or greater than or equal to 0.1mV in other leads) on the admission ECG.
2. NSTEMI is defined as an ACS in which there is cardiac marker evidence of myocardial necrosis (e.g., positive troponin, CK-MB or CPK) without new ST-segment elevation.

Either of the following (in the absence of ST elevation):

a. ST-segment depression or T-wave abnormalities

b. Ischemic symptoms in the presence or absence of chest discomfort.

Ischemic symptoms may include:

(1) Unexplained nausea and vomiting or diaphoresis

(2) Persistent shortness of breath secondary to left ventricular failure

(3) Unexplained weakness, dizziness, lightheadedness, or syncope

3. Unstable angina is defined as angina pectoris (or equivalent type of ischemic discomfort) with any 1 of the 3 following features:

    a. Angina occurring at rest and prolonged, usually greater than 20 minutes

    b. New-onset angina of at least CCS classification III severity

    c. Recent acceleration of angina reflected by an increase in severity of at least 1 CCS class to at least CCS class III

The patient must also not have any biochemical evidence of necrosis.

a. Definite/probable unstable angina: Patients with clinical history consistent with the diagnosis of unstable angina as described above, in whom ischemia has been confirmed by the presence of ST-segment changes on the initial ECG or in association with recurrent rest pain, by a positive stress test, or by the presence of small elevations of troponin that do not meet criteria for MI

b. Possible unstable angina is present when an acute ischemic process has not been excluded as a possible cause of the presenting symptoms, or the clinical history is consistent with unstable angina but no diagnostic test (noted above) was performed to confirm the diagnosis

3.2.2 Patients with CHF

Framingham Criteria for Congestive Heart Failure

Diagnosis of CHF requires the simultaneous presence of at least 2 major criteria or 1 major criterion in conjunction with 2 minor criteria.

1. Major criteria:

a. Paroxysmal nocturnal dyspnea

b. Neck vein distention

c. Rales

d. Radiographic cardiomegaly (increasing heart size on chest radiography)

e. Acute pulmonary edema

f. S3 gallop

g. Increased central venous pressure (>16 cm H_2O at right atrium)

h. Hepatojugular reflux

i. Weight loss >4.5 kg in 5 days in response to treatment

2 Minor criteria:

j. Bilateral ankle edema

k. Nocturnal cough

l. Dyspnea on ordinary exertion

m. Hepatomegaly

n. Decrease in vital capacity by one third from maximum recorded

o. Tachycardia (heart rate>120 beats/min.)

Minor criteria are acceptable only if they can not be attributed to another medical condition (such as pulmonary hypertension, chronic lung disease, cirrhosis, ascites, or the nephrotic syndrome). The Framingham Heart Study criteria are 100% sensitive and 78% specific for identifying persons with definite congestive heart failure.

3.2.3 Disease controls

Thirty one disease control subjects from community with matched age and gender were recruited on the presence or absence of hypertension or smoking status which have been proved to influence ROCK activity.[229-231] All the diseas controls were processed coronary angiography and showed normal results.

3.2.4 Normal controls

Twenty normal subjects from community with matched gender were also recruited, how had:

(1) No history of cardiovascular or systemic illness

(2) Normal physical examination including blood pressure, as well as normal hemoglucostix and ECG

(3) No echocardiographic evidence of structure or functional heart disease

(4) No need for regular medications

3.3 Definition of other clinical elements

3.3.1 GRACE score

The more recent Global Registry of Acute Coronary Events (GRACE) score was developed from the registry,[232] with a population of patients across the entire spectrum of ACS. (Table 3.1 & 3.2)

Table 3.1 Calculation of GRACE score

GRACE	(0–258)
Age (yrs)	
<40	0
40–49	18
50–59	36
60–69	55
70–79	73
≥80	91
Heart rate (bpm)	
<70	0
70–89	7
90–109	13
110–149	23
150–199	36
>200	46
Systolic BP (mmHg)	
<80	63
80–99	58
100–119	47
120–139	37
140–159	26
160–199	11
>200 0	0
Creatinine (mg/dl)	
0–0.39	2
0.4–0.79	5
0.8–1.19	8
1.2–1.59	11
1.6–1.99	14
2–3.99	23
>4	31
Killip class	
Class I	0
Class II	21
Class III	43
Class IV	64
Cardiac arrest at admission	43
Elevated cardiac markers	15
ST-segment deviation	39

Table 3.2 6-month death and MI predictive ratio to GRACE score

GRACE Score	6 Month Death & MI predictive ratio
<1	0.00
1≤ <19	0.04
19≤ <34	0.05
34≤ <47	0.06
47≤ <58	0.07
58≤ <68	0.08
68≤ <76	0.09
76≤ <85	0.10
85≤ <92	0.11
92≤ <99	0.12
99≤ <105	0.13
105≤ <111	0.14
111≤ <117	0.15
117≤ <122	0.16
122≤ <127	0.17
127≤ <132	0.18
132≤ <136	0.19
136≤ <139	0.20
139≤ <143	0.21
143≤ <147	0.22
147≤ <151	0.23
151≤ <155	0.24
155≤ <159	0.25
159≤ <162	0.26
162≤ <166	0.27
166≤ <169	0.28
169≤ <172	0.29
172≤ <175	0.30
175≤ <179	0.31
179≤ <182	0.32
182≤ <185	0.33
185≤ <188	0.34
188≤ <190	0.35
190≤ <193	0.36
193≤ <196	0.37
196≤ <199	0.38
199≤ <201	0.39
201≤ <227	0.40
227≤ <249	0.50
249≤ <271	0.60
271≤ <294	0.70
294≤ <323	0.80
323≤	0.90

3.3.2 Developed evidence of new CHF after ACS admission

1 None (absence of rales over the lung fields)

2 Mild CHF (rales over 50% or less of the lung fields). Evidence of new pulmonary vascular congestion on chest radiograph also meets the definition.

3 Severe CHF (rales over more than 50% of the lung fields). Evidence of pulmonary edema on chest radiograph would also meet this definition.

3.3.3 Killip class

Killip class of the patient at the time of hospital admission:

1. Class 1: Absence of rales over the lung fields and absence of S3
2. Class 2: Rales over 50% or less of the lung fields or the presence of an S3
3. Class 3: Rales over more than 50% of the lung fields
4. Class 4: Shock (see also Outcomes section for full definition)

3.3.4 Type of ECG changes

1 ST-segment elevation indicates greater than or equal to 1 mm (0.1 mV) elevation in 2 or more contiguous leads

2 ST-segment depression of at least 0.5 mm (0.05 mV) in 2 or more contiguous leads (includes reciprocal changes)

3 T-wave inversion of at least 1 mm (0.1 mV) including inverted T waves that are not indicative of acute MI

4 Q waves refer to the presence of Q waves that are greater than or equal to 0.03 second in width and greater than or equal to 1 mm (0.1 mV) in depth in at least 2 contiguous leads

3.3.5 Maximum stenosis by vessel (left anterior descending coronary artery [LAD], left circumflex [LCx], right coronary artery [RCA], left main [LM], graft)

Stenosis represents the percentage occlusion, from 0 to 100%, associated with the identified vessel systems. Percent stenosis at its maximal point is estimated to be the amount of reduction in the diameter of the "normal" vessel proximal to the lesion. For the denominator, take the maximum internal lumen diameter proximal and distal to the lesion. In instances where multiple lesions are present, enter the highest percentage stenosis noted. The systems of interest are as follows and should include major branch vessels of greater than 2 mm diameter:

- Greatest stenosis assessed in the LAD or any major branch vessel
- Greatest stenosis assessed in the LCx or any major branch vessel
- Greatest stenosis assessed in the RCA or any major branch vessel
- Greatest stenosis assessed in the LM
- Greatest stenosis assessed in bypass graft

3.3.6 Cardiogenic shock

Experienced cardiogenic shock. Clinical criteria for cardiogenic shock are hypotension (a systolic blood pressure of less than 90 mmHg for at least 30 minutes or the need for

supportive measures to maintain a systolic blood pressure of greater than or equal to 90 mmHg), end-organ hypoperfusion (cool extremities or a urine output of less than 30 ml/h, and a heart rate of greater than or equal to 60 beats per minute). The hemodynamic criteria are a cardiac index of no more than 2.2 l/min per square meter of body-surface area and a pulmonary-capillary wedge pressure of at least 15mmHg.[233]

3.3.7 Smoking

History confirming cigarette smoking in the past. Choose from the following categories:

1. Current: Smoking cigarettes within 1 month of this admission
2. Recent: Stopped smoking cigarettes between 1 month and 1 year before this admission
3. Former: Stopped smoking cigarettes greater than 1 year before this admission
4. Never: Never smoked cigarettes

3.3.8 New York Heart Association (NYHA) class

I. Asymptomatic: Patients with cardiac disease but without resulting limitations of physical activity. Ordinary physical activity does not cause undue fatigue, palpitations, dyspnea, or angina.

II. Mildly Symptomatic: Patients with cardiac disease resulting in slight limitation of physical activity. Comfortable at rest, ordinary physical activity results in fatigue, palpitation, dyspnea, or angina.

III. Moderately symptomatic: Patients with cardiac disease resulting in marked limitation of physical activity. Comfortable at rest, less than ordinary activity causes fatigue, palpitation, dyspnea or angina.

IV. Severe/Symptoms at rest: Patients with cardiac disease resulting in inability to carry on any physical activity without discomfort. Symptoms of cardiac insufficiency or of angina syndrome may be present even at rest. Any physical activity leads to increased discomfort.

3.4 Laboratory testing

3.4.1 NT-proBNP testing

Blood samples were collected in the first 24 h after admission, usually the next morning at 8:00 with the patient fasting. All samples were collected into EDTA-Na tubes without stasis and were centrifuged at 2, 200g for 20 min within 30 min of sample collection. Plasma and serum samples were collected into aliquots and stored at -80°C until batch analysis. Plasma samples were used for measurement of NT-proBNP, using a Roche Diagnostic proBNP assay on an Elecsys 2010 analyser (Roche Diagnostics, Mannheim, Germany). Total assay precision ranges from 1.8% at 800 pmol/L to 2.7% at 20.7 pmol/L and the detection limits are 0.6 and 4130 pmol/L (to convert pmol/L to pg m/L, multiply pmol/L values by 8.457).

3.4.2 hs-CRP testing

10 ml EDTA whole blood was taken from ACS patients within 24 hours from admission. Plasma and serum samples were collected into aliquots and stored at -80°C until batch analysis. Afinion^{TM} AS100 Analyzer was used to test the C-reactive protein (CRP) in human by plasma. Measuring range for plasma is 5-160mg/L. If the CRP concentration was above the measuring range, plasma samples was diluted with 0.9% saline up to 4 times and re-tested.

3.4.3 Assay for leukocyte Rho-Kinase activity

3.4.3.1 Leukocyte preparation

To isolate human leukocytes, 20 ml of blood sample was mixed with Hanks balanced salt solution (HBSS) in a 50-ml citrate-containing tube. Ten milliliters of Histopaque (Sigma, Histopaque-1077) was layered with 25 ml of diluted blood in two tubes and centrifuged at room temperature for 30 min at 1400–1500 rpm. The supernatant containing the leukocytes was aspirated, mixed with HBSS, diluted with 2% dextran (1:1 ratio), and allowed to sit at room temperature for 30 min. The top layer was then aspirated, mixed with HBSS, and centrifuged at room temperature for 5 min at 1400–1500 rpm. The supernatant was discarded, and the pellet containing the leukocytes was resuspended in 3 ml of cold PBS. After swirling the tubes for 30 s, HBSS was added to stop the lysis. After centrifugation, the supernatant was discarded and the pellet was resuspended with 5 ml of M199. After determining cell yield and viability by using the Trypan blue exclusion test (usually 4–8 x 10^6 viable cells with a viability of more than

95%), the suspension was diluted with HBSS to achieve 5 x 10^6 cells/ml. Then, 400 ml of the leukocyte suspension was transferred to four sterile 1.5-ml tubes. 100 ml of fixative solution (see earlier discussion) was added to each tube. To avoid over phosphorylation, 1 mM of hydroxyfasudil was added to the TCA fixative solution. After vortexing and centrifuging the samples at 4° for 5 min at 12,000 rpm, the supernatant was removed and HBSS was added. The samples were centrifuged again at 4° for 1 min at 12,000 rpm. The supernatant was removed with a micropipette. The remaining leukocyte pellets were stored at -80° until use.

3.4.3.2 ROCK activity detection

ROCK activity was determined by the level of MBS phosphorylation. Making two 7.5% separating gels with 1.5-mm spacers requires a 20-ml solution consisting of 9.6 ml H_2O, 5 ml 30% acrylamide/bisacrylamide, 5 ml Tris (1.5 M, pH 8.8), 200 µl 10% SDS, 200 µl 10% ammonium persulfate, and 12 µl tetramethylethylenediamine (TEMED). After the separating gels solidify (approximately 30 to 60 min), the stacking gel solution (5% acrylamide) is added to the top of the separating gel. For two gels, prepare a 5-ml solution consisting of 3.44 ml H_2O, 833 µl 30% acrylamide/bisacrylamide, 625 µl Tris (1 M, pH 6.8), 50 µl 10% SDS, 50 µl 10% ammonium persulfate, and 5 µl TEMED.

We use SDS-PAGE buffer as the electrophoresis buffer (for recipe, see later). After centrifugation, pellets are dissolved in 10 µl of 1 mol/liter Tris and mixed with 100 µl of extraction buffer (8 mol/liter urea, 2% SDS, 5% sucrose, 5% 2-mercaptoethanol, 0.02%

bromphenol blue). A SDS-PAGE protein standard (i.e., Bio-Rad, Richmond, CA) should be loaded on each gel in a separate lane. To avoid the interference by different exposure durations and variable membrane conditions, we use lipopolysaccharidepretreated NIH/3T3 cell lysates as a positive control and also to standardize results between different experiments. Standard size gels are electrophoresed at 130 V at 23° for 1.5 h. After complete electrophoresis, the proteins are then transferred to polyvinylidene fluoride membranes (Immobilon P, Millipore Bedford, MA). The membrane is then soaked for 5 s in methanol and washed briefly in H_2O. The gel, transfer membrane, and filter paper are then soaked in transfer buffer (for recipe, see later) for 5 min.

For transferring, mount the following layers in order from bottom (anode of transfer apparatus) to top (cathode of transfer apparatus): one buffer-soaked thick filter paper, the transfer membrane, the gel, and one buffer-soaked thick filter paper. Air bubbles between these layers should be avoided and removed by gently rolling a glass pipette over the transfer membrane. Negatively charged proteins will move downward (from the gel into the membrane). The proteins are transferred at 105 V for about 105 min at 4°C. Blocking of unspecific binding sites is achieved by incubating the membrane in PBS with 0.1% Tween and 5% milk for 0.5 h at room temperature or overnight at 4°C.

The membranes are then incubated with rabbit antiphospho-specific Thr^{853}-MBS polyclonal antibody (Biosource) and rabbit anti-MBS polyclonal antibody (Covance). Bands are visualized using the ECL detection kit (Amersham Corp./New England

Nuclear). ROCK activity is expressed as the ratio of pMBS in each sample per pMBS in each positive control divided by MBS in each sample per MBS in each positive control.

3.4.3.3 Reagents

Reagents Fixative solution: 50% trichloroacetic acid (Sigma), 50 mM dichlorodiphenyltrichloroethane (Sigma), protease inhibitors (Calbiochem, EMD Biosciences, Inc., Darmstadt, Germany), 1 mM phenylmethylsulfonyl fluoride, and 1 mM NaF. The last three substances should be added immediately before use.

SDS-PAGE buffer: 10x (use dilution 1x): 250 mM Tris, 1.92 M glycine, and 1% SDS, pH 8.3.

Western blot transfer buffer: 1x: 25 mMTris, pH 8.3, 190 mMglycine, and 10% methanol.

PBST: pH 7.4: 0.1% Tween 20 dissolved in PBS.

3.5 Statistics

Categorical variables are expressed as percentages of the corresponding population and continuous variables as means ± standard deviation. Values of $p<0.05$ were considered to indicate statistical significance. One-Way Analysis of Variance (ANOVA) was used for comparing of mean values of continuous variables among groups, and post-hoc analysis was performed by Scheffe's test to examine for inter-group differences. ROCK

activity was adjusted for age between different groups as healthy control subjects were inevitably younger than other diseased controls. Univariate linear regression (Pearson and Spearman's correlation) models were used to assess the relation between parametric clinical variables and ROCK activity. All variables with a significant association but did not exhibit excessive collinearity with each other were evaluated for inclusion in a stepwise multiple regression analysis model using ROCK activity as the dependent variable. Receiver operating characteristics (ROC) analysis was performed to determine the best cutoff value of ROCK activity and other biomarkers to following up outcomes in the patient corhort. Multivariate Cox regression analysis was performed to investigate for independent predictors of death outcomes. Event-free survival (days alive) was estimated by the Kaplan – Meier method and compared between groups by the log-rank test. All statistical analyses were conducted with the SPSS statistical package for Vista version 15.0 (SPSS Inc., Chicago, Illinois).

CHAPTER 4

ROCK ACTIVITY

IN

ACUTE CORONARY SYNDROME

CHAPTER 4. ROCK ACTIVITY IN ACUTE CORONARY SYNDROME

4.1 Introduction

Patients presenting with acute coronary syndrome (ACS) have a wide spectrum of risk for death and new ischemic events [234, 235], given that this wide definition includes both ST elevation myocardial infarction (STEMI), non-ST elevation myocardial infarction (NTSEMI) and unstable angina (UA). Several scores have been proposed to assess this heterogeneous risk amongst ACS patients. One of the most frequently used is the GRACE risk score for ACS patients, which is based on clinical and ECG variables, with troponin T (TnT) and creatinine phosphokinase as biomarkers. Nowadays, several cardiac biomarkers have been explored as useful predictors of risk in patients diagnosed of ACS. The most widely studied biomarkers are C-reactive protein (CRP), an acute-phase reactant produced by hepatocytes in response to stimulation by inflammatory cytokines and amino terminal pro–B-type NPs (NT-proBNP) which is released when increased myocardial stretch secondary to ischemia induced left ventricular systolic and/or diastolic dysfunction[236].

Being discovered in 1996, Rho-kinase (ROCK) is one of the effectors of the small GTP-binding protein Rho which regulates a wide range of fundamental cell functions such as contraction, motility, proliferation, and apoptosis.[237 238] In endothelial cells, Rho/ROCKs activation was reported to play a role in oxidized LDL-induced endothelial cell contractility[26] and in modulation of endothelial fibrinolytic activity.[27] In vascular smooth muscle cells, the Rho/ROCKs system is involved in smooth muscle cells proliferation

and migration, AII-induced expression of monocyte chemoattractant protein-1[57] and plasminogen activator inhibitor type-1.[70] In fibroblasts and inflammatory cells, activation of Rho signaling pathways has been shown to induce the transcriptional activity of serum response factor, activator protein-1 and nuclear factor kappa-light-chain-enhancer of activated B cells, which are potentially important in atherogenesis.[239, 240] In addition, a recent study indicated that ROCK1 plays a key role in macrophage chemotaxis, cholesterol uptake, and foam cell formation, all of which are hallmark events in the pathogenesis of atherosclerosis.[64] Inflammation plays a critical role in endothelial cell function that promotes systemic or local thrombosis. It is characterized by the activation of vascular wall cells and circulating leukocytes leading to recruitment and infiltration of inflammatory cells into the vessel wall[241] and this process has been proven to be mediated by ROCK1.[65] Growing evidence suggests that the Rho/ROCK system may play an important role in the pathogenesis of atherosclerosis involving in vascular smooth muscle cells contraction,[12] platelet aggregation and activation,[242] regulation of endothelial nitric oxide synthase synthesis[243] and other important steps in the inflammatory process,[65, 241] all of which may lead to acute coronary syndromes (ACS).[68, 201, 244] ROCK activity in leukocytes was proved increased by smoking habit which may reflect ROCK activity in vascular endothelial cells and endothelial function, suggesting an interaction between ROCK activity and endogenous NO.[245] After acute myocardial infarction cardiomyocytes within the infarcted areas, as well as morphologically normal cardiomyocytes in the border zones of infarction, lose their membrane phospholipid asymmetry.[246] Recently, a novel signaling route of Rho-ROCK-flippase signaling was proved to maintain asymmetrical membrane phospholipid

distribution in cardiomyocytes.[88] Thus, ROCK is emerging as a potential therapeutic target in cardiovascular disease. Preclinical studies showed that inhibition of Rho/ROCK pathway can limit early atherosclerotic plaque development,[66] reduce the size of ischemic-reperfusion injury[90] and protect against vasopressin.[77] However, it is uncertain whether ROCK activity is elevated in patients during the acute phase of myocardial ischemia. Therefore, the objective of the present study was to investigate whether ROCK activity is elevated in ACS patients and factors associated with increased ROCK activity in ACS were examined. We also hypothesized that a multimarker approach with the simultaneous assessment of CRP, NT pro-BNP and ROCK would provide complementary information to GRACE risk score in terms of prognosis in a population of patients with the diagnosis of ACS.

4.2 Method

4.2.1 Study subjects

173 consecutive patients (61% men; aged 68±13) admitted to a university teaching hospital for ACS were enrolled between December 2007 and May 2009. ACS was diagnosed based on the ACC/AHA guideline.[247] Patients were divided into ST-elevation myocardial infarction (STEMI), non-STEMI (NSTEMI) and unstable angina (UA) groups. Patients received standard management as recommended for ACS, with regard to aspirin, clopidogrel, low molecular weight heparin, glycoprotein IIb, IIIa inhibitors, b-blockers, statins and ACE inhibitors, as appropriate. Calculation of the GRACE score based on clinical history, ECG and laboratory values upon first arrival to the CCU or the

acute medical admissions unit.[248] All the patients were followed up till 15th March 2010 (mean: 15.4±7.6 months, from 0.5 month to 27.5 months) or until the occurrence of major events (death from any cause, readmission with ACS or admission with congestive heart failure). Congestive heart failure was defined as hospitalization for a clinical syndrome involving at least two of the following: paroxysmal nocturnal dyspnea, orthopnea, elevated jugular venous pressure, pulmonary crackles, third heart sound and cardiomegaly or pulmonary oedema on chest x-ray. If the patient is rehospitalized for ACS or CHF, but finally dying during hospitalization, the event will be recorded as ACS or CHF. The clinical signs and symptoms must have represented a clear change from the normal clinical status, requiring intravenous diuretics, inotropic support or vasodilator therapy. Fifty-one volunteers were subdivided into disease control group (n=31) (76% men; aged 69±8 yrs) and healthy control group (n=20) (33% men; aged 57±8 yrs) depending on the presence or absence of hypertension or smoking status which have been proved to influence ROCK activity.[229-231] All disease and normal control subjects had normal epicardial coronary arteries on angiography. Effect of statins was statistically adjusted. Written informed consents were obtained from all subjects.

4.2.2 Laboratory

Blood samples were collected in the first 12 hours after admission, usually the next morning at 8:00 with the patient fasting. All samples were collected into EDTA-Na tubes without stasis and were centrifuged at 2, 200g for 20 min within 30 min of sample collection. Plasma and serum samples were collected into aliquots and stored at -80°C

until batch analysis. Plasma samples were used for measurement of NT-proBNP, using a Roche Diagnostic proBNP assay on an Elecsys 2010 analyser (Roche Diagnostics, Mannheim, Germany). Total assay precision ranges from 1.8% at 800 pmol/L to 2.7% at 20.7 pmol/l and the detection limits are 0.6 and 4130 pmol/l (to convert pmol/l to pg m/L, multiply pmol/l values by 8.457).

4.2.3 Assay for leukocyte Rho-Kinase activity

Leukocytes were isolated from 10 ml peripheral blood at the admission following a validated and standardized protocol.[249] The leukocytes were frozen and stored at -80°C until all samples were collected. The ROCK assays were performed on all leukocytes samples at the same time. The samples were analyzed by Western blotting for the phosphorylation of the myosin-binding subunit (MBS) of myosin light-chain phosphatase with an antibody that specifically recognizes phosphorylated Ser853 MBS.[249] Inter-experimental results were standardized to lysophosphatidic acid–induced MBS phosphorylation (positive control).

4.2.4 Statistical analysis

All statistical analyses were conducted with the SPSS statistical package for Vista version 15.0 (SPSS Inc., Chicago, Illinois). Patients were divided into four quartilies of NT-proBNP, ROCK activity and GRACE score at the time of admission. One-Way Analysis of Variance (ANOVA) was used for comparing of mean values of continuous variables among groups, and post-hoc analysis was performed by Scheffe's test to

examine for inter-group differences. Dummy variable was used to adjusted age effect between different groups because healthy control subjects were inevitably younger than other diseased controls and ACS subgroups. Univariate linear regression (Pearson and Spearman's correlation) models were used to assess the relation between parametric clinical variables and ROCK activity. Multivariate Cox regression analysis using the enter method was used to look at the independent predictors of clinical endpoint. We compared the predictive accuracy of the NT-proBNP, GRACE score, ROCK activity and combined NT-proBNP/ROCK activity score using receiver operating characteristic (ROC) curves, analyses are under the curve (AUC). MedCalc version 11.4 was used for the comparison of different areas under the curve. To do an ROC curve analysis using NT-proBNP and ROCK activity together, we had to calculate weighted scores for each as follows: ($\beta 1$ x NT-proBNP) + ($\beta 2$ x ROCK activity), where $\beta 1$ and $\beta 2$ denote estimates of β coefficient for the NT-proBNP and ROCK activity obtained from the multivariate cox regression model. Event rates for clinical outcomes were also determined using the Kaplan-Meier method and compared using the log rank test. Data were expressed as mean±SD and two-sided p value of $p<0.05$ was considered statistically significant.

4.3 Results

4.3.1 Clinical characteristics

The baseline clinical characteristics and laboratory parameters of the subjects in different groups were summarized in Tables 4.1. All the ACS groups (STEMI, NSTEMI

and UA) and the disease control group were matched for age, gender, smoking status. No significant difference of the medications was observed among ACS groups. Systolic blood pressure was similar in the ACS groups and disease control group. There was no significant difference among the ACS groups, disease control and normal control groups for their body mass index, diastolic blood pressure and heart rate. The left ventricular ejection fraction of the STEMI and NSTEMI groups were significantly lower than the UA group, disease control group and the healthy control group (all $p<0.05$). The fasting glucose, white blood cell (WBC) level, plasma creatinine level, peak cardiac troponin T and peak creatine phosphokinase (CPK) were higher in all the ACS groups (all $p<0.05$) than the disease control and normal control groups. In contrast, the eGFR was lower among the ACS patients (all $p<0.05$).

Table 4.1 Baseline Characteristics of Acute Coronary Syndrome and Control Subjects

Baseline Characteristic	**STEMI (n=81)**	**NSTEMI (n=68)**	**UA (n=27)**	**Disease Control (n=31)**	**Normal Control (n=20)**	**ANOVA *p***
Age(yrs)	63±14†	72±10	72±10	69±8	57±8*	<0.001
Gender (male)	41(68%)	38 (63%)	12 (46%)	16 (76%)	10 (33%)‡	0.003
Current Smoker	18 (30%)	9 (15%)	4 (15%)	4 (19%)	0	NA
Medications						
Hypertension	31 (52%)	39 (65%)	15 (58%)	17 (81%)	0	NA
DM	17 (28%)	20 (33%)	9 (34%)	0	0	NA
Hyperlipidemia	9 (15%)	10 (17%)	6 (23%)	0	0	NA
CVA	4 (7%)	3 (5%)	3 12%)	0	0	NA
Chronic renal failure	5 (8%)	3 (5%)	2 (8%)	0	0	NA
LVEF (%)	46.5±9.6§	50.2±12.0	58.0±10.0	64.2±4.1	69.5±5.5	<0.001
SBP (mmHg)	135±29	147±29	151±30	143±20	128±23‖	0.007
DBP (mmHg)	76±18	77±18	77±15	82±10	76±11	0.857
HR (/minute)	80±21	87±29	78±19	69±13	87±12	0.080
BMI (kg/m^2)	23.9±3.4	24.2±3.6	25.8±2.9	25.3±8.5	23.6±3.2	0.629
Heart failure symptom on presentation	11 (16%)	21 (31%)	4 (15%)	NA	NA	NA
Laboratory test						
Fasting glucose	7.6±1.9§	6.6±1.7	5.8±0.9	5.2±0.8	5.1±0.3	<0.001
TC (mmol/l)	4.9±1.1	4.7±1.2	4.2±0.9	4.8±0.8	5.1±0.7	0.050
LDL-C (mmol/l)	2.8±1.0	2.9±1.0	2.4±0.8	2.9±1.0	3.0±0.5	0.119
TG (mmol/l)	1.7±1.3	1.6±1.0	1.6±0.8	1.7±1.9	1.3±0.6	0.317

HDL-C (mmol/l)	1.3±0.5	1.2±0.4	1.2±0.3	1.4±0.4	1.5±0.4	0.051
WBC (10^9/l)	13.1±3.8#	11.5±4.4§	8.6±3.3	8.4±3.7	5.5±1.1	<0.001
Creatinine (µmol/l)	154±187	155±97	183±184	91±13**	72±16**	0.031
eGFR	67.2±31.3	57.4±26.5	53.4±25.3	79.8±2.5**	82.8±4.1**	0.023
Peak cTnT (µg/l)	3.80±5.77†	0.76±1.12	0.023±0.03	NA	NA	<0.001
Peak CPK (U/l)	2392.7±2026.0*	658.0 ± 1397.0	110.7±80.1	127.3±62.9	NA	<0.001

*p<0.05 vs NSTEMI, UA & Disease Control

† p<0.05 vs NSTEMI & UA

‡ p<0.05 vs ETEMI & Disease Control

§ p<0.05 vs UA, Disease Control & Normal Control

|| p<0.05 vs NSTEMI

# p<0.05 vs NSTEMI, UA, Disease control & Normal Control

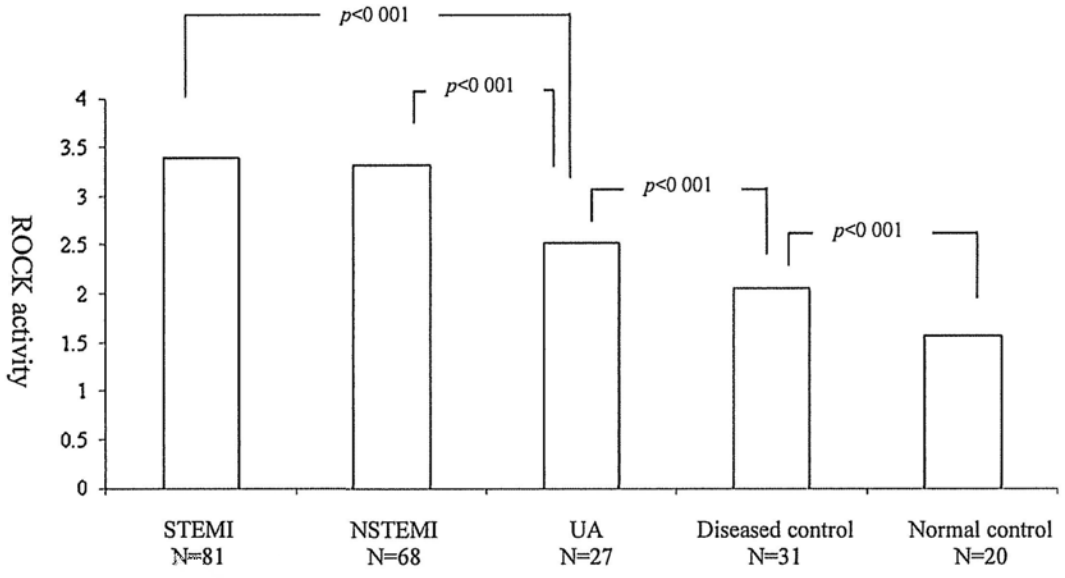
** p<0.05 vs STEMI, NSTEMI & UA

All results are presented as mean ± SD.

4.3.2 ROCK Activity in ACS and control subjects

In all the ACS subgroups, the ROCK activity (STEMI=3.31±0.93, NSTEMI=3.36±1.04 and UA =2.52±0.59) was significantly higher than the disease control and normal control groups (disease control=2.06±0.38 and normal control=1.57±0.43) (all $p<0.001$). Interestingly, there was no significant difference of ROCK activity between the STEMI and NSTEMI groups, though they were significantly highly than the UA group (both $p<0.001$). Although the disease control group showed a mild but significant elevation of ROCK activity than the normal control group ($p<0.001$), it remained much lower than all the 3 ACS groups (figure 4.1).

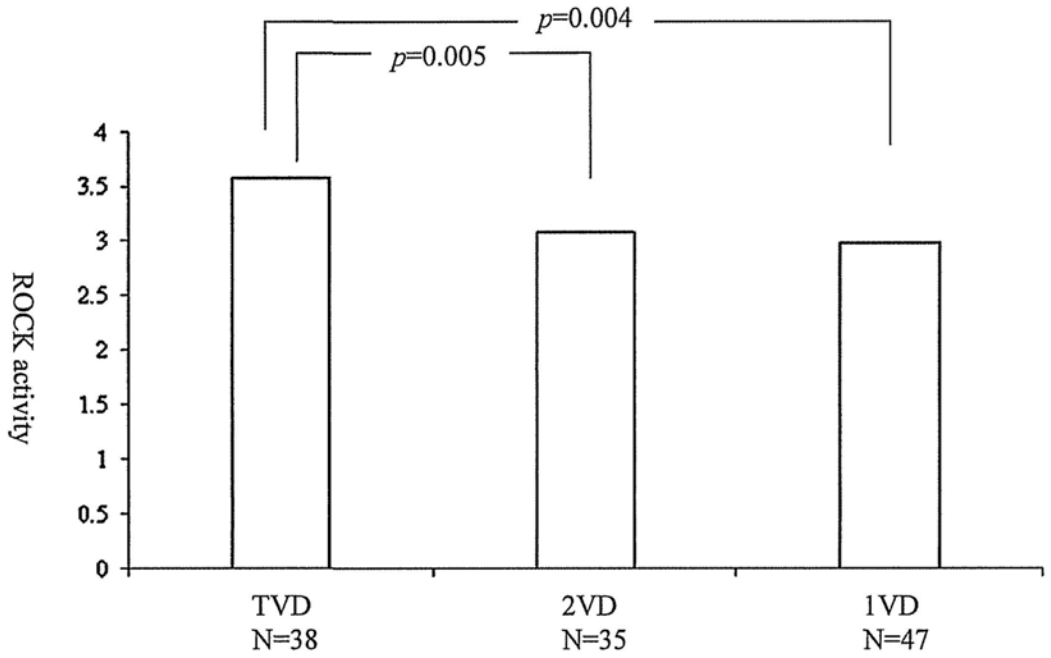
Figure 4.1 Comparison of different ACS groups, disease control and normal control. ROCK activity in different ACS groups, disease control and normal control groups (Mean ± SD). Leukocyte ROCK activity was measured as percent staining of phosphorylated (p- and phospho-) MBS of myosin light-chain phosphatase (pThr853-MBS) relative to the staining of total (t) MBS.



4.3.3 ROCKs activity and number of diseased coronary arteries

Among ACS patients, those with triple-vessel disease (3.58±1.08) had significantly higher ROCK activity than those with two-vessel (3.01±0.79) and single-vessel disease (2.97±0.88) (both $p<0.05$). However, no difference was observed between the latter 2 groups (Figure 4.2).

Figure 4.2 Comparison of ROCK activity in ACS subgroups with different diseased vessel number.



4.3.4 Independent predictors for ROCK activity in ACS subjects

Univariate analysis demonstrated positive correlation of ROCK activity with age (r=0.285; $p<0.001$), current smoking status (r=0.143; $p=0.05$), heart failure symptom on presentation (r=0.211; p=0.01), peak cTnT (r=0.403; $p<0.001$), peak CPK (r=0.301; $p<0.001$), plasma creatinine (r=0.260; $p<0.001$), fasting glucose (r=0.164; $p=0.04$), baseline WBC (r=0.375; $p<0.001$), low-density lipoprotein-cholesterol (LDL-C) (r=0.270; $p=0.001$), CRP (r=0.277; $p<0.001$), NT-proBNP (r=0.296; $p<0.001$) and number of diseased coronary vessel (r=0.383; $p<0.001$) (Table 2). In contrast, there were negative correlations of ROCK activity with LVEF (r=-0.387; $p<0.001$) and eGFR (r=-0.209; $p=0.01$). On multivariate analysis, heart failure symptom on presentation ($p=0.002$), LDL-C level ($p=0.001$), number of diseased coronary vessels ($p=0.048$) were independent predictors for the ROCK activity in ACS patients (Table 4.2).

Table 4.2 Prediction of ROCK Activity in Univariate and Multivariate regression models

Variables	Univariate		Multivariate (Stepwise)	
	Coefficient	*p*	β	*p*
Age (yrs)	0.285	<0.001	-	-
Gender (male)	0.056	0.410	-	-
Current smoker	0.143	0.050	-	-
Heart failure symptom on presentation	0.211	0.010	0.287	0.002
LVEF (%)	-0.387	<0.001	-	-
cTnT (μg/l) (peak)	0.403	<0.001	-	-
CPK (U/l) (peak)	0.301	<0.001	-	-
Creatinine (μmol/l)	0.260	<0.001	-	-
eGFR (60ml/min/1.73m^2)	-0.209	0.010	-	-
Fasting Glucose	0.164	0.040	-	-
WBC (x10^9/l)	0.375	<0.001	-	-
LDL-C (mmol/L)	0.270	0.001	0.315	0.001
TC (mmol/l)	0.082	0.250	-	-
CRP	0.277	<0.001	-	-
NT-proBNP	0.296	<0.001	-	-
Number of diseased coronary vessels	0.383	<0.001	0.227	0.048
Current usage of Statin	-0.118	0.137	-	-

4.3.5 Univariate and multivariate predictors of clinical endpoint

Totally, there were 23 deaths, 33 readmissions with ACS and 13 admissions with CHF within 2 years. Figure 4.3 shows distribution of 2 years cardiovascular event according to different quartilies of NT-proBNP, ROCK activity and GRACE score. The highest ROCK activity and NT-proBNP consistently predicted clinical endpoint. As shown in figure 4.3, the distribution of 2 years endpoint rates in the different NT-proBNP, ROCK activity or the GRACE score quartilies demonstrated a consistent gradient of risk. Only the quartily 1 of GRACE score group has higher event rate than quartily 2 group (38.5% vs 22.9%). Table 4.3 shows the univariate correlation between different clinical variables and long-term cardiovascular endpoint. PCI treatment in admission (HR: 0.253; 95% CI: 0.118-0.543), ROCK activity (HR: 1.383; 95%CI: 1.010-1.894), NT-proBNP (per 200pg/ml increased) (HR: 1.003; 95%CI: 1.001-1.005) and Statins usage after discharge (HR: 0.426; 95%CI: 0.213-0.851), were independent predictors for long-term clinical endpoint (table 4.4).

Figure 4.3 Comparison of event rate of NT-proBNP, ROCK activity and GRACE score in different quartily.

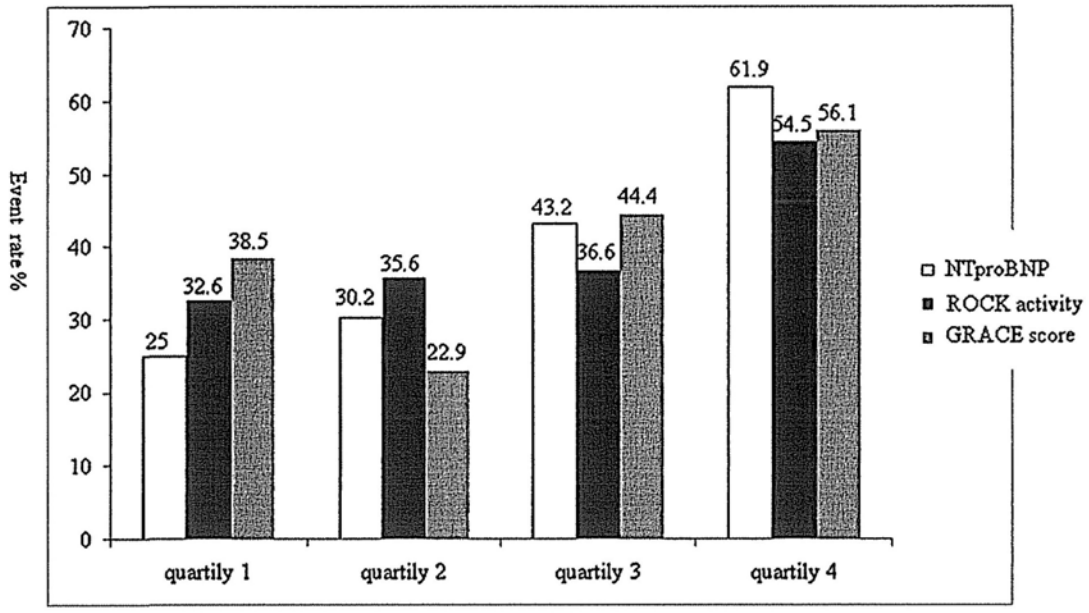


Table 4.3 Baseline clinical characteristics between no event and event groups

Baseline Characteristic	**No event (n=104)**	**Event (n=69)**	**ANOVA *p***
Age(yrs)	65±12	73±13	<0.001
Gender(male)	73 (78%)	37 (59%)	0.020
Current Smoker	24 (23%)	12 (17%)	0.406
Heart failure on admission	8 (8%)	27 (39%)	<0.001
PCI done during hospitalization	75 (72%)	20 (29%)	<0.001
Medical history			
Hypertension	59 (57%)	38 (55%)	0.578
DM	20 (19%)	29 (42%)	0.001
Hyperlipidemia	17 (16%)	13 (19%)	0.393
CIHD	14 (13%)	20 (29%)	0.011
Clinic Test			
LVEF (%)	51±10	47±13	0.028
SBP (mmHg)	145±27	142±32	0.868
DBP (mmHg)	80±18	75±17	0.045
HR (/minute)	83±24	82±24	0.864
BMI (kg/m^2)	24±3	24±4	0.737
Laboratory test			
Fasting glucose (mmol/l)	6.8±1.7	6.8±2.0	0.893
TC (mmol/l)	4.8±1.2	4.5±1.1	0.018
LDL-C (mmol/l)	3.0±1.0	2.5±0.9	0.004
TG (mmol/l)	1.7±1.2	1.7±1.0	0.998
HDL-C (mmol/l)	1.3±0.4	1.2±0.4	0.493
WBC ($10^9/l$)	11.8±4.3	11.6±4.2	0.768
Creatinine (μmol/l)	111±46	218±202	<0.001
eGFR (ml/min/1.73 m^2)	61±21	46±32	<0.001
TnT (peak)	1.7±2.9	2.5±5.6	0.440
CPK (peak) (U/l)	1721±2049	830±1485	0.002
ROCK activity	3.07±0.83	3.49±1.17	0.011
NT-proBNP (pg/ml)	3169±7479	15749±31329	<0.001
CRP (mg/l)	26.2±36.7	37.9±49.2	0.074
GRACE score	0.29±0.15	0.34±0.16	0.026

Table 4.4 Univariate and multivariate analyses of clinical endpoint

Variables	Univariate		Multivariate (enter)	
	Coefficient	***p***	**HR**	**95%CI**
Gender (male)	-0.169	0.020	-	-
PCI treatment in admission	-0.420	<0.001	0.253	0.118-0.543
DM past medical history	0.248	0.001	-	-
CIHD past medical history	0.191	0.011	-	-
LVEF (%)	-0.177	0.028	-	-
Baseline ROCK activity	0.208	0.011	1.383	1.010-1.894
NT-proBNP (per 200pg/ml increased)	0.312	<0.001	1.003	1.001-1.005
GRACE score	0.190	0.026	-	-
Statins	-0.321	<0.001	0.426	0.213-0.851
Regular Nitrates	0.241	0.002	-	-
Diuretics	0.297	0.001	-	-

4.3.6 Predictive accuracy of ROCK activity and NT-proBNP levels (ROC and incremental statistic analysis)

We compared the predictive accuracy of the ROCK activity, NT-proBNP and the combined use of NT-proBNP/ROCK activity by using ROC curves. Individual AUC values were obtained for different combinations of clinical endpoint (Table 4.5). NT-proBNP demonstrated better discriminatory accuracy (AUC: 0.684; 95% CI: 0.601-0.767) in predicting the majority of clinical endpoint compared to the ROCK activity (AUC: 0.599; 95% CI: 0.511-0.687) and GRACE score (AUC: 0.612; 95% CI: 0.535 - 0.685). Interestingly, the combined use of ROCK activity and NT-proBNP (AUC: 0.692; 95% CI: 0.607-0.773) always performed better than ROCK activity, GRACE score and NT-proBNP alone (table 4.5). Combined ROCK activity and NT-proBNP showed significantly higher AUC than GRACE score alone (p=0.041) (Table 4.6 and Figure 4.4). We also used another method adjusting PCI, statinusage, LVEF, eGFR, GRACE score, DM past history and gender. The areas under the ROC curve values of old (NT-proBNP) and new (NT-proBNP combined with ROCK activity) models for long-term event were 0.815 and 0.825 respectively (Figure 4.5). The p valure is 0.0364(Figure 4.5). From the incremental statistics results, combination of ROCK activity and NT-proBNP significantly predicted long-term outcome more accurately when comparing with ROCK activity or NT-proBNP alone (p=0.022) (Figure 4.6).

Table 4.5 Area under the ROC curve values for prediction of clinical endpoint

	AUC	**95% CI**	**Sensitivity (%)**	**Specificity (%)**	***p***
ROCK activity	0.599	0.522 - 0.673	56.5	56.7	0.028
GRACE score	0.612	0.535 - 0.685	59.3	61.5	0.013
NT-proBNP	0.684	0.609 - 0.752	63.8	67.3	<0.001
ROCK&NT-proBNP	0.692	0.615 - 0.758	65.2	64.4	<0.001

Table 4.6 Comparison of areas under the ROC curve values for prediction of clinical endpoint

AUC comparison	**Difference between areas**	***p***
GRACE vs NT-proBNP	0.0722	0.065
GRACE vs ROCK activity	0.0123	0.790
GRACE vs ROCK&NT-proBNP	0.0783	0.041
NT-proBNP vs ROCK activity	0.0844	0.105
NT-proBNP vs ROCK&NT-proBNP	0.0061	0.414
ROCK activity vs ROCK&NT-proBNP	0.0906	0.060

Figure 4.4 Comparison of areas under the ROC curve values of ROCK activity, GRACE score, NT-proBNP and combined ROCK activity and NT-proBNP for long-term event.

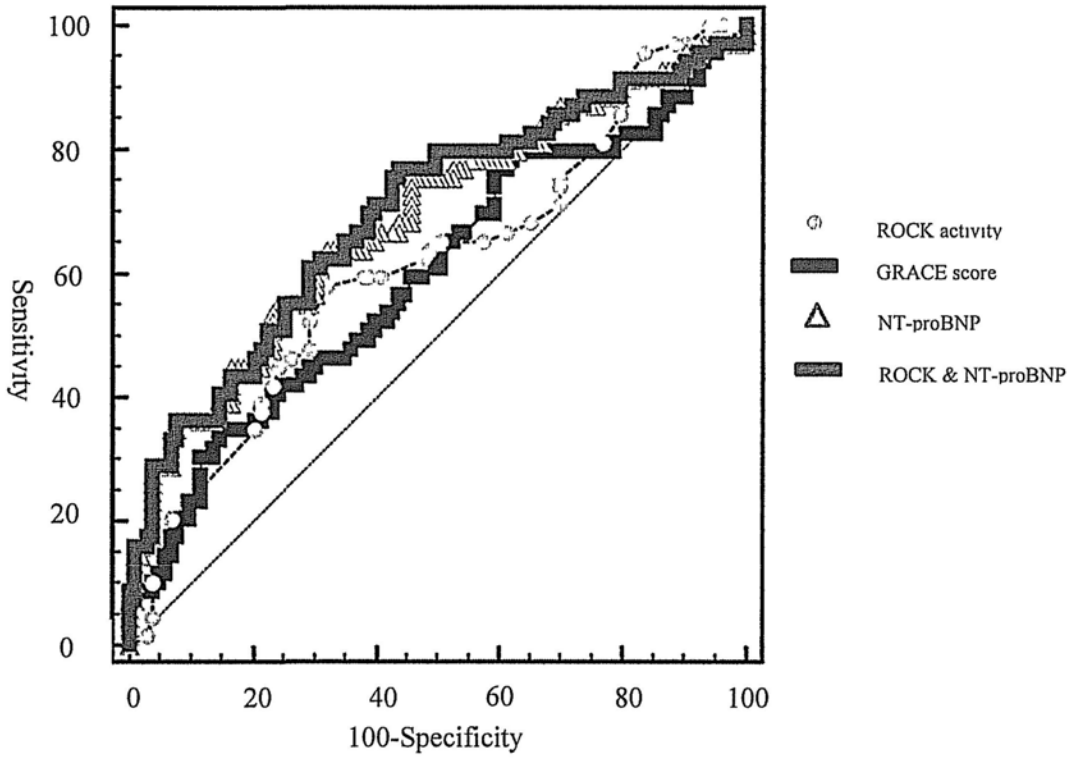


Figure 4.5 Comparison of areas under the ROC curve values of old (NT-proBNP) and new (NT-proBNP combined with ROCK activity) models for long-term event. The blue line is represented the old model adjusting PCI, statinusage, LVEF, eGFR, GRACE score, DM past history and gender. The red line is represented the new model adjusting statinusage, LVEF, eGFR, GRACE score, DM past history and gender.

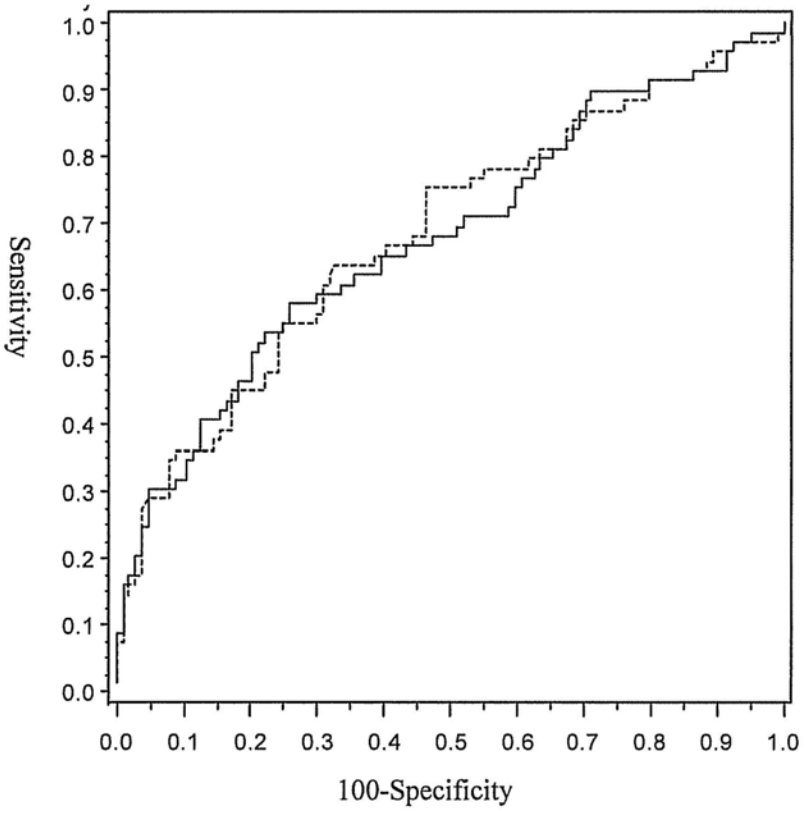
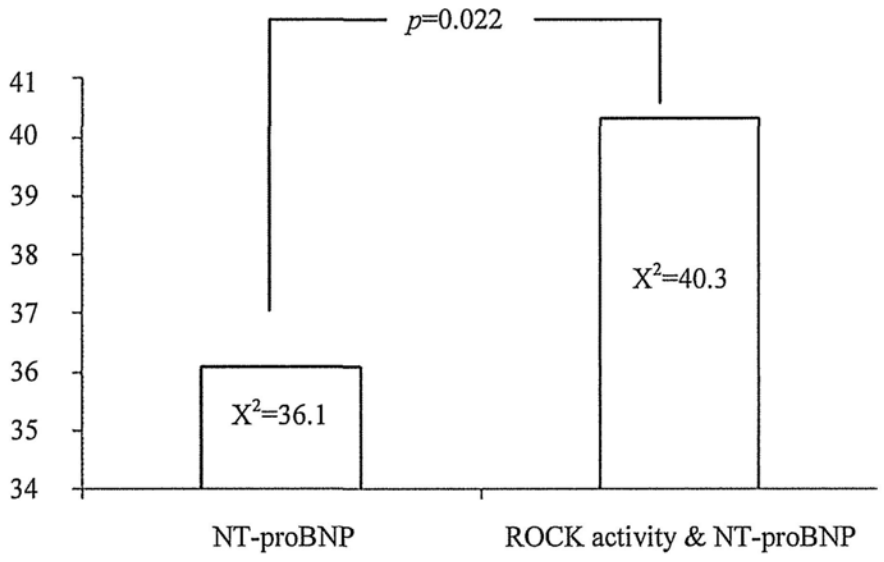


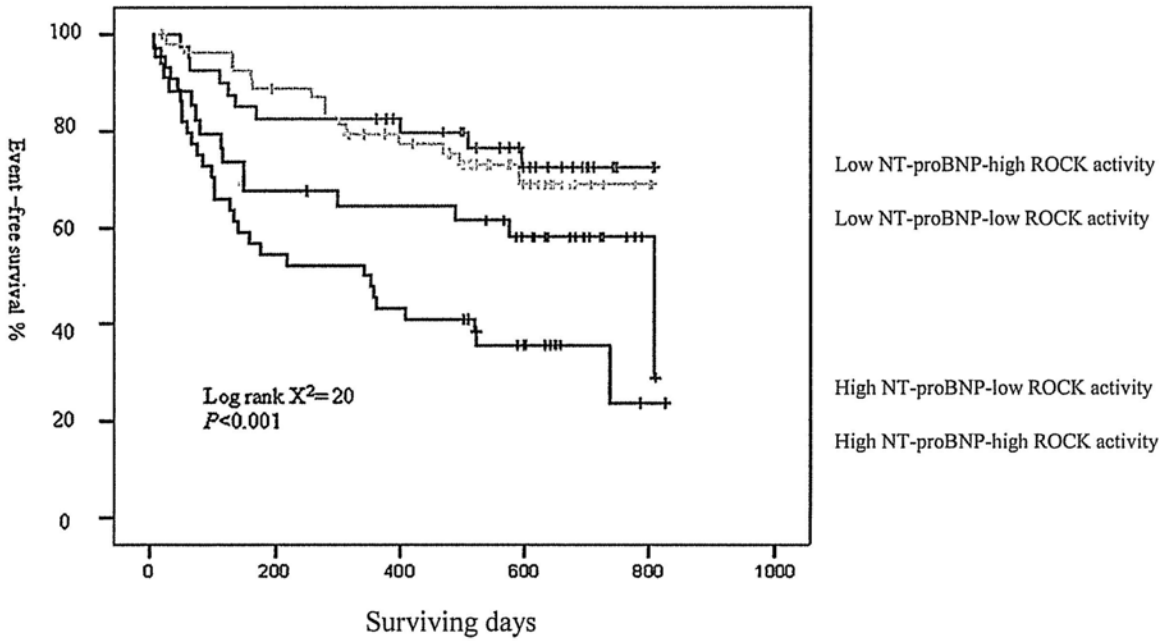
Figure 4.6 Incremental predictive valve of combining the NT-proBNP and ROCK activity for long-term event on top of the individual NT-proBNP predictor.



4.3.7 The ROCK activity and NT-proBNP as a composite measure

In this analysis, we assessed whether using ROCK activity and NT-proBNP synergistically would improve risk prediction. We used a NT-proBNP cut-off level of 1986 pg/ml and a ROCK activity cut-off value of 3.03 in our study population (best cut-off value for long-term event). As demonstrated by the Kaplan-Meier survival curves (figure 4.7), patients with a high NT-proBNP -high ROCK activity on admission were approximately five times more likely to experience a cardiovascular event at around two years (RR: 5.156; 95% CI: 2.180-12.191) compared to those with low NT-proBNP and low ROCK activity. In addition, patients with high NT-proBNP-high ROCK activity were also more likely to die or experience a cardiovascular event at two years compared to those with high NT-proBNP-low ROCK activity (RR: 2.624; 95% CI: 1.035-6.651) (figure 4.7).

Figure 4.7 Kaplan-Meier survival curves for cardiovascular events. High NT-proBNP defined as 1986 pg/ml (sensitivity is 63.8% and specificity is 67.3%) and high ROCK activity defined as 3.03 (sensitivity is 56.5% and specificity is 56.7%). High NT-proBNP-high ROCK activity group has higher risk (RR: 5.156; 95% CI: 2.180 to 12.191) to low NT-proBNP-low ROCK activity group. Similarly, high NT-proBNP-high ROCK activity group has higher risk (RR: 2.624; 95% CI: 1.035 to 6.651) to high NT-proBNP-low ROCK activity group. Log-rank=20, $p<0.001$.



4.3.8 Diagnostic power and predictive power

According to the sample size, SD and mean, the diagnositic and predictive powers were calculated in acute coronary symdrone study. If sample size is 10, the diagnostic power in this study is more than 96%. If sample size is 10, the diagnostic power in this study is more than 83%

4.4 Discussion

Our study demonstrated that peripheral leukocyte ROCK activity was increased in the ACS than normal or at-risk subjects, especially in those with elevated cTnT $\geq$0.1μg/l. In addition, heart failure symptom on admission, LDL-C level and number of diseased coronary vessels were independent predictors for ROCK activity in the ACS subjects. We also defined the utility of the combined measurement of baseline ROCK activity and NT-proBNP as biomarkers to predict adverse events. These findings support the hypothesis that the activation of ROCK activity may play a role in the pathogenesis of ACS and suggest that therapies that can inhibit ROCK may be clinically useful in the ACS management.

4.4.1 ROCK activity across spectrum of coronary artery disease

Elevated ROCK activity was proven to involve in cerebral and coronary vasospasm,[80, 83] hypertension,[183] pulmonary hypertension,[106] atherosclerosis[250] and metabolic

syndrome.[251] Studies also showed that ROCK activity is involved in the cellular and molecular events of ischemic cascade, such as inflammation,[65, 241, 252] artherosclerotic plaque,[244] coronary vasospasm and ischemic-reperfusion injury[90, 93] and many of these effects can be blocked by pharmacological inhibition of ROCK in animal models.[1, 172] In addition, Feska and colleagues have firstly demonstrated that ROCK activity was also increased in acute stroke patients, suggesting its pathogenesis role in acute ischemia.[253] Our findings corroborate and extend the above by demonstrating that after adjusting for age, there is a gradation of ROCK activity from being normal in the healthy control, mildly elevated in at-risk group without ACS to those with unstable angina and myocardial infarction. Interestingly, there was no significant difference in ROCK activity between STEMI and NSTEMI in presence of difference in peak cTnT levels or myocardial injury. Previous study has provided that ROCK-2 phosphorylated the Tn complex, most likely at cTnT, which means Rho/ROCK pathway involved during the whole progress of myocardial injury.[19] Our results could not demonstrate a difference of ROCK activity between STEMI and NSTEMI, probably because ROCK activation is nonspecific, and occurs in many thrombotic, inflammatory, and malignant neoplastic disorders, including acute stroke.[253] However, it does support the common pathophysiology underlying both subsets of MIs with perhaps similar disease burden and inflammation.

4.4.2 Relation of ROCK activity with LDL-cholesterol and severity of coronary artery disease

One of our novel findings was that LDL-cholesterol and severity of coronary artery disease were another two independent predictors for ROCK activity in the ACS patients. Triple-vessel disease may represent more severe underlying atherosclerosis and inflammation. Accumulating evidence indicates that Rho-kinase is involved in the vascular effects of various vasoactive factors, including angiotensin II,[254] thrombin,[255] endothelin-1[256] and serotonin.[53] Thus, Rho-kinase may play an important role in the pathogenesis of arteriosclerosis directly by enhancing the process through activation of its substrates and indirectly by mediating the signal transduction of various vasoactive mediators. ROCK activity was proved enhanced by smoking habit which causes inflammation and oxidative stress in vascular wall. ROCK activity also was indicated a predictor of endothelial function.[245] Recently, ROCK1 has been proved playing a key role in macrophage chemotaxis, cholesterol uptake, and foam cell formation, all of which are hallmark events in the pathogenesis of atherosclerosis.[64] In LDL receptor knockout mice, atherosclerotic plaque developed after high-fat cholate-free diet was attenuated by inhibition of Rho kinase.[66] Furthermore, long-term inhibition of ROCKs has been shown to cause marked regression of coronary arteriosclerosis and disappearance of coronary vasospastic activities in vivo in a pig model.[257] Rho/ROCK signaling inhibition by HMG-CoA reductase inhibitors (statins) offers a potential mechanism for some of the pleotropic effects of these agents.[258, 259] Importantly, Nohira et al. (2008) were first to demonstrate that statins inhibit ROCKs activity and improve endothelial function in patients with stable atherosclerosis.[68]

4.4.3 Combination of NT-proBNP and ROCK activity can identify a subset of ACS patients at particularly high risk

Multivariate analyses showed that both ROCK activity and NT-proBNP were independent predictors for long-term cardiovascular event. From the AUC and Kaplan-Meier curves suggest that NT-proBNP significantly predicts a poor prognosis in ACS patients over and above the ROCK activity. This suggests that both the ROCK activity and NT-proBNP may reflect somewhat different risk attributes in ACS which is supported by there being only a partial correlation between them. We also demonstrated that a high NT-proBNP level and a high ROCK activity when used together identified a subset of ACS patients who were at especially high risk of developing future cardiovascular event.

In this study, the GRACE score and CRP were also studied. The GRACE score was developed initially to predict in-hospital mortality[232] across the entire spectrum of ACS patients but recently its predictive power has also been demonstrated for longer term risk of death and myocardial infarction in this same patient population.[260] From our study, the GRACE score was significantly correlated with long-term event but it was not an independent predictor. It might because our sample size was small. On the other hand, in our corhort, in-hospital PCI was an independent predictor and strongly correlated with the long-term outcome. It appears to be better than the GRACE score in predicting long-term outcomes. In this study, clopidogrel was routinely used on those patients with the

treatment of PCI. So PCI treatment in hospital as a variable was put into the Cox regression but not clopidogrel. This might explain why the GRACE score was not chosen to be an independent predictor for long-term cardiovascular events. In this study, the GRACE model which was chosen for prediction was for the period from admission to 6 months but not from discharge to 6 months. In the former model, PCI done in the hospital and CIHD medical history were not involved. On the other hand, among these ACS patients, the usage of statins after discharge had a greater protection against death or major cardiovascular events, which is consistent with previous study.[261] However, the predictive value of GRACE score may be increased when combined with other biomarkers, such as NT-proBNP[262] or adrenomedullin.[263] It is probable that biomarkers might be more meaningful than GRACE score alone for predicting long-term outcome. Similarly, no correlation between CRP and long-term outcome was found. This might also due to the small sample size as previously this kind of acute response marker has been shown to be a strong independent predictor of future cardiovascular events.[264] CRP also correlates with the number of angiographically complex coronary artery stenosis. Similar results were found in this study between ROCK activity and the severity of coronary arterial disease. This probably indicates that ROCK activity might be a stronger predictor than CRP for future cardiovascular events.

Our study showed that both ROCK activity and NT-proBNP are the independent predictors for future cardiovascular event. NT-proBNP reflects ischemia as well as haemodynamics and the magnitude and duration of the increase in plasma concentrations of NT-proBNP after ACS are proportional to myocardial infarct size and

the degree of left ventricular dysfunction.[265] Clinical studies have clearly demonstrated that NT-proBNP levels are increased after episodes of ischemia: elevated NT-proBNP levels have been observed in patients with unstable angina[266] and during and after percutaneous coronary intervention. In the study by Ang DS, et al.[262] the AUC of BNP was around 0.701 which is a little higher than in ours. This might be because their sample size was larger and their following up period was short (only 6 months). The combination of NT-proBNP with GRACE score only increased the AUC area from 0.701 to 0.707 which is a smaller increase than ours (from 0.684 to 0.692). Here we analyzed our data in three different but complementary ways because each analysis had its strengths and weaknesses in this complex situation. The multiple regression analysis is best for determining whether ROCK activity independently adds to the NT-proBNP. The ROC curve helps determine how big an extra contribution ROCK activity makes to the NT-proBNP predictability. ROCK activity plus NT-proBNP makes an extra contribution to risk assessment over the NT-proBNP alone in multivariate analyses, and the ROC and incremental analyses shows that this extra contribution is significant. ROC analysis and incremental analyses are really designed to compare two single predictors and to compare a combination of predictors versus a single predictor. The Kaplan Meier curves emphasized combination of these two biomarkers can separate patients under different potential risks. Taking all three analyses into account, the message is that ROCK activity plus NT-proBNP predicts risk better than NT-proBNP alone. In summary, ROC analysis, multivariate analyses and Kaplan-Meier curves illustrate that ROCK activity plus NT-proBNP can identify risk better than NT-proBNP alone in the

majority of ACS patients. This is especially for the ACS patients who have high NT-proBNP but low ROCK activity.

Our observation that ROCK activity predicted clinical endpoint independent of the NT-proBNP suggests that ROCK activity represent different aspect from NT-proBNP in ACS patients. In CHF patients, ROCK was not an independent predictor but also can increase the predictive value for long-term mortality. One possibly relevant mechanism here is that inflammation and atherosclerosis are the main underlying mechanisms in ACS but not CHF so that the predicted value of ROCK activity in ACS seems better than in CHF.

Before these data, it might have been anticipated that NT-proBNP would predict mainly heart failure events while CRP would predict mainly ACS events. Interestingly, only NT-proBNP and ROCK activity appear to predict both ACS and heart failure events. Our study also demonstrates for the first time that patients with an elevation in only one of NT-proBNP or ROCK activity have a risk intermediate between those with a high NT-proBNP and ROCK activity and those with low values for both.

This study might help future research into tomorrow's ACS. In addition, we also demonstrated that both NT-proBNP and ROCK activity can be used synergistically to identify a subset of ACS patients who are at a particularly high risk of future cardiovascular events. Obviously further data are required to confirm these findings.

4.5 Future work

Although ROCK activity has been shown increased in many disease states including hypertension, angina, metabolic syndrome and stroke, there is a need to further define its role and prognostic values in various clinical diseases. There are still no data about ROCK activity of predicting future cardiovascular events and the changes of ROCK activity during long-term follow up. In this study, peripheral blood was collected at the time point of 6 months follow up. Further examinations of the variation of ROCK activity are pending. Furthermore, a longer period of follow up than 2 years in the current study should be further explored. It might be meaningful to clarify the precise role of ROCK activity in diagnosis and prognosis in other vascular diseases.

The established chemical antagonists of ROCK (Y-27632, H-1152, Wf-536, fasudil, and hydroxyfasudil) are non-specific ROCK1 and ROCK2 inhibitors, and they exhibit some nonspecific inhibition of other protein kinases. It is, therefore, not clear how ROCK1 and ROCK2 differ in their regulation and function. Future experiments utilizing gene targeting of ROCK1 and ROCK2 are necessary to provide more direct evidence of the role of these proteins in regulating biological processes in the EC, SMC or leukocyte. It is better to understand the differences between ROCK1 and ROCK2 that can lead to the development of more specific and effective therapeutics. For clinical development of future ROCK inhibitors in ischemic heart disease, therapeutic window and range of dosage will need to be further defined.

4.6 Limitation

ACS patients and subjects at-risk were older than healthy control subjects. Aging is known to associate with increased ROCK activity, due to excessive oxidative stress on peripheral vasculature.[231] We adjusted for age in our statistical analysis as healthy subjects >70 years old were difficult to find. Another main limitation of this study is patient numbers and the follow up period could be extended.

4.7 Conclusions

ACS is associated with increased ROCK activity for which heart failure symptom on presentation, LDL-C level and the number of diseased coronary vessel are independent predictors. In addition, we also demonstrated that both ROCK activity and NT-proBNP can be used synergistically to identify a subset of ACS patients who are at a particularly high risk of future cardiovascular events. A combination of these two biomarkers might inform us a better prediction for long-term event free survival than only NT-proBNP. This implies that both the NT-proBNP and ROCK activity reflect somewhat different risk attributes when predicting adverse prognosis in ACS and their synergistic use can enhance risk stratification in ACS to a small but potentially useful extent. Further studies are required to confirm if it is associated with therapeutic benefits in inhibition of ROCK activity in ACS patients.

CHAPTER 5

ROCK ACTIVITY

IN

CONGESTIVE HEART FAILURE

CHAPTER 5. ROCK ACTIVITY IN CONGESTIVE HEART FAILURE

5.1 Introduction

Cardiac hypertrophy leading to congestive heart failure (CHF) is a leading cause for human morbidity and mortality, and the incidence of heart failure has been constantly increasing during the past decades. Cardiac hypertrophy is an adaptive response of the heart to pressure or volume overload. This initial adaptive response becomes maladaptive, switching the heart from a compensated to decompensated state and finally leading to heart failure or sudden death due to decompensation.[267] The molecular response to pressure overload is complex and may include modulation of various intracellular signal pathways. Furthermore, pressure overload leads to the secretion of vasoactive peptides, such as angiotensin II and endothelin 1, which play pivotal roles in the induction of these hypertrophic responses.[111, 112] Recent studies suggest that the hypertrophic process is also mediated, in part, by an increase in myocardial oxidative stress.[202, 268] In the myocardium, Ras, Rho, and Rac are involved in the hypertrophic response.[269-271] On the other hand, vasoconstrictor neurohumoral systems, such as the renin-angiotensin-aldosterone system and the sympathetic nervous system, are important in the pathophysiology of congestive heart failure.[272] Studies have also revealed the importance of the Rho proteins and their associated kinases, Rho kinase (ROCK), plays a critical role in mediating the effects of RhoA on stress fiber formation, smooth muscle contraction, cell adhesion, membrane ruffling, cell motility and apoptosis.[273,274] Currently, there are 2 isoforms of ROCK, ROCK1 and ROCK2. Pharmacological inhibition of ROCK suggests an in vivo role for ROCK in the pathogenesis of cardiac

hypertrophy and remodeling.[275] Activation of ERK1/2 and of the cardiac transcription factor GATA-4 is identified as downstream nuclear mediators of ROCKs during myocardial cell hypertrophy.[121]

However, these inhibitors do not distinguish between ROCK1 and ROCK2, and could also have non-selective effects.[74] Recent genetic studies indicated that ROCK1 and ROCK2 have distinct non-redundant functions in cardiac hypertrophy and remodeling.[123, 124] Previously, many studies relied heavily on pharmacological inhibitors, and not much on gene deletion. Recently, ROCK1 was proved contributing to the development of cardiac fibrosis and induction of fibrogenic cytokines in cardiomyocytes in response to pathological stimuli via ROCK1knockout (ROCK1-/-) mice.[45] ROCK1 was proved inhibiting several pathological events including cardiomyocyte apoptosis in compensated hypertrophic hearts via genetic deletion. In addition, ROCK1 deficiency could prevent the transition from hypertrophy to heart failure from transgenic mice.[125] ROCK1 deficiency attenuated progression into heart failure by preserving chamber dimension and contractile function. [125] ROCK1 deficiency also suppressed increase in cardiomyocyte apoptosis and cardiac fibrosis while preserving cardiomyocyte hypertrophy.[125] Overexpression of ROCK1 alone was not sufficient to cause significant LV remodeling and cardiac dysfunction, but markedly increased animal death of Gαq mice under unstressed condition (without pregnancy) associated with increased cardiomyocyte apoptosis, cardiac fibrosis, and contractile dysfunction.[125] It supports the long-term beneficial effects of ROCK1 deficiency in hypertrophic decompensation and

suggests that ROCK1 may be an attractive therapeutic target to limit heart failure progression.

These findings suggest that ROCK-dependent signaling pathways may potentially contribute to the mechanism of systemic and renal vasoconstriction and cardiac hypertrophy in CHF. Indeed, cardiac-specific over expression of RhoA in mice resulted in a lethal form of heart failure, characterized by atrial enlargement, conduction defects, contractile failure, and generalized edema.[276] Similarly, Kobayashi et al.[117] demonstrated the importance of ROCK pathways in the induction of cardiac dysfunction and remodeling in the failing hearts of Dahl salt-sensitive rats with CHF, and Kishi et al.[185] proved that ROCK is involved in the increased forearm vascular resistance and impaired vasodilatation in patients with heart failure. The hypothesis of this study was to clarify the involvement of ROCK in CHF patients and whether ROCK predicts long-term mortality.

5.2 Method

5.2.1 Study subjects

Consecutive patients (52% men; aged 74 ± 12 yrs) admitted to a university teaching hospital (the Prince of Wales Hospital in Hong Kong) for CHF were enrolled between December 2007 and January 2009. 178 patients were recruited. CHF was diagnosed based on the ACC/AHA guideline.[277] All the patients were followed up till 1st February

2010 (14.4±7.2, 0.5M to 26M) or until the occurrence of cardiac death. Fifty-one volunteers were subdivided into disease control group (n=31) (76% men; aged 69±8 yrs) and healthy control group (n=20) (33% men; aged 57±8 yrs) depending on the presence or absence of hypertension or smoking status which have been proved to influence ROCK activity.[229-231] All disease and normal control subjects had normal epicardial coronary arteries on angiography. Effect of statins was statistically adjusted. Written informed consents were obtained from all subjects. The study was approved by the Institution's Ethics Committee.

5.2.2 Assay for leukocyte Rho-Kinase activity

Leukocytes were isolated from 10 ml peripheral blood at the admission following a validated and standardized protocol.[249] The leukocytes were frozen and stored at -80°C until all samples were collected. The ROCK assays were performed on all leukocytes samples at the same time. The samples were analyzed by Western blotting for the phosphorylation of the myosin-binding subunit (MBS) of myosin light-chain phosphatase with an antibody that specifically recognizes phosphorylated Ser853 MBS.[249] Inter-experimental results were standardized to lysophosphatidic acid–induced MBS phosphorylation (positive control).

5.2.3 Statistical analysis

Categorical variables are expressed as percentages of the corresponding population and continuous variables as means ± standard deviation. Values of $p<0.05$ were considered to indicate statistical significance. One-Way Analysis of Variance (ANOVA) was used for comparing of mean values of continuous variables among groups, and post-hoc analysis was performed by Scheffe's test to examine for inter-group differences. ROCK activity was adjusted for age between different groups as healthy control subjects were inevitably younger than other diseased controls. Univariate linear regression (Pearson and Spearman's correlation) models were used to assess the relation between parametric clinical variables and ROCK activity. All variables with a significant association but did not exhibit excessive collinearity with each other were evaluated for inclusion in a stepwise multiple regression analysis model using ROCK activity as the dependent variable. Receiver operating characteristics (ROC) analysis was performed to determine the best cutoff value of ROCK activity and NT-proBNP to following up outcomes in the patient corhort. Multivariate Cox regression analysis was performed to investigate for independent predictors of death outcomes. Event-free survival (days alive) was estimated by the Kaplan – Meier method and compared between groups by the log-rank test. All statistical analyses were conducted with the SPSS statistical package for Vista version 15.0 (SPSS Inc., Chicago, Illinois).

5.3 Results

5.3.1 Baseline Characteristics

Baseline clinical features and biochemical profiles are summarized in table 5.1. A comparison of baseline clinical and biochemical parameters between the heart failure and disease control groups did not show any statistically significant differences at age, gender and smoking status and medical history of hypertension. The normal controls showed no risk factors while the age (57 ± 8 yrs) was significantly lower than that of heart failure (74 ± 12 yrs) and disease controls (69 ± 8 yrs) (p<0.001). Compared with disease and normal controls, the heart failure patients had lower left ventricular ejection fraction (LVEF) (47.5 ±14.3 vs 64.2 ±4.1 and 69.5 ± 5.5, %, p<0.001), total cholesterol (TC) (4.3 ± 1.3 vs 4.8 ± 0.8 and 5.1 ± 0.7, mmol/l, p=0.003) and low-density lipoprotein cholesterol (LDL-C) (2.4 ± 1.1 vs 2.9 ± 1.0 and 3.0 ± 0.5, mmol/l, p=0.011). On contract, heart failure patients had higher systolic blood pressure (SBP) (148 ± 31 vs 128 ± 23, mmHg, p=0.002), fasting glucose (6.5 ± 1.8 vs 5.1 ± 0.3, mmol/l, p=0.002) and white blood cells (WBC) (9.2 ± 4.0 vs 5.5 ± 1.1, 10^9/l, p=0.004) than normal controls.

Table 5.1 Baseline characteristics of congestive heart failure, disease control and normal control subjects.

Baseline Characteristic	**CHF (n=178)**	**Disease Control (n=31)**	**Normal Control (n=20)**	**ANOVA *p***
Age(yrs)	74±12	69±8	57±8*	<0.001
Gender(male)	92 (52%)	16 (76%)	10 (33%)‡	0.003
Current Smoker	18 (10%)	4 (19%)	0	NA
Medications				
Hypertension	113 (63%)	17 (81%)	0	NA
Diabetes Mellitus	71 (40%)	0	0	NA
Hyperlipidemia	46 (26%)	0	0	NA
Chronic renal failure	36 (20%)	0	0	NA
Clinic				
LVEF (%)	47.5±14.3‡	64.2±4.1	69.5±5.5	<0.001
SBP (mmHg)	148±31§	143±20	128±23	0.003
DBP (mmHg)	79±20	82±10	76±11	0.622
HR (/minute)	88±24	69±13	87±12	0.080
BMI (kg/m^2)	24.8±4.1	25.3±8.5	23.6±3.2	0.375
Laboratory				
Fasting glucose (mmol/l)	6.5±1.8‡	5.2±0.8	5.1±0.3	0.007
TC (mmol/l)	4.3±1.3§	4.8±0.8	5.1±0.7	0.003
LDL-C (mmol/l)	2.4±1.1§	2.9±1.0	3.0±0.5	0.011
TG (mmol/l)	1.6±1.5	1.7±1.9	1.3±0.6	0.317
HDL-C (mmol/l)	1.3±0.6	1.4±0.4	1.5±0.4	0.204
WBC ($10^9/l$)	9.2±4.0§	8.4±3.7	5.5±1.1	0.052
Creatinine (umol/l)	173±148	91±13	72±16	0.086

*p<0.001 vs CHF and Disease control

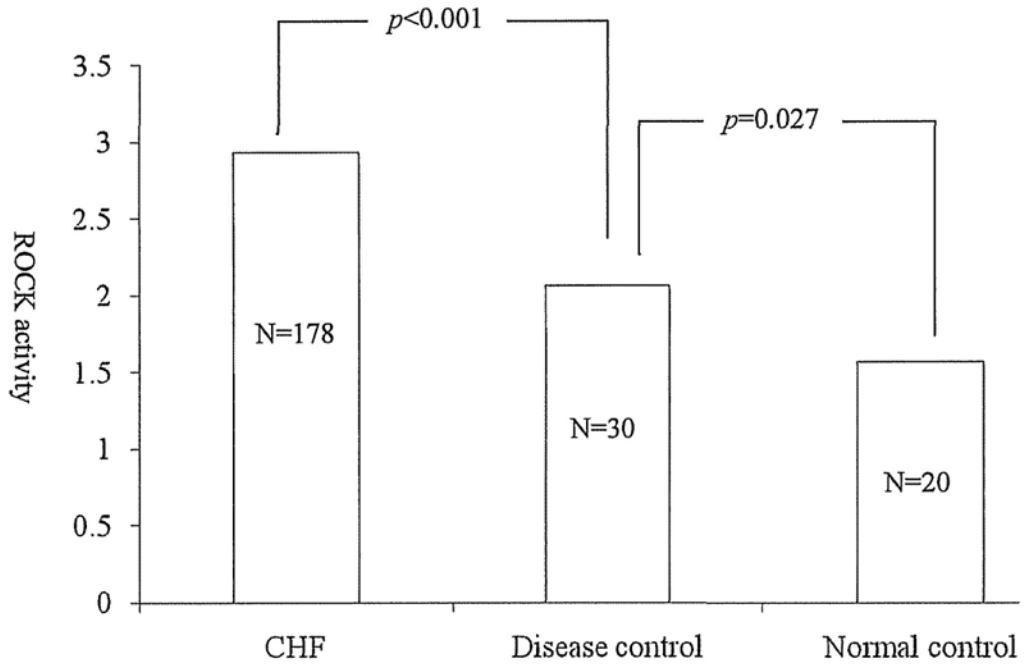
‡p<0.001 vs Disease control can Normal control

§p<0.05 vs Normal control

5.3.2 ROCK activity in CHF and control groups

The ROCK activity (2.93±0.87, number (n) =178) was significantly higher than that of the disease control (2.06±0.38, n=31, $p<0.001$) and normal control groups (1.57±0.43, n=20, $p<0.001$) (Figure 5.1).

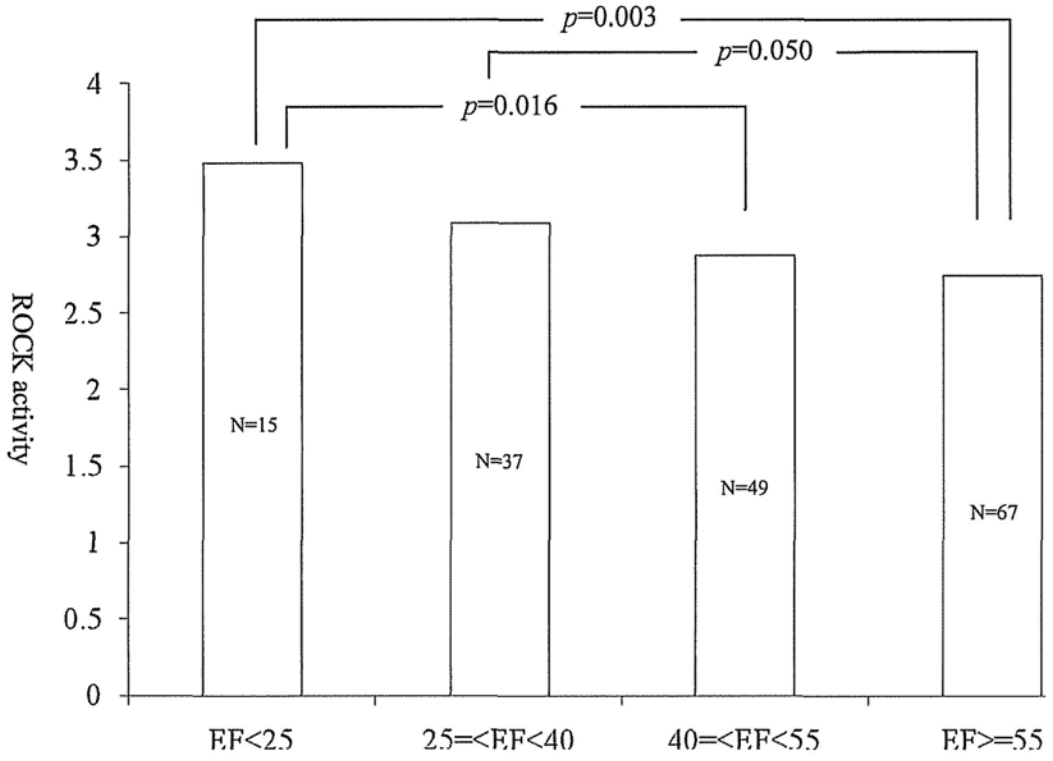
Figure 5.1 Comparison of ROCK activity in CHF, disease and normal control groups.



5.3.3 ROCK activity in different heart failure subgroups

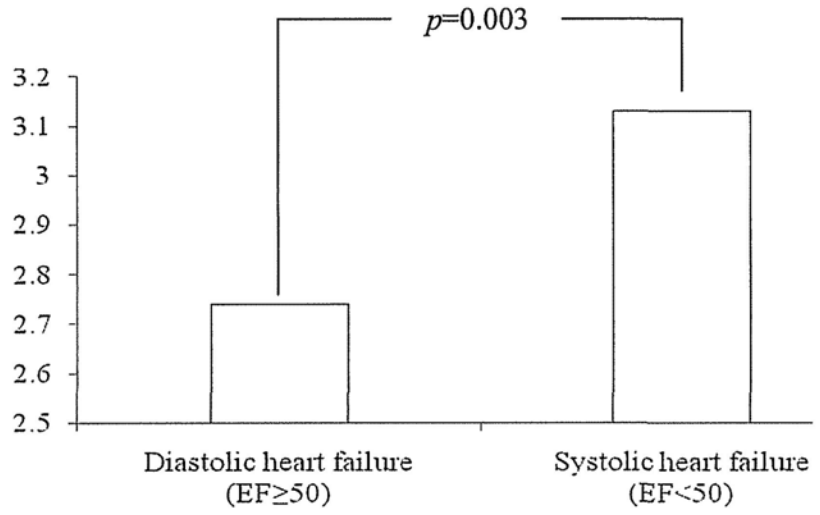
In the CHF cohort, patients were divided into four subgroups according to LVEF on admission: severe (<25%), moderate (25% to 40%), mild (40% to 55%) and normal EF (>55%). ROCK activity in severe EF group (3.48±1.36, n=15) was significantly higher than that in mild (2.88±0.74, n=49; p=0.003) and normal EF (2.75±0.85, n=67; p=0.016) groups. Patients with moderate EF (3.09±0.65, n=37) had higher ROCK activity than those with normal EF (p=0.050) (Figure 5.2).

Figure 5.2 Comparison of ROCK activity in different groups of CHF with severe (<25%), moderate (25% to 40%), mild (40% to 55%) and normal EF (>55%)



On the other hand, ROCK activity in systolic heart failure (3.189±0.132) was obviously higher than that of diastolic heart failure (2.741±0.099) (figure 5.3) (p=0.003).

Figure 5. 3 Comparison of diastolic and systolic heart failure groups



There was no difference of ROCK activity between NYHA1 (N=24, 2.51±0.79) and NYHA2 (N=51, 2.57±0.62) groups (p=0.758). Apparently, ROCK in NYHA3 (N=59, 3.06±0.63) and NYHA4 (N=41, 3.51±1.05) were both significantly higher than in NYHA1 (p=0.003, p<0.001) and NYHA2 (p=0.001, p<0.001) groups (figure 5.4). Similarly, those patients occurred acute decompensated heart failure had higher ROCK activity (N=109, 3.23±0.94) than those with stable heart failure (N=69, 2.61±0.83) (p<0.001) (figure 5.5).

Figure 5. 4 Comparison of ROCK activity in different New York Heart Association (NYHA) groups

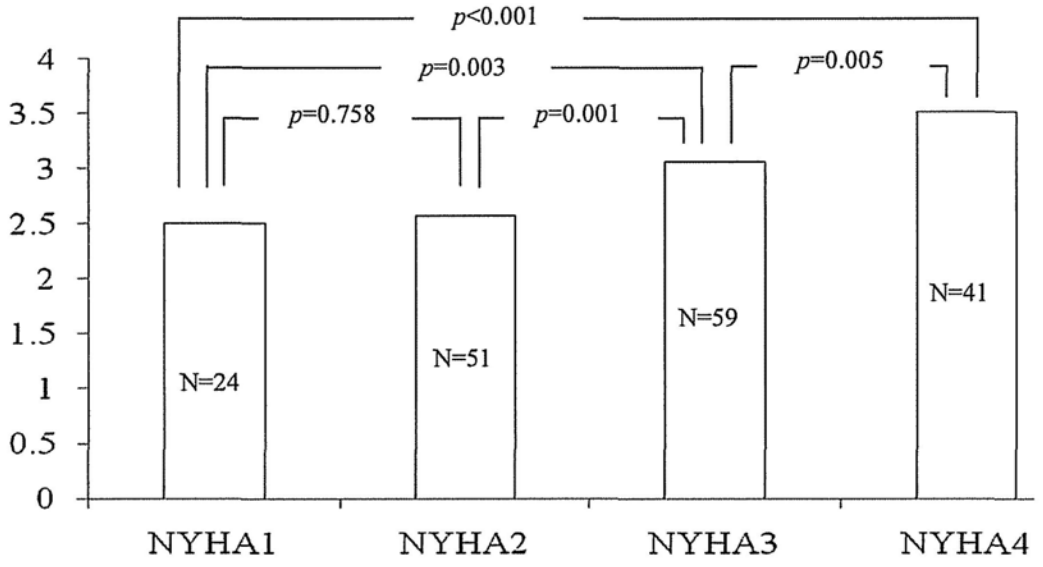
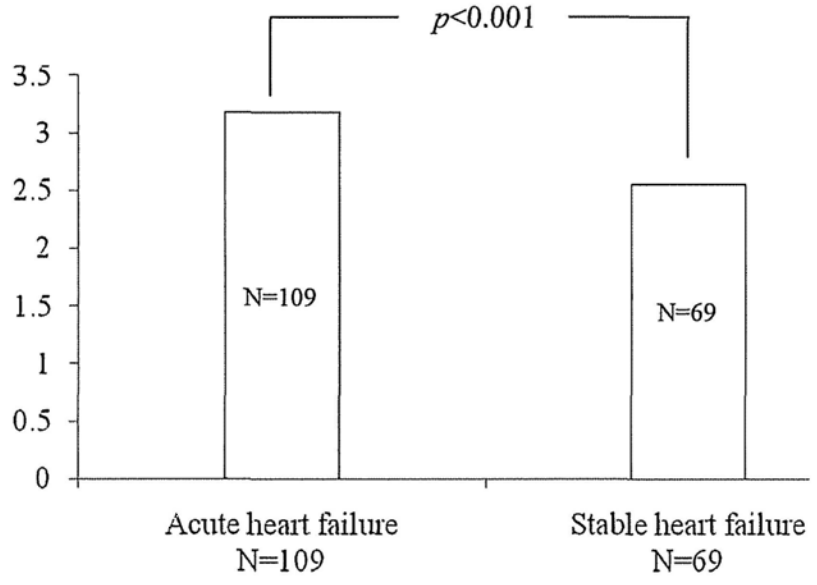


Figure 5. 5 Comparison of acute and stable heart failure groups



5.3.4 Predictors of ROCK Activity in CHF measured at baseline

Univariate association analysis showed the correlation coefficient of ROCK activity with components of CHF (Table 5.2).

Table 5.2 Nonparametric Correlations between clinical and biochemical parameters with ROCK

Covariants	**ROCK activity**	
	Coefficient	*p*
Age (yrs)	0.094	0.197
Gender (male)	0.091	0.365
Current smoker	0.120	0.210
Heart failure status (acute)	0.443	<0.001
NYHA class	0.435	<0.001
Heart failure with MI on admission	0.423	<0.001
Dyspnea at rest	0.411	<0.001
Medical history		
Heart failure	0.182	0.020
Hypertension	0.018	0.384
Diabetes mellitus	0.053	0.882
Hyperlipidemia	0.023	0.066
Ischemic heart disease	0.254	0.030
Renal failure	0.321	0.028
Blood pressure, (mmHg)		
SBP (mmHg)	0.085	0.264
DBP (mmHg)	0.032	0.703
HR (/minute)	0.298	0.001
BMI	0.167	0.064
LVEF (%)	-0.587	<0.001

Six-minute Hall-Walk (feet)	-0.604	<0.001
Laboratory values		
Sodium (mmol/l)	-0.169	0.025
Urea (mmol/l)	0.176	0.019
Creatinine (umol/l)	0.361	<0.001
Total protein	-0.155	0.044
TG (mmol/l)	-0.041	0.637
LDL-C (mmol/l)	0.128	0.677
TC (mmol/l)	0.121	0.736
HDL-C (mmol/l)	-0.091	0.292
Fasting Glucose (mmol/l)	0.112	0.304
HbA1c	0.032	0.819
Platelet	-0.064	0.402
WBC (10^9/l)	0.128	0.102
Total hospital length of stay (days)	0.118	0.208

Among the associated factors, heart failure status (r=0.443, p<0.001), dyspnea at rest (r=0.411, p<0.001), NYHA class (r=0.435, p<0.001), heart failure with myocardial ischemia (MI) on admission (r=0.423, p<0.001), history of ischemic heart disease (r=0.254, p=0.030) or heart failure (r=0.182, p=0.020), heart rate (r=0.298, p=0.001), urea (r=0.176, p=0.019) and creatinine (r=0.360, p<0.001) were all positively associated with increased levels of ROCK activity. In contrast, left ventricular ejection fraction (r=-0.587, p<0.001) and sodium (r=-0.169, p=0.025) were negatively associated with ROCK activity. Multivariate regression models (stepwise) showed that NYHA class (β=0.349, p<0.001), low LVEF (β=-0.277, p<0.001) and high creatinine (β=0.202, p=0.006) predict baseline ROCK activity in CHF (Table 5.3).

Table 5.3 Prediction of ROCKs Activity in Univariate and Multivariate regression models.

Variables	Univariate		Multivariate (Stepwise)	
	Coefficient	*p*	β	*p*
Heart failure status (acute or stable)	0.443	<0.001	-	-
Dyspnea at rest	0.411	<0.001	-	-
NYHA class	0.435	<0.001	0.349	<0.001
Heart failure with MI on admission	0.423	<0.001	-	-
History of ischemic heart disease	0.254	0.030	-	-
History of heart failure	0.182	0.020	-	-
History of renal failure	0.321	0.028	-	-
Left Ventricular Ejection Fraction (%)	-0.587	<0.001	-0.277	<0.001
Heart rate (bpm)	0.298	0.001	-	
Sodium (mmol/l)	-0.169	0.025	-	-
Urea (mmol/l)	0.176	0.019	-	-
Creatinine (umol/l)	0.361	<0.001	0.202	0.006

5.3.5 Clinical outcome and predictors of long-term event-free survival

The mean duration of follow up was 14.4 (±7.2) months (range 0.5 to 26 months). 112 patients (82%) were followed up for more than one year and forty-five patients (25.3%) reached the primary end point of death. Accordingly, event-free survival days were from 382 to 783 days. Further investigation was performed by Cox regression survival analysis for a long-term event outcome including the following baseline variables which were significantly correlated with death: age (r=0.177, p=0.019), serum sodium (r=-0.180, p=0.018), heart rate (r=0.183, p=0.020), creatinine concentrations (r=0.263, p<0.001), blood urea (r=0.275, p<0.001) and ROCK activity (r=0.178, p=0.019) and NT-proBNP (r=0.352, p=0.005) (Table 5.4).

Table 5.4 Relation between clinical and laboratory characteristics and the occurrence of death during follow up

Baseline Characteristic	**No death (n=133)**	**Death (n=45)**	**ANOVA *p***
Age(yrs)	72.5±12.2	77.2±9.7	0.019
Gender(male)	71 (53%)	20 (44%)	0.301
Current Smoker	11 (8%)	2 (4%)	0.465
Heart failure with MI on admission	35 (26%)	15 (33%)	0.321
NYHA class	2.41±1.14	2.86±0.92	0.402
Medical history			
Hypertension	84 (63%)	30 (67%)	0.431
Diabetes Mellitus	54 (41%)	19 (42%)	0.678
Hyperlipidemia	34 (26%)	10(22%)	0.706
Clinical and Echocardiography			
LVEF (%)	48.0±14.4	45.7±14.7	0.389
SBP (mmHg)	148±31	152±35	0.540
DBP (mmHg)	80±21	79±20	0.743
HR (/minute)	85.3±23.2	95.4±24.4	0.020
BMI	24.8±4.0	25.3±4.6	0.610
RFP	1.61±0.92	1.57±0.65	0.900
E'	4.46±1.63	3.89±1.37	0.186
E/E'	24.21±11.16	25.71±13.11	0.642
Laboratory			
Sodium (mmol/l)	138.9±3.7	137.4±3.7	0.018
Fasting glucose (mmol/l)	6.46±1.94	6.50±1.54	0.931
TC (mmol/l)	4.36±1.27	4.17±1.27	0.453
LDL-C (mmol/l)	2.50±1.13	2.10±0.89	0.047
TG (mmol/l)	1.40±0.67	2.01±3.00	0.052
HDL-C (mmol/l)	1.28±0.52	1.28±0.66	0.997
WBC (10^9/l)	8.88±3.77	10.22±4.60	0.066
Creatinine (umol/l)	166.7±117.6	260.0±216.1	<0.001
Urea (mmol/l)	10.4±5.5	14.8±9.3	<0.001
ROCK activity	2.85±0.78	3.21±1.04	0.019
NT-proBNP (pg/ml)	6729±11369	17533±31475	0.005

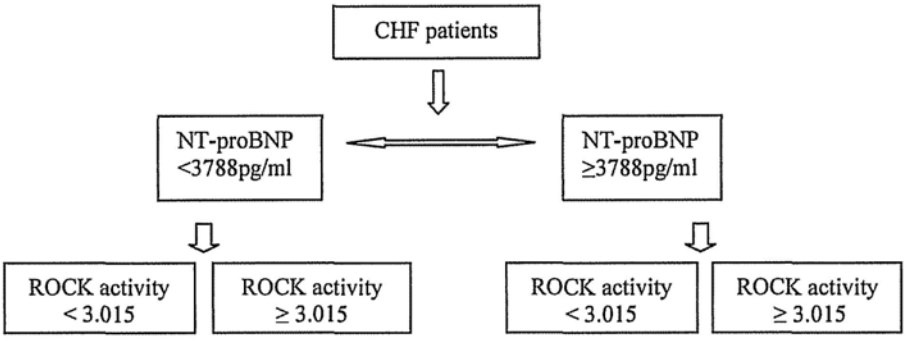
Of all variables tested, age (HR=1.038, 95%=1.001-1.076, p=0.044), sodium level (HR=0.894, 95%=0.825-0.969, p=0.007), heart rate at admission (HR=1.020, 95%=1.006-1.034, p=0.006) and NT-proBNP level (HR=1.200, 95%=1.000-1.002, p=0.038) were the independent predictors for the long-term mortality (Table 5.5).

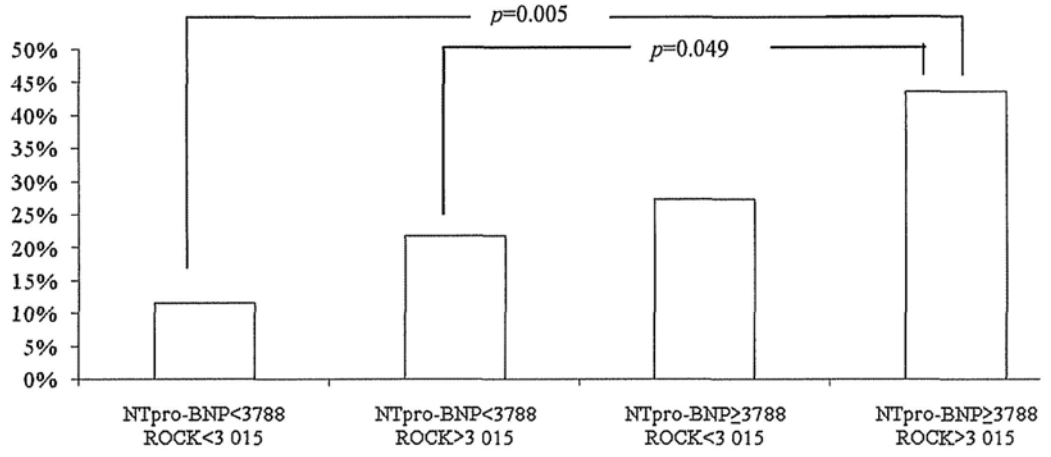
Table 5.5 Multivariate Cox regression of baseline ROCK activity to long-term mortality

Covariate	**Multivariate (forward)**		
	HR	**95%CI**	***p***
Age (years)	1.038	1.001-1.076	0.044
Sodium (mmol/l)	0.894	0.825-0.969	0.007
Heart rate (beats per minute)	1.020	1.006-1.034	0.006
Creatinine (umol/l)	-	-	0.195
Urea (mmol/l)	-	-	0.126
ROCK activity	-	-	0.563
NT-proBNP (pg/ml)	1.200	1.000-1.002	0.038

In our study, the best cutoff value for ROCK activity to predict long-term mortality was 3.015, with sensitivity and specificity rates of 58% and 60% respectively. The area under the curve (AUC) was 0.61, p=0.037. The best cutoff value for NT-proBNP to predict long-term mortality was 3788pg/ml, with sensitivity and specificity rates of 65% and 59% respectively. The AUC was 0.59, p=0.012. CHF patients were separated into four subgroups according to these two cut off points. Patients with more than 3788pg/ml NT-proBNP and 3.015 ROCK activity had 44% mortality over 2 years, which is significantly higher than that of the group with less than 3788pg/ml NT-proBNP and 3.015 ROCK activity (12%, p=0.005). The mortality of the group with low NT-proBNP but high ROCK activity (22%) was also significantly lower than that of the group with high NT-proBNP and high ROCK activity (44%, p=0.049) (Figure 5.6). Another statistic method, the Kaplan-Meier curves for event-free survival within 2 years according to NT-proBNP combining with ROCK activity, also showed significant difference within groups (Log rank x^2=11.62, p=0.009) (Figure 5.7).

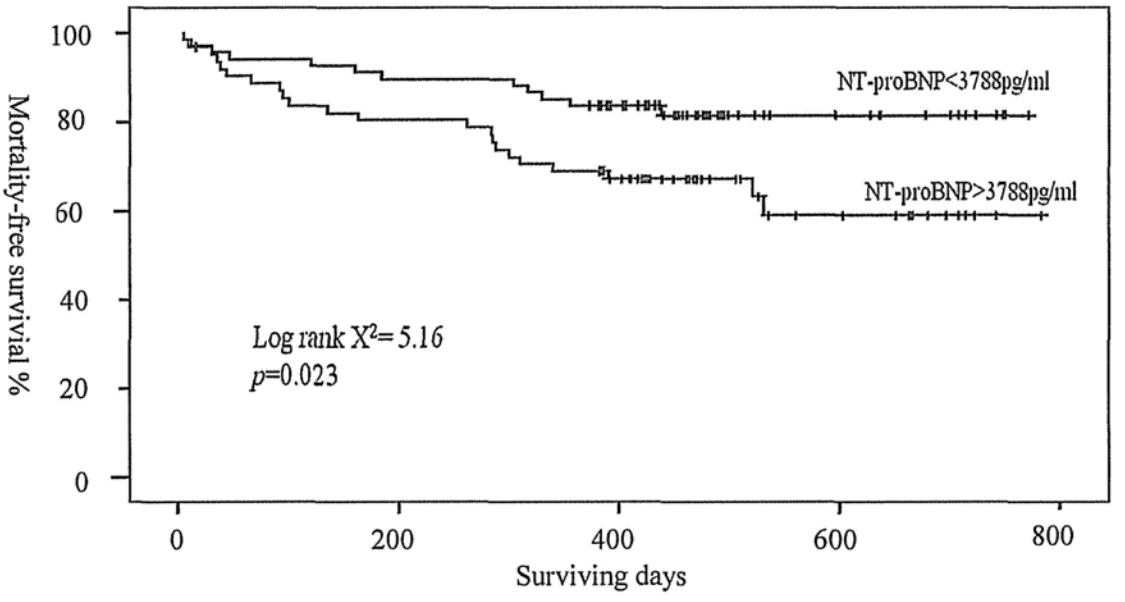
Figure 5.6 Comparison of mortality in different subgroups according to the different cutoff point of NT-proBNP and ROCK activity

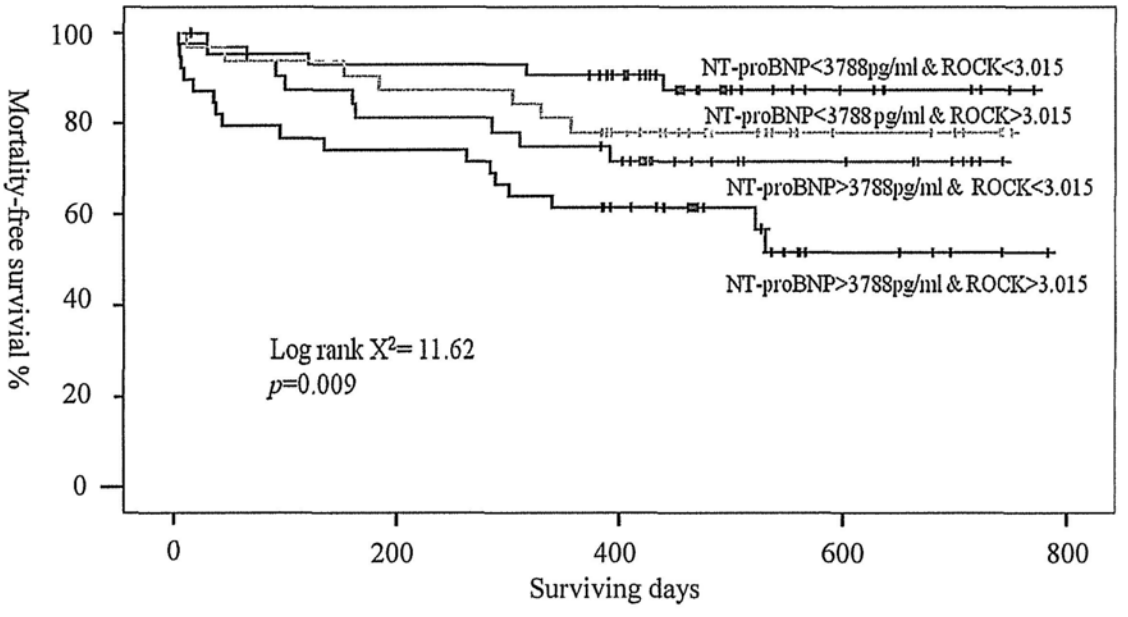




Total No.	43	32	33	39
Dead No.	5	7	9	17
Mortality	12%	22%	27%	44%

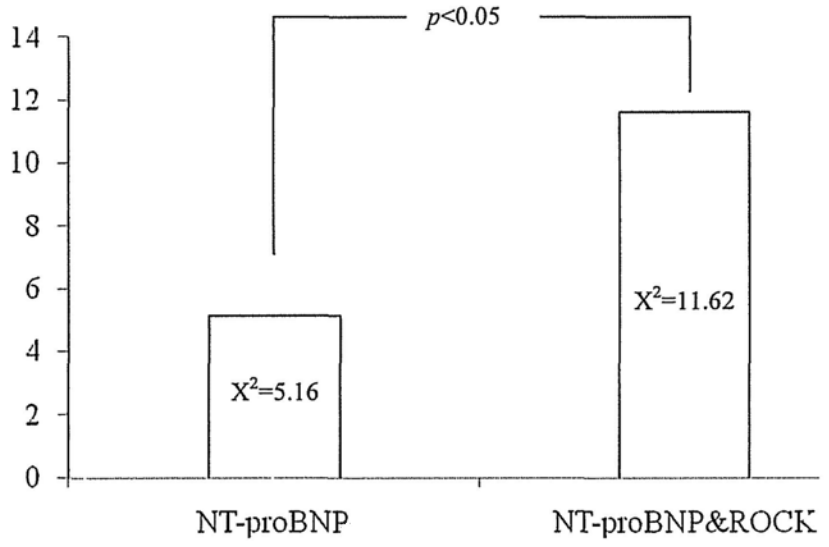
Figure 5.7 The upper figure is the Kaplan-Meier curves for event-free survival within 2 years according to NT-proBNP (Log rank x^2=5.16, p=0.023). The lower figure is the Kaplan-Meier curves for event-free survival within 2 years according to NT-proBNP combining with ROCK activity (Log rank x^2=11.62, p=0.009).





Furthermore, although only NT-proBNP has prognostic value, combining both NT-proBNP and ROCK activity was significantly superior in predicting mortality when comparing with only a single factor ($p<0.05$) (Figure 5.8).

Figure 5.8 Incremental predictive value of combining the NT-proBNP and ROCK activity for long-term mortality on top of the individual NT-proBNP predictor.



5.3.6 Diagnostic power and predictive power

According to the sample size, SD and mean, the diagnositic and predictive powers were calculated in heart failure study. If sample size is 10, the diagnostic power in this study is more than 98%. If sample size is 10, the diagnostic power in this study is more than 98%

5.4 Discussion

In this study, we have shown for the first time that ROCK activity is higher in CHF subjects than disease control and normal control groups after adjusting for age. Higher baseline ROCK activity was associated with several features of CHF, such as more severe symptoms on admission (heart failure status, NYHA class), MI on admission, history of ischemic heart disease history, heart failure or renal failure, poor systolic cardiac function (LVEF and heart rate) and lower renal function (sodium, urea and creatinine). In addition, NYHA class, low LVEF and high creatinine predicted the baseline ROCK activity in CHF. Combining ROCK activity and NT-proBNP had an incremental value in predicting long-term mortality. These findings indicate that ROCK activity might be a risk marker for CHF and suggest the involvement of increasing ROCK activity in the pathopysiology and progression of CHF.

5.4.1 Increased ROCK activity in CHF

It has been shown that ROCK may act as a downstream effector in the intracellular signaling of several G protein-coupled receptors, including those of angiotensin II, norepinephrine, and endothelin-1, the activities of which are known to be elevated in CHF.[173, 254, 278] In addition, the ROCK system has been implicated in the mediation of endothelin-1- and mechanical stress-induced hypertrophic responses in cardiac myocytes.[279] These findings suggest that ROCK-dependent signaling pathways may potentially contribute to the mechanism of systemic and renal vasoconstriction and cardiac hypertrophy in CHF.

Although this is the first study to find evidence of increased ROCK activity in humans with CHF, many studies in animal models have confirmed the potential involvement of Rho/ROCK in heart failure. In a dog model of tachypacing-induced heart failure, the Ca^{2+}-sensitizing mechanism of a conduit artery (femoral artery) is augmented, resulting in an enhanced vasoconstrictor response to norepinephrine.[173] Y-27632, which is the inhibitor of ROCK, attenuates this response without a significant change in intracellular Ca^{2+} concentrations in VSMC, suggesting the Rho/ROCK pathway is involved in the increased vasoconstrictor response in heart failure.[173] Transgenic mice that over express RhoA in the heart develop loss of systolic function and develop dilated cardiomyopathy.[276] Several neurohormonal factors, such as AII, are believed to participate in ventricular hypertrophy and to the transition to heart failure. Rho-kinase is involved in the pathogenesis of left ventricular remodeling and dysfunction after MI in a mouse model in vivo.[113] Long-term inhibition of ROCK by fasudil, treatment reduces the AII-induced cardiomyocyte hypertrophy in wild-type as well as in apoE-KO mice.[118]

In addition, ROCK inhibition improves cardiac function by preventing AII-induced decrease in ventricular contractility, cardiac output, and cardiac stoke volume.[118] In Dahl salt-sensitive hypertensive rats, the ventricular hypertrophy and function is significantly ameliorated by ROCK inhibition.[116, 117] It has been suggested that the cardioprotective effect of ROCK inhibition involved upregulation of the downregulated eNOS and the reduction of oxidative stress through the inhibition of NAD(P)H oxidase and lectin-like oxidized low-density lipoprotein receptor-1 expression.[116] Recently, deletion of ROCK1 was reported inhibited several pathological events including cardiomyocyte apoptosis in compensated hypertrophic hearts. ROCK1 deficiency could also prevent the transition from hypertrophy to heart failure.[125]

In human, intra-arterial infusion of fasudil causes a preferential increase in forearm blood flow as compared with control subjects, suggesting an involvement of Rho/Rho-kinase pathway in increasing peripheral vascular resistance in heart failure.[185] However, the long-term effects of fasudil as a vasodilator therapy in the treatment of heart failure remain to be examined. We previously demonstrated that ACS patients with heart failure symptoms on admission had much higher ROCK activity than those without. Although the ROCK activity in systolic heart failure was significantly higher than that of diastolic heart failure, both systolic and diastolic heart failure patients had higher ROCK activity than that of control subjects. In this study, ROCK activity showed high association with the ratio of left ventricular ejection fraction. Our study was corroborated by a mice model of diastolic heart failure demonstrating a potential involvement of ROCK1 in the

development of diastolic heart failure and structural remodeling.[280] Another study suggested the possibility that the RhoA-ROCK pathway plays an important role in the process of hypertension-induced left ventricular hypertrophy leading to contractile dysfunction (such as decrease in left ventricular ejection fraction).[275]

5.4.2 ROCK activity associated with poor renal function

Rho/ROCK pathway was involved in the pathogenesis of nephrosclerosis in severely hypertensive rats. ROCK inhibition by Y-27632 and fasudil results in relaxation of afferent as well as efferent arterioles in vitro[134] and in vivo hydronephrotic kidney models.[135] In addition to a critical role of ROCK activity in the renal microvasculature, Rho proteins are important endogenous regulators of several types of renal tubular functions, including proliferation, migration, and apoptosis.[136] Recent studies revealed that specific Rho-kinase inhibitors, Y-27632 or fasudil, significantly attenuate the tubulointerstitial fibrosis in the kidney induced by unilateral ureteral obstruction.[147] In addition, fasudil attenuates glomerulosclerosis in salt-induced hypertensive rats partly by the inhibition of the transforming growth factor beta/collagen cascade.[149] Recently, fasudil has been demonstrated to partly reverse hypertensive glomerulosclerosis in spontaneously hypertensive rats by multiple mechanisms including inhibition of extracellular matrix production, oxidative stress, adhesion molecule production, and antifibrinolysis.[281] Furthermore, Nichikimi et al. also elucidated the mechanisms of the Rho/Rho-kinase pathway participating in the pathogenesis of nephrosclerosis in

spontaneously hypertensive rats (SHR, a model for hypertensive glomerulosclerosis) independently of blood pressure-lowering activity.[153]

Interestingly in this study, poor renal function also independently predicted high ROCK activity in the patients with heart failure. Although many in-vitro observations strongly suggest substantial involvement of ROCK in mediating the progression of renal injury, only a couple of studies have provided direct in-vivo evidence for the contribution of ROCK to the development of renal disease, especially in human. It is the first time to demonstrate the association of renal function and ROCK activity in heart failure patients in whom the 6-month mortality risk increased with decreasing renal function.[282] Furthermore, renal dysfunction causes further congestion and neurohormonal activation, which are factors associated with adverse outcomes in patients with heart failure.[283] Thus, these studies support a key role of Rho/ROCK pathway in heart failure because of the relationship of ROCK activity and renal function.

Noma K., et al. (2005)[229] demonstrated that not only endothelial dysfunction but also activated ROCK in vascular smooth muscle cells were found in healthy young male smokers compared with nonsmokers. This suggests that smoking is involved in not only endothelial dysfunction but also activation of ROCK in vascular smooth muscle cells in forearm circulation. In our study, we did not find any relationship between smoking and ROCK activity in CHF. This might because that impaired systolic cardiac function and renal failure were the main causes of increasing ROCK activity in severe heart failure.

5.4.3 Subjects with high baseline NT-proBNP and high ROCK activity have worse long-term outcome

In this study, we demonstrated for the first time the prognostic value of ROCK and NT-proBNP in patients with CHF. In those who subsequently died, ROCK activity and NT-proBNP were significantly higher than in the survivors. NT-proBNP is a well-established marker for the diagnosis of heart failure[284] and which can also be used as a prognostic tool[285] and for monitoring treatment.[286] In our CHF cohort, NT-proBNP was an independent predictor of long-term mortality whereas ROCK activity was not. However, the combination of ROCK activity and NT-proBNP was more useful for predicting mortality in patients with CHF than NT-proBNP alone. This suggests that although NT-proBNP is a more sensitive biomarker than ROCK activity in CHF, ROCK is more representative of the inflammation, endothelial function and vasoconstriction. In fact, NT-proBNP has been combined with other clinic biomarkers previously to improve its predictive power for mortality in heart failure. Such combinations with NT-proBNP include cardiac troponin I in systolic heart failure,[287] cardiac troponin T and NT-proBNP in decompensated heart failure,[288] NT-proBNP and eGFR after acute myocardial infarction predicting heart failure event,[289] and NT-proBNP and copeptin in chronic heart failure.[290] Therefore, simultaneous measurement of ROCK activity and NT-proBNP could provide complementary information and a simple multimarker strategy that categorizes the patients with advanced CHF based on the number of elevated biomarkers, may provide rapid risk stratification. There may be a therapeutic aspect to

ROCK as Winaver et al. demonstrated the possible beneficial anti-hypertrophic properties of ROCK in heart failure rats.[291]

5.5 Future work

From our study, peripheral blood was collected at the time point of 6 months follow up. Further collection will be done within a day, day-to-day or week-to-week. It is probably more useful to evaluate change of serial ROCK levels during short-term (inpatient) and long-term (outpatient). For inpatient monitoring, comparison of ROCK activity and NT-proBNP or BNP should be explored, although studies have suggested that serial BNP or NT-proBNP measurements are useful for detecting short-term cardiac improvements. The changes of ROCK activity after long-term treatment should be another interesting point to compare it with NT-proBNP. Further studies are required to evaluate the prognostic value of ROCK and NT-proBNP for the prediction of outcomes after longer period than only around 2 years.

A major challenge for the inhibitor studies is to determine if ROCK truly represents a viable target for the treatment of human disease and whether ROCK1 and ROCK2 mediate different cellular functions. ROCK1 mRNA level was proved to be induced and ROCK activity was increased in transgenetic mice, but ROCK2 expression remained

unchanged under all the condition tested and may not play a significant role in hypertrophic decompensation. These observations further support an isoform specific role of ROCK1 in pathological remodeling and preventing the development of heart failure.[125] In particular, it will be of interest to determine *1*) if ROCK1 deficiency could inhibit heart failure progression in other decompensated heart failure settings, *2*) if inducible deletion of ROCK1 could reverse or attenuate cardiac remodeling in failing hearts, and *3*) if specific inhibition of ROCK1 activity in human heart failure patients represents a valid therapeutic approach.

5.6 Limitation

CHF patients and subjects at-risk were older than healthy control subjects. Aging is known to be associated with increased ROCK activity, due to excessive oxidative stress in peripheral vasculature.[21] We adjusted for age in our statistical analysis as healthy subjects >70 years old were difficult to find. On the other hand, the number of total subjects could increase more and the period could be prolonged.

5.7 Conclusion

ROCK activity is elevated in CHF. Bad NYHA class, low LVEF and high creatinine predict baseline ROCK activity in CHF. In addition, ROCK activity combined with NT-proBNP was a good predictor for long-term event-free survival in CHF. Further studies

would be helpful to elucidate whether reduction of ROCK activity is associated with therapeutic benefits in patients with CHF.

CHAPTER 6

SUMMARY

CHAPTER 6. SUMMARY

The Rho/ROCK pathway has recently attracted a great deal of attention in various research areas, particularly in cardiovascular diseases.[1] It has already been demonstrated that increased ROCK activity is present in cerebral ischemia, coronary vasospasm, hypertension, vascular inflammation, atherosclerosis, erectile dysfunction, and cardiac hypertrophy in which the Rho/ROCK pathway plays an important role in various cellular functions.[292] However, clinical data of ROCK activity in the settings of acute myocardial ischemia and heart failure are scarce. The aims of this study were, therefore, to examine the expression and characteristics of ROCK activity in patients with ACS and heart failure as well as the prognostic values of ROCK activity for long-term clinical events.

6.1 Increasing ROCK activity in ACS and heart failure

In the first part of this thesis, ROCK activity was investigated in ACS patients divided into STEMI, NSTEMI and UA subgroups. Overall, they had significantly higher ROCK activity compared to the disease-controlled patients without ACS that in turn demonstrated mildly but significantly elevated activity than the normal control subjects. Both STEMI and NSTEMI patients had similar ROCK activity, though their activities were significantly higher than that of the UA group. The ROCK activity was elevated not only during and after myocardial infarction damage but also involved in the pathophysiology of apoptosis and hypertrophy. The ROCK activity in heart failure

patients was studied in the subsequent part. Similar to ACS patients, the ROCK activity was increased in patients with heart failure compared to normal and disease control subjects. Thus, our studies confirmed that over- expression of ROCK activity is present in both ACS and heart failure populations.

6.2 Independent predictors of ROCK activity in ACS and heart failure

LDL-C level and number of diseased coronary vessels, which are the main determinant of myocardial infarction severity, were independent predictors of ROCK activity in ACS patients. In addition, ACS patients with heart failure symptoms on presentation (e.g. shortness of breath (typically worse when lying flat, which is called orthopnea), coughing, chronic venous congestion, ankle swelling, and exercise intolerance) had higher ROCK activity when comparing with those with either ACS or heart failure alone. Furthermore, heart failure symptom on presentation was an independent predictor of ROCK activity. On the other hand, NYHA class, low LVEF and high creatinine, which represent severity of heart failure, were independent predictors of the baseline ROCK activity in heart failure.

6.3 Comparison of different independent predictors of ROCK activity in ACS vs heart failure

Although the major role of the treatment in ACS is to reduce the myocardial infarct size and the infarct size sometimes is one of the contributions to the development and prognosis of heart failure, the predictors in these two diseases should be different. Atherosclerosis and inflammation are the main pathological damage while cardiac hypertrophy and apoptosis are dominant in heart failure. Level of serum LDL and the number of diseased coronary vessels should represent the severity of the heart disease and the burden of atherosclerosis and inflammation. These are also recognized as independent predictors for long time cardiovascular events in ACS. It can be explained those ACS patients with heart failure symptom had higher ROCK activity because of more damage of myocardial infarction and the development of myocardial apoptosis. In this study, for those patients only having heart failure symptom (bad NYHA class), LVEF and renal function were proved independent predictors for ROCK activity, which differed from ACS. The most important factor affecting the severity of heart failure is renal function. A critical role of ROCK in the renal microvasculature was investigated in previous studies that Rho proteins are important endogenous regulators of several types of renal tubular functions, including proliferation, migration, and apoptosis. In our study, we did not find any relationship between smoking and ROCK activity in heart failure. This might because that impaired systolic cardiac function and renal failure were the main causes of increasing ROCK activity in severe heart failure. Same results were also

found in ACS patients. It might due to mildly endothelial disfunction induced by smoking was not the main cause for atherosclerosis and inflammation in ACS.

6.4 Prognostic value of ROCK activity in ACS versus heart failure

In the follow-up study, ACS patients with a high N-terminal pro-B-type natriuretic peptide (NT-proBNP) and a high ROCK activity on admission had a five-fold risk to experience a cardiovascular event, when compared to those with low NT-proBNP and low ROCK activity. Moreover, patients with high NT-proBNP and high ROCK activity were also more likely to die or experience a cardiovascular event at two years when comparing to those with high NT-proBNP and low ROCK activity. Although ROCK activity was not an independent predictor for long-term mortality in our heart failure cohort, combining ROCK activity and NT-proBNP had an incremental value in predicting long-term mortality over NT-proBNP alone. Although NT-proBNP was proved a good biomarker in helping diagnosing heart failure and predicting long-term cardiac outcomes in ACS and heart failure, there is still room for further improving diagnostic and prognostic accuracy. Accumulating evidence suggests that combination of multiple biomarkers can improve the diagnostic accuracy. ROCK activity may serve a novel biomarker in the armamentarium of diagnostic and prognostic markers for heart failure or ACS.

6.5 Comparison of ROCK activity with other biomarkers in ACS and heart failure

In the last few years, many circulating biomarkers including traditional (e.g. hs-CRP, NT-proBNP, white blood cells, Interleukin-6) and novel genetic biomarkers (e.g. miR-494) were widely examined in cardiovascular diseases. Recent studies have suggested the possibility of measuring other hormones such as proadrenomedullin, C-terminal endothelin-1, and midregional pro-A–type natriuretic peptide.[293] Some of these markers have shown to a lower individual biologic variability than BNP and NT-proBNP. There is an evolving trend of utilizing multiple biomarkers in improving diagnostic accuracy or prognostication in cardiovascular disease. As demonstrated in our study, ROCK activity is an independent predictor for long-term outcomes in ACS but not in heart failure patients. However, combination of ROCK activity and NT-proBNP increased the prognostic value in both of these clinical entities. It might be a shifting clinical paradigm in future to focus on development of multiple biomarkers for cardiovascular diagnosis and prognosis.

CHAPTER 7. REFERENCE LIST

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