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REVIEW

Basic Science for the Clinician

2014 Williams Harvey Lecture: importance of coronary vasomotion abnormalities—from bench to bedside†

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Coronary vasomotion abnormalities play important roles in the pathogenesis of ischaemic heart disease, in which endothelial dysfunction and coronary artery spasm are substantially involved. Endothelial vasodilator functions are heterogeneous depending on the vessel size, with relatively greater role of nitric oxide (NO) in conduit arteries and predominant role of endothelium-derived hyperpolarizing factor (EDHF) in resistance arteries, where endothelium-derived hydrogen peroxide serves as an important EDHF. The functions of NO synthases in the endothelium are also heterogeneous with multiple mechanisms involved, accounting for the diverse functions of the endothelium in vasomotor as well as metabolic modulations. Cardiovascular abnormalities and metabolic phenotypes become evident when all three NO synthases are deleted, suggesting the importance of both NO and EDHF. Coronary artery spasm plays important roles in the pathogenesis of a wide range of ischaemic heart disease. The central mechanism of the spasm is hypercontraction of vascular smooth muscle cells (VSMCs), but not endothelial dysfunction, where activation of Rho-kinase, a molecular switch of VSMC contraction, plays a major role through inhibition of myosin light-chain phosphatase. The Rho-kinase pathway is also involved in the pathogenesis of a wide range of cardiovascular diseases and new Rho-kinase inhibitors are under development for various indications. The registry study by the Japanese Coronary Spasm Association has demonstrated many important aspects of vasospastic angina. The ongoing international registry study of vasospastic angina in six nations should elucidate the unknown aspects of the disorder. Coronary vasomotion abnormalities appear to be an important therapeutic target in cardiovascular medicine.

Keywords Coronary vasomotion • Endothelial cells • Vascular smooth muscle • Coronary spasm • Rho-kinase

Introduction

Coronary vasomotion is an important determinant of coronary vascular resistance and thus coronary blood flow, which is regulated by multiple mechanisms, including the endothelium, vascular smooth muscle cells (VSMCs), myocardial metabolic demand, autonomic nervous system, and inflammation.[1–8] In the current era of coronary intervention, much attention has been paid to structural abnormalities of the coronary artery and less attention to its functional abnormalities. However, ~10% of all patients with and up to 30% of female patients with acute coronary syndrome[9] and more than 60% of patients with chest pain undergoing elective coronary angiography[10] have no evidence of organic coronary artery stenosis, indicating the involvement of functional coronary vasomotion abnormalities. Indeed, the coronary artery (and other arteries as well) should be regarded as functional conduit rather than structurally rigid pipes. This review article will briefly summarize the 2014 William Harvey Lecture by the author. In this article, the translational research on coronary vasomotion abnormalities in the author's laboratory will be briefly reviewed, with a special reference to endothelial modulation of vascular tone and coronary artery spasm.

Endothelial modulation of vascular tone

The endothelium plays an important role in the modulation of vascular tone by synthesizing and releasing several vasodilator substances, collectively named as endothelium-derived relaxing factors (EDRFs), including vasodilator prostaglandins [mainly prostaglandin I_2 (PGI_2)], nitric oxide (NO), and endothelium-derived hyperpolarizing factor (EDHF; *Figure 1A*).[11–16] The term EDRF(s) is currently used in two

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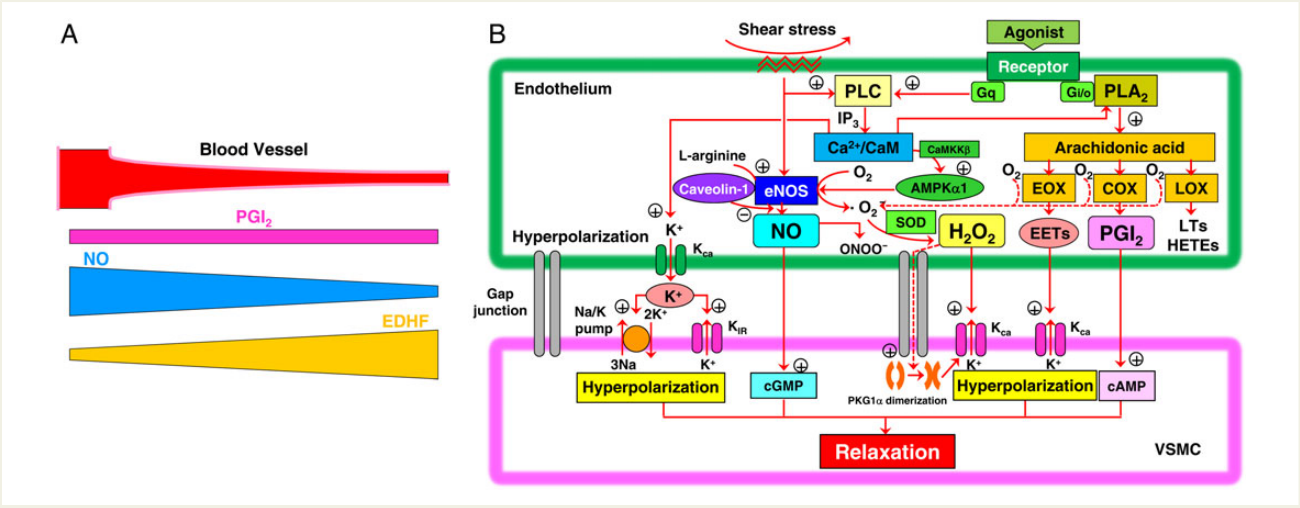

Figure 1 Endothelial control of vascular tone. (*A*) Relative contribution of endothelium-derived relaxing factors as a function of vessel size. (*B*) Intracellular mechanisms of the synthesis of endothelium-derived relaxing factors and their mechanisms of vascular smooth muscle relaxation.

different ways; one is strictly related to NO based on the original report[11] and the another is related to all vasodilating substances released from the endothelium.[12,13] In this article, the author takes the latter position.

Relative importance of endothelium-derived relaxing factors as a function of vessel size

Endothelium-derived NO mediates vascular relaxation of relatively large, conduit arteries (i.e. aorta and epicardial coronary arteries), while EDHF plays an important role in modulating vascular tone of resistance arteries (e.g. coronary microvessels) (*Figure 1A*).[17,18] Endothelium-derived hyperpolarizing factor causes vascular relaxation by opening calcium-activated K (K_{Ca}) channels and then hyperpolarizes membrane of VSMC (*Figure 1B*).[12,13,19] Endothelium-derived hyperpolarizing factor is synthesized not only upon stimulation by agonists but also by shear stress as in the case of NO.[20]

Nature of endothelium-derived hyperpolarizing factor(s)

Although the nature of EDHF has not been fully elucidated, different EDHFs could exist depending on species, blood vessels, and the size of blood vessels tested.[12,21] Since the first report on the existence of EDHF,[22,23] several candidates have been proposed for the nature of EDHF, including epoxyeicosatrienoic acids (EETs), metabolites of arachidonic P450 epoxygenase pathway,[24,25] K^+ ions,[26] and electrical communication through myoendothelial gap junctions (*Figure 1B*).[27] Based on the similarities between NO and EDHF in terms of susceptibility to atherosclerotic risk factors and responses to medications, we postulated that endothelial NO synthase (eNOS) might be a source of not only NO but also EDHF.[28] Using eNOS-deficient ($eNOS^{-/-}$) mice, we were able to identify that endothelium (eNOS)-derived hydrogen peroxide (H_2O_2) is an EDHF in mouse mesenteric arteries.[28] We then confirmed that this is also the case in human mesenteric arteries[29] and porcine[30] and canine[31] coronary microvessels (*Figure 1B*). Other investigators also reported that endothelium-derived H_2O_2 is an EDHF in piglet pial arterioles[32] and human coronary microvessels.[33] We further demonstrated that endothelial Cu,Zn superoxide dismutase (SOD) plays an important role in the synthesis of H_2O_2 in mouse[34] and human[35] mesenteric arteries. In order to fully understand the role of endothelial NOSs in the H_2O_2/EDHF-mediated responses, we generated mice that are deficient of all three NOS isoforms, including eNOS, neuronal NOS (nNOS), and inducible NOS (iNOS).[36] Interestingly, although NO responses were abolished in the aorta of singly $eNOS^{-/-}$ mice (*Figure 2A*), the EDHF responses of mesenteric arteries were progressively reduced as the number of deleted NOS gene increased and were finally abolished in triply $NOSs^{-/-}$ mice (*Figure 2B*), whereas vasodilator and hyperpolarizing responses of VSMC per se were preserved.[37] These results provided us with the novel concept that endothelial NOSs system plays an important role as the EDHF-generating system in microvessels, while the system acts as the NO-generating system in large conduit arteries in its original meaning (*Figure 2C*).[37] In contrast, the contribution of other oxygenases to EDHF responses may be minimal as pharmacological or genetic blockade of those oxygenases had no effects on the responses (*Figures 1B* and *2C*).[38]

Vasodilating mechanisms of hydrogen peroxide/endothelium-derived hyperpolarizing factor

Hydrogen peroxide has been reported to cause vasodilatation through several mechanisms, including cGMP, cAMP, cyclooxygenase, and several K channels, depending on blood vessels tested.[21] Importantly, as an EDHF in microcirculation, H_2O_2 rapidly reaches VSMC, stimulates the 1α isoform of cGMP-dependent protein kinase (PKG1α) to form disulfide form and opens K_{Ca} channels with subsequent VSMC hyperpolarization and relaxation (*Figure 3*).[39] Hydrogen peroxide/EDHF plays an important role in blood pressure regulation as mice with dysfunctional PKG1α exhibit hypertension.[39]

Mechanisms for enhanced endothelium-derived hyperpolarizing factor responses in microvessels

Endothelium-derived hyperpolarizing factor responses appear to be the back-up system for NO responses that are easily impaired by

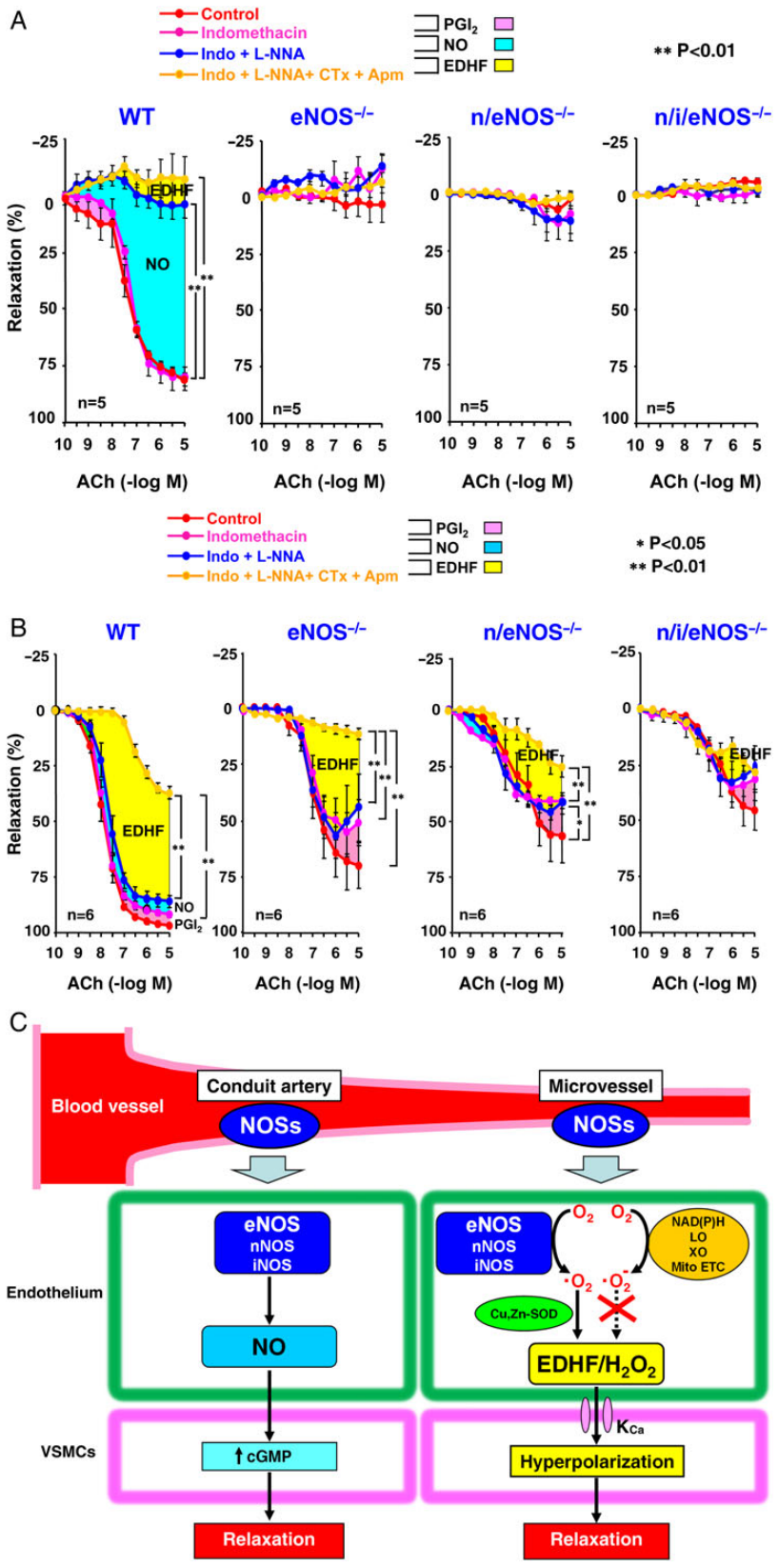

Figure 2 Endothelium-dependent relaxations in $NOS^{-/-}$ mice. (*A*) In the mouse aorta, endothelium-dependent relaxations to acetylcholine were abolished in singly $eNOS^{-/-}$ mice. (*B*) In the mouse mesenteric artery, endothelium-dependent relaxations were progressively reduced as the number of deleted NOS genes increased and were finally abolished in triply $NOS^{-/-}$ mice. (*C*) The vasodilator functions of the NOS system in the endothelium appears to be heterogeneous depending on the size of blood vessels. (*A* and *B*, from Ref.[37] with permission.)

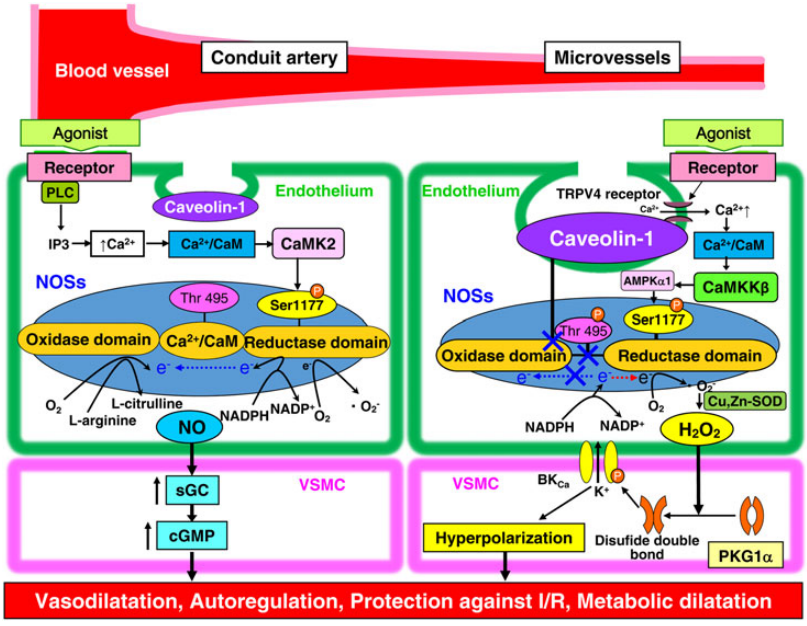

Figure 3 Mechanisms for the enhanced endothelium-derived hyperpolarizing factor responses in microvessels. Multiple mechanisms are involved for the enhanced endothelium-derived hyperpolarizing factor responses in microvessels, including functional inhibition of endothelial NO synthase through caveolin-1 and Ca^{2+}/calmodulin-dependent protein kinase kinase β in the endothelium and enhanced PKG1α-mediated vascular smooth muscle cells relaxation in response to hydrogen preoxide/endothelium-derived hyperpolarizing factor.

atherosclerotic risk factors.[21] Thus, we aimed to elucidate the molecular mechanisms for enhanced EDHF responses in microvessels in mice in order to seek for a new strategy for atherosclerosis. The results showed that when compared with the aorta, eNOS is functionally suppressed in microvessels, for which Ca^{2+}/calmodulin-dependent protein kinase kinase β (CaMKKβ) and caveolin-1 are involved and that relaxation responses of VSMC to H_2O_2 are enhanced through PKG1α-mediated mechanism (*Figure 3*).[40] Thus, multiple mechanisms are involved in the enhanced EDHF responses in microvessels (*Figure 3*).[21,40] We have recently demonstrated the important role of the bone marrow (BM) in modulating microvascular endothelial and metabolic functions.[41] In this study, reduced microvascular endothelial and metabolic functions in $eNOS^{-/-}$ mice were improved by transplantation of wild-type BM but not $eNOS^{-/-}$-BM and that those improvements were absent in doubly $eNOS^{-/-}$/$adiponectin^{-/-}$ or e/$nNOS^{-/-}$ mice.[41] Thus, the BM plays an important role in modulating microvascular endothelial and metabolic functions, for which adiponectin and nNOS may be involved.[41] Recently, we have also demonstrated that endothelial AMP-activated protein kinase (AMPK), an important metabolic regulator, plays a crucial role in EDHF responses in microvessels (but not in the aorta), regulating blood pressure and coronary flow responses in mice *in vivo* (*Figure 3*).[42]

Clinical importance of hydrogen peroxide/endothelium-derived hyperpolarizing factor

Since EDHF responses are defined as the remaining responses after the blockade of those mediated by vasodilator PGs and NO, it is not so easy to precisely evaluate the *in vivo* importance of EDHF, especially in humans. However, the existence of EDHF-mediated responses has been repeatedly documented in isolated human arteries[29,33] and human forearm circulation *in vivo*.[43–47] In the canine coronary microcirculation *in vivo*, endothelium-derived H_2O_2 exerts important cardioprotective effects, including coronary autoregulation,[31] myocardial protection against ischaemia/reperfusion injury,[48] and metabolic coronary dilatation (*Figure 3*).[49] Furthermore, EDHF responses are abolished when all three NOS isoforms are absent in the triply $NOS^{-/-}$ mice.[21,37] Importantly, those mice exhibit typical characteristics of metabolic syndrome in humans, including visceral obesity, hypertension, glucose intolerance, and dyslipidaemia with a reduced survival (mainly due to myocardial infarction), indicating the important roles of the NOSs system to maintain cardiovascular and metabolic homeostasis (*Figure 4A* and *B*).[36,50,51] It has been reported that EDHF responses are impaired in postmenopausal women[43] and patients with coronary artery disease[44] and are improved by short-term oestrogen-replacement therapy and long-term oral treatment with eicosapentaenoic acid, respectively.

Coronary artery spasm

Coronary artery spasm plays an important role in a wide variety of ischaemic heart diseases not only in variant angina but also in unstable angina, myocardial infarction, and sudden death.[52–54] Since coronary artery spasm can be induced by a variety of stimuli with different mechanisms of action (even in the same patient), the occurrence of the spasm appears to be due to local hyperreactivity of the coronary artery rather than to an enhanced stimulation with a single mechanism of action.[55,56] To elucidate the cellular and molecular

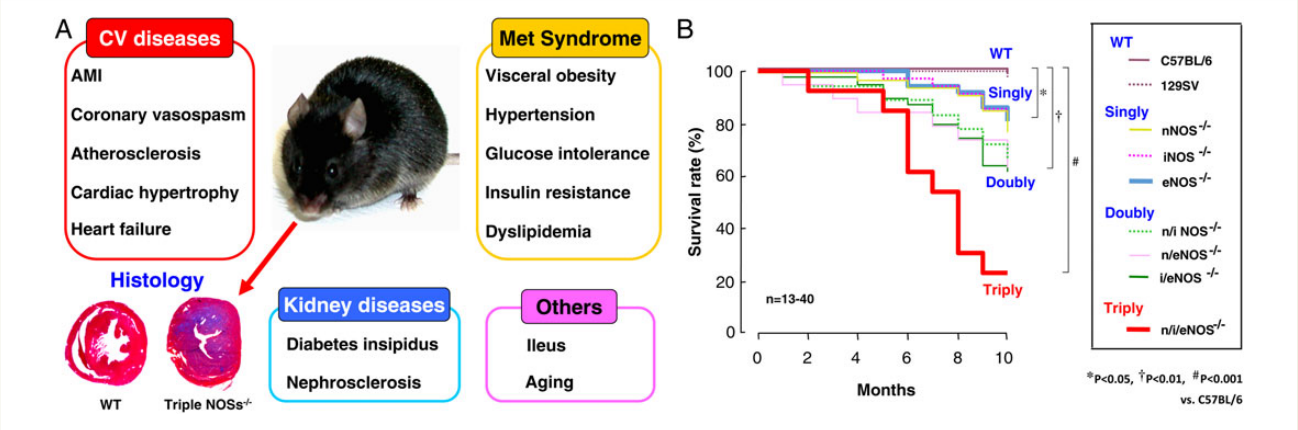

Figure 4 Metabolic phenotypes and reduced survival of triply NOS$^{-/-}$ mice. (*A*) In mice, multiple metabolic phenotypes become evident only when all three NO synthases are deleted. (*B*) The long-term survival becomes deteriorated as the number of deleted NO synthases increases. (*B*, from Ref.[36] with permission.)

mechanisms of the spasm, we have developed the animal models of the spasm.

Animal models of coronary artery spasm

Based on the clinical observations that coronary artery spasm frequently occurs at the angiographically atherosclerotic lesions of the coronary artery, we first examined whether experimental atherosclerotic coronary lesion, induced by a combination of balloon endothelium removal and high-cholesterol feeding, exhibits hyperresponsiveness to vasoconstrictor agents in pigs *in vivo*.[57,58] Although the degree of the atherosclerotic lesion was too mild to detect angiographically, intracoronary administration of serotonin or histamine repeatedly induced coronary artery spasm at the atherosclerotic lesion (*Figure 5A*), and there was a close topological correlation between the spastic site and atherosclerotic lesion (*Figure 5B*).[57,58] These results provided the first experimental evidence for the close relationship between coronary artery spasm and coronary atherosclerosis,[57,58] which was subsequently confirmed in patients who underwent coronary balloon angioplasty[59] and those with vasospastic angina.[60] In this first porcine model, however, endothelial dysfunction is inevitable because of endothelial regeneration after endothelium removal[61,62] and it was practically difficult to separate the role of endothelial dysfunction and VSMC hypercontraction in this first model.[63]

Based on the pathological reports that the spastic human coronary artery had extensive adventitial inflammation and perivascular nerve lesions,[64–66] we then examined whether experimental adventitial inflammation could cause vasospastic activity of the coronary artery without endothelium removal in pigs *in vivo*. Two weeks after the adventitial application of interleukin-1β (IL-1β), coronary angiography showed the development of mild stenotic lesion, where intracoronary administration of serotonin, histamine or platelet activating factor (PAF) repeatedly caused coronary spasm but not at the control site (*Figure 5C*).[67–71] Histological examination showed adventitial accumulation of inflammatory cells, mild neointimal formation, and a marked reduction in vascular cross-sectional area (negative remodelling) (*Figure 5D*)[67–71] and VSMC phenotypes were altered towards dedifferentiation at the spastic site.[70] In this second porcine model, endothelial vasodilator functions were fairly preserved as expected.[72] These vascular effects were not specific for IL-1β, because the same adventitial treatment with other inflammatory cytokines (e.g. IL-1α and TNF-α) also induced the similar histological and functional alterations of the porcine coronary artery.[70] These results provided the first experimental evidence for the role of adventitial inflammation in the pathogenesis of coronary artery spasm, indicating that among the components of atherosclerosis, inflammatory changes play a major role in the pathogenesis of the spasm.

Endothelial dysfunction vs. vascular smooth muscle cell hypercontraction

Accumulating evidence has indicated that VSMC hypercontraction plays a major role in the pathogenesis of coronary spasm, whereas the role of endothelial dysfunction may be minimal (*Table 1*). First, coronary spasm occurs at a given site of the atherosclerotic coronary artery, whereas endothelial dysfunction is rather generalized throughout the epicardial coronary arteries and systemic arteries throughout the body.[9,73] Second, vasodilating responses to bradykinin[74] or substance P,[75–77] both of which are endothelium-dependent vasodilators, are fairly preserved at the spastic coronary segment in patients with variant angina. Third, long-term treatment with eicosapentaenoic acid, a major component of fish oils that is known to improve endothelial vasodilator function,[78,79] failed to suppress coronary vasospastic activity in patients with variant angina.[80] Fourth, the racial difference in the prevalence of vasospastic angina with greater prevalence in Japanese than in Caucasian population[53,54,81] cannot be simply explained by endothelial dysfunction. Fifth, marked diurnal changes of coronary spasm with high prevalence from night to early morning[82,83] cannot be explained by simple endothelial dysfunction, although circadian variation of VSMC hyperreactivity remains to be fully elucidated. Sixth, fluctuation of spastic location[84] and even spontaneous remission of the spasm[85] occur in some patients with vasospastic angina despite the persistence of atherosclerotic coronary lesion, which again cannot be explained by simple endothelial dysfunction. Seventh, nitrates are effective to

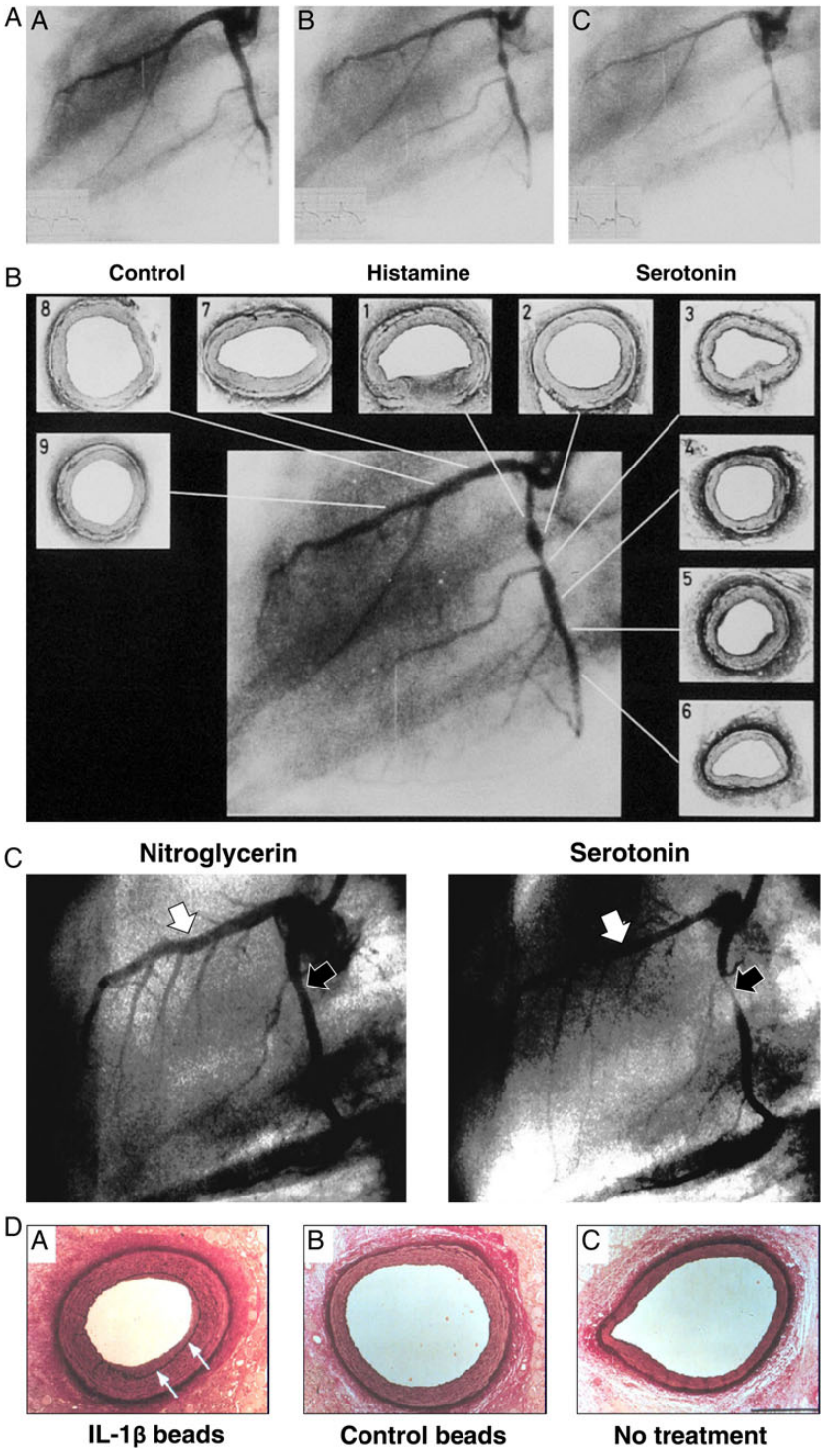

Figure 5 Coronary artery spasm induced in two porcine models *in vivo*. (*A* and *B*) Coronary artery spasm was induced in atherosclerotic miniature pigs induced by balloon endothelial injury and high-cholesterol feeding (*A*), where topological correlation was noted between the spastic sites and the early atherosclerotic lesions (*B*). (*A*, from Ref.[57] with permission). (*C* and *D*) Coronary artery spasm was induced in pigs with adventitial inflammation (*C*), where intimal thickening and negative remodelling were noted (*D*). (*C* and *D*, from Ref.[67] with permission.)

acutely abolish coronary spasm by VSMC relaxation but have no acute beneficial effect on endothelial dysfunction, indicating that coronary spasm is a phenomenon of VSMC hypercontraction.[86] Finally, as discussed later, there are lines of direct evidence that coronary spasm is caused by VSMC hypercontraction mediated by Rho-kinase activation (*Table 1*). Indeed, it was shown that contractility of

Table 1 Evidence for the primary role of vascular smooth muscle cells hypercontraction but not endothelial dysfunction in the pathogenesis of coronary artery spasm

Coronary artery spasm (VSMC hypercontraction)	Endothelial dysfunction
Local	Systemic
Evidence of coronary VSMC hypercontraction	Preserved endothelium-dependent responses to BK and SP
—	Failure of EPA with improved endothelial function to suppress coronary spasm
Racial difference	No racial difference
Marked diurnal change	Less diurnal change
Fluctuation and spontaneous remission	No fluctuation or spontaneous remission
Acute effects of vasodilators	No acute effects of vasodilators

BK, bradykinin; EPA, eicosapentaenoic acid; SP, substance P; VSMC, vascular smooth muscle cells.

coronary VSMC is augmented at the spastic coronary segment in a patient with variant angina[87] and as mentioned later in detail, in the porcine models of coronary artery spasm.[88,89] Furthermore, it has been demonstrated in the second porcine model with IL-1β that VSMC hypercontraction plays a primary role while endothelial vasodilator function is fairly well preserved both *in vivo* and *in vitro*.[67,72]

Mechanism of vascular smooth muscle cells hypercontraction

When agonists (e.g. serotonin and histamine) bind to their receptors, phospholipase C is activated, leading to the formation of inositol 1,4,5-triphosphate (IP_3) and diacylglycerol by the hydrolysis of phosphatidyl-inositol 4,5-bis-phosphate (*Figure 6A*).[90,91] Inositol 1,4,5-triphosphate then binds to an IP3 receptor on the membrane of the sarcoplasmic reticulum (SR) to mobilize the stored calcium ions (Ca^{2+}) from the SR into the cytosol. Diacylglycerol activates protein kinase C (PKC), causes vasoconstriction and augments Ca^{2+} sensitivity of contractile proteins.[90] Thus, both the intracellular Ca^{2+} store and the PKC-mediated pathway could contribute to the pathogenesis of coronary spasm, although the relative importance of the two mechanisms remains to be clarified. It has been demonstrated that several mechanisms are involved in the Ca^{2+} sensitivity of myosin filaments, including myosin phosphatase[92] and the small GTPase Rho and its target, Rho-kinase (*Figure 6A*).[93,94]

Vascular smooth muscle cells hypercontraction and coronary artery spasm

Coronary artery spasm is caused primarily by VSMC hypercontraction. However, the vasocontracting response to increasing Ca^{2+} concentrations is unaltered in VSMC from spastic coronary artery in our first porcine model.[88] Subsequently, it was also reported that vasocontracting response to increasing concentrations of Ca^{2+} is unaltered in aortic VSMC from Watanabe hereditary hyperlipidaemic rabbits as compared with normal rabbits.[95] These results suggest that Ca^{2+} sensitivity of contractile proteins per se is unaltered at the spastic site and that the key mechanism for coronary spasm is present somewhere between receptors and contractile proteins in the signal transduction pathway for VSMC contraction (*Figure 6A*). Furthermore, in the second porcine model with IL-1β, coronary hypercontraction is mediated primarily by the 5-HT_{2A} serotonergic receptor, whereas the expression or functions (receptor affinity and number) of the 5-HT_{2A} receptor are not significantly altered when compared with normal coronary arteries.[96] These results suggested that the key mechanism for the spasm is present somewhere below the receptors and above the contractile proteins in the signal transduction pathway for VSMC contraction (*Figure 6A*).

In addition to the receptor-mediated stimulation by serotonin or histamine, direct activation of PKC by phorbol esters also causes coronary spasm while inhibition of PKC (by PKC inhibitors, such as staurosporine and sphingosine) suppresses it in both the first[97] and the second porcine models.[98] Coronary artery spasm induced by serotonin or histamine was also inhibited by the PKC inhibitors.[97,98] The inhibitory effects of the PKC inhibitors were specific because they did not inhibit the coronary contractions induced by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$).[97,98] These results indicated that the PKC-mediated pathway is substantially involved in the pathogenesis of coronary spasm (*Figure 6A*). In our porcine models of coronary spasm, the spasm was also induced by Bay K 8644, a direct opener of L-type Ca channel, which was also inhibited by the PKC inhibitors (*Figure 6A*).[97,98] These results suggested that Ca^{2+} entry through L-type Ca channel into VSMC is the initial trigger for coronary spasm and that Ca^{2+} entry might be augmented via PKC-dependent mechanism (*Figure 6A*).[97,98] Indeed, it has been demonstrated that L-type Ca channel is functionally up-regulated at the spastic site in our first porcine model.[99]

Enhanced myosin light-chain phosphorylations and coronary artery spasm

Phosphorylation of MLC is one of the most important steps for VSMC contraction.[92,100] Vascular smooth muscle cells contraction is initiated by Ca^{2+}/calmodulin-activated MLC kinase (MLCK) with subsequent phosphorylation of the 20-kDa regulatory MLC.[92,100] Phosphorylation of the regulatory MLC then activates myosin Mg^{2+}-ATPase and permits cross-bridge cycling, which leads to force generation and contraction.[92,100] The level of MLC phosphorylation is determined by a balance between MLC phosphorylation by MLCK and dephosphorylation by MLC phosphatase (*Figure 6A*).[92,100] It was reported that MLC phosphorylation is augmented in canine vasospastic cerebral artery after experimental subarachnoid hemorrhage[101] and in hyperplastic rabbit carotid artery after balloon injury.[102]

In our porcine model with IL-1β, MLC monophosphorylation was enhanced at the spastic coronary segment and MLC diphosphorylation, which was never observed in the normal coronary artery, was also induced during serotonin-induced coronary spasm.[89] There was a positive correlation between the serotonin-induced coronary vasocontractions and MLC mono- and diphosphorylations.[89] Fasudil, an inhibitor of protein kinases with 10 times more potent inhibitory effect against PKC (Ki = 3.3 μmol/l) than against MLCK (Ki = 36.0 μmol/l), caused dose-dependent inhibition in both serotonin-induced coronary hypercontractions and enhanced MLC

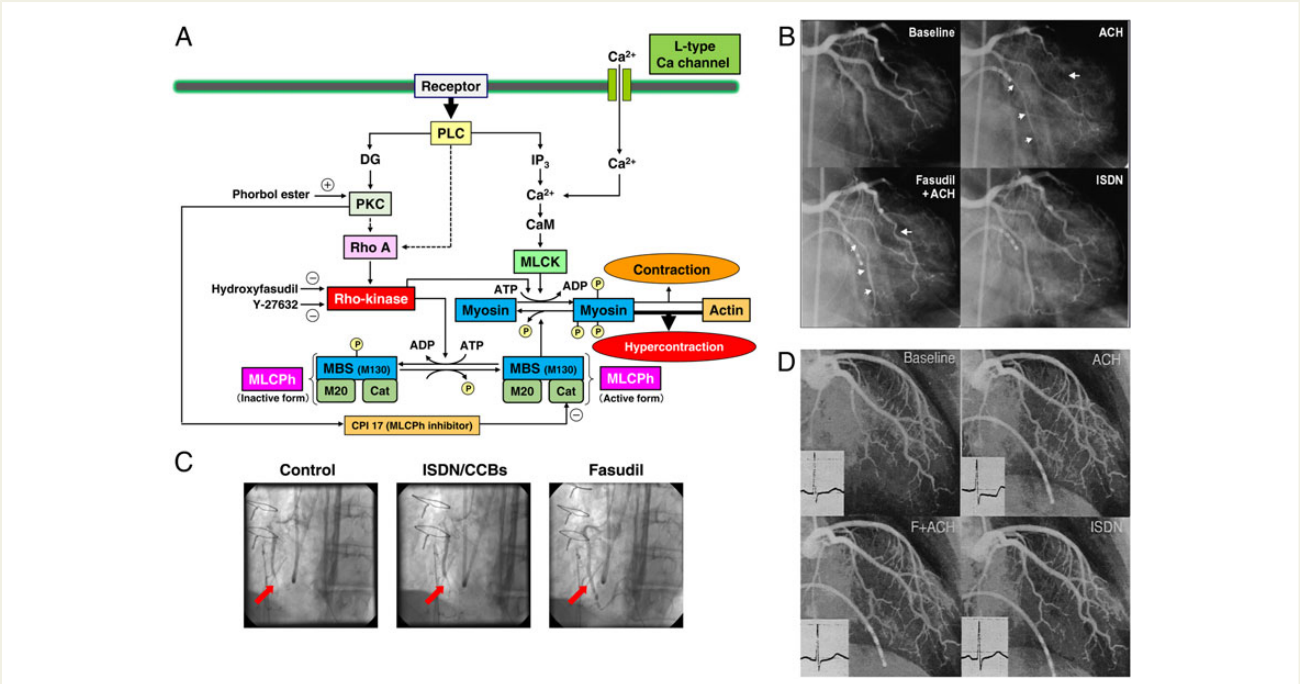

Figure 6 Molecular mechanisms of vascular smooth muscle cells hypercontraction for coronary spasm. (*A*) The central molecular mechanism of vascular smooth muscle cells hypercontraction for coronary spasm is Rho-kinase-mediated enhancement of myosin light chain phosphorylations through inhibition of myosin light chain phosphatase. (From Ref.[91] with permission). (*B*) Inhibitory effects of intracoronary administration of fasudil, a Rho-kinase inhibitor, on acetylcholine-induced multi-vessel coronary spasm in a patient with vasospastic angina. (From Ref.[120] with permission.) (*C*) Inhibitory effects of intracoronary fasudil on refractory angina resistant to nitrates or CCBs in a patient undergoing coronary artery bypass surgery. (Quoted from Ref.[121] with permission.) (*D*) Inhibitory effects of intracoronary fasudil on ischaemic ECG changes in a patient with microvascular angina. (Quoted from Ref.[122] with permission.)

phosphorylations in the spastic coronary segment.[89] Phosphorylation of the second site of MLC is known to further increase the actin-activated Mg^{2+}-ATPase activity of myosin *in vitro*.[103,104] These results indicated that enhanced MLC phosphorylations play a central role in the pathogenesis of coronary spasm in our porcine model. The phosphorylated site of MLC is MLCK-dependent Ser19 for MLC monophosphorylation and MLCK-dependent Ser19/Thr18 for MLC diphosphorylation.[105]

Phenotype modulation of VSMC (from growth-arrested type to actively growing type) was noted in the neointimal regions of the atherosclerotic artery.[106] In cultured VSMC, MLC diphosphorylation is enhanced in actively growing cells compared with growth-arrested cells.[107] In the second porcine model, the phenotype of VSMC (myosin heavy chain isoforms) is altered towards dedifferentiation.[70] These results suggest that MLC diphosphorylation occurs only in the actively growing cells in the spastic coronary artery. The phenotype change of arterial VSMC may thus be one of the important mechanisms of coronary artery spasm.

The generation of diphosphorylated MLC is caused in part by inhibition of MLC phosphatase in VSMC.[102] The treatment with calyculin A, a protein phosphatase inhibitor, potently induces MLC diphosphorylation in VSMC without an increase in intracellular Ca^{2+} levels.[107] In permeabilized porcine aortic VSMC, the increase in intracellular Ca^{2+} levels causes MLC monophosphorylation alone, whereas additional treatment with GTP-γ S, which is thought to inactivate MLC phosphatase, causes both mono- and diphosphorylation of MLC.[108] These results suggest that inhibition of MLC phosphatase activity is essential for induction of MLC diphosphorylation in VSMC. The mechanism of the inhibition of MLC phosphatase, however, remains to be clarified. Several important reports have been published on this issue. A novel inhibitor of MLC phosphatase that is potentiated by PKC (C-kinase-activated phosphatase inhibitor, CPI-17) has been isolated from porcine aortic media.[109] Rho-kinase phosphorylates the 130-kDa subunit of MLC phosphatase and reduces its activity.[90] All these mechanisms may be involved in the inhibition of MLC phosphatase at the spastic coronary segment (*Figure 6A*).

Rho-kinase and vascular smooth muscle cell hypercontraction

Studies *in vitro* demonstrated that a GTP-binding protein regulates the receptor-mediated sensitization of the MLC phosphorylation[110] and that small GTPase Rho is involved in the GTP-enhanced Ca^{2+} sensitivity of VSMC contraction.[93,108] Importantly, Rho regulates MLC phosphorylation through its target, Rho-kinase, and the myosin-binding subunit (MBS) of myosin phosphatase.[94,111] Smooth muscle MLC phosphatase consists of 38-kDa catalytic subunit, the 130-kDa MBS, and the 20-kDa subunit.[112,113] The MBS serves as a target subunit of MLC phosphatase to myosin and enhances the activity of the enzyme towards myosin.[112] Activated Rho interacts

with Rho-kinase to activate it. The activated Rho-kinase subsequently phosphorylates the MBS, thereby inactivating myosin phosphatase.[94] Rho-kinase itself might also phosphorylate MLC at the same site that is phosphorylated by MLCK, and activate myosin ATPase *in vitro*.[111] Activated form of Rho-kinase enhances MLC phosphorylation[114] and induces VSMC contraction,[115] stress fibre formation and neurite retraction[116] (*Figure 6A*). Both pathways, inhibition of myosin phosphatase and direct phosphorylation of MLC, may be involved in the increase in MLC phosphorylations.[115]

Hydroxyfasudil, an active metabolite of fasudil after oral absorption, preferentially inhibits Rho-kinase compared with MLCK or PKC (at least 100 times more potent at IC50 levels).[105] Hydroxyfasudil causes dose-dependent inhibition of the serotonin-induced coronary spasm in the porcine model with IL-1β both *in vivo* and *in vitro* through suppression of serotonin-induced increases in MLC mono- and diphosphorylations.[105] Thus, the hydroxyfasudil-sensitive Rho-kinase-mediated pathway plays an important role in the enhanced MLC phosphorylations in the spastic coronary artery (*Figure 6A*).

In order to further elucidate the molecular mechanism of coronary spasm in our porcine model, experiments were performed to examine whether or not Rho-kinase is up-regulated at the spastic site and if so, how it induces VSMC hypercontraction.[117] RT–PCR analysis demonstrated that the expression of Rho-kinase mRNA and, to a lesser extent, that of RhoA mRNA were significantly up-regulated in the spastic than in the control segment.[117] Western blot analysis showed that during the serotonin-induced contractions, the extent of MBS phosphorylation was significantly greater in the spastic than in the control segment. Furthermore, another Rho-kinase inhibitor, Y-27632,[118] also inhibited not only serotonin-induced hypercontractions *in vivo* and *in vitro* but also the increase in MBS phosphorylations.[117] Importantly, there was a highly significant positive correlation between the extent of MBS phosphorylations and that of contractions in the spastic but not in the control segments.[117] These results indicate that Rho-kinase is up-regulated at the spastic site and plays a key role in inducing VSMC hypercontraction by inhibiting MLC phosphatase through MBS phosphorylation in our porcine model (*Figure 6A*).[117,119] Indeed, subsequent clinical studies showed that intracoronary fasudil is effective in almost all patients with epicardial coronary spasm (*Figure 6B*).[120] Importantly, intracoronary fasudil is also effective in relieving intractable coronary spasm resistant to nitrates and Ca channel blockers (*Figure 6C*).[121] Moreover, fasudil is effective to suppress microvascular coronary spasm in approximately two-thirds of

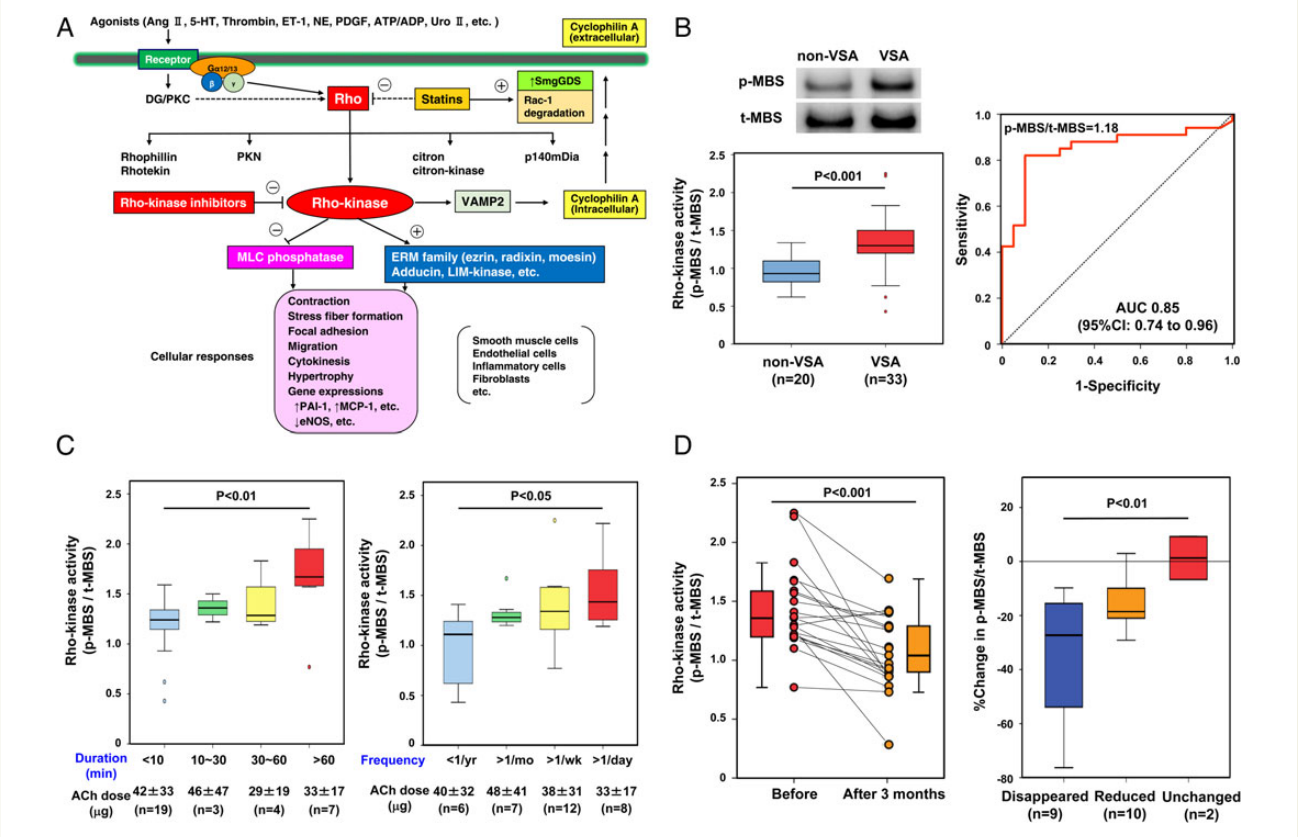

Figure 7 The important roles of Rho/Rho-kinase pathway in the pathogenesis of cardiovascular diseases. (*A*) The Rho/Rho-kinase pathway plays important roles in the pathogenesis of vasospastic disorders as well as atherosclerotic cardiovascular diseases in general. (Quoted from Ref.[91] with permission.) (*B–D*) Rho-kinase activity in circulating leucocytes may be a useful biomarker for the diagnosis of vasospastic angina (*B*), and assessment of disease activity (*C*) and efficacy of medical treatment (*D*). (Quoted from Ref.[131] with permission.)

patients with microvascular angina (*Figure 6D*).[122] These results indicate the usefulness of Rho-kinase inhibitors for the treatment of coronary vasospastic disorders.[91,123,124]

Rho-kinase and cardiovascular diseases

Accumulating evidence has indicated that Rho-kinase plays important roles in the pathogenesis of a wide range of cardiovascular diseases in general and coronary vasomotion abnormalities in particular (*Figure 7A*).[91,123,124] Indeed, the Rho/Rho-kinase pathway not only mediates VSMC hypercontraction thorough inhibition of MLC phosphatase as mentioned above but also promotes atherosclerotic process through enhancing cell responses towards the disorder (*Figure 7A*).[91,123,124] One of the recent topics on the Rho-kinase pathway is that the secretion of cyclophilin A (CyPA), which is a novel mediator of oxidative stress, is mediated by Rho-kinase (*Figure 7A*).[125,126] We have recently identified that small GTP-binding protein dissociation stimulator (SmgGDS) plays a central role of the pleiotropic effects of statins independent of the Rho-kinase pathway (*Figure 7A*).[127] Furthermore, in a series of studies, we have demonstrated that the Rho-kinase pathway plays a crucial role in the pathogenesis of coronary hyperconstricting responses induced by drug-eluting stents (DESs) in pigs[128] and humans[129] and that long-term treatment with a long-acting nifedipine suppresses DES-induced coronary vasomotor abnormalities through indirect inhibition of Rho-kinase pathway.[130]

We have recently demonstrated that Rho-kinase activity in circulating leucocytes is a useful biomarker for coronary artery spasm, not only for the diagnosis of the disorder (*Figure 7B*) but also for the assessment of disease activity (*Figure 7C*) and efficacy of medical treatment (*Figure 7D*)[131] and that there is a circadian change of the activity with a peak noted in the early morning associated with chest symptoms.[132] Rho-kinase activity in circulating leucocytes of patients is also elevated in pulmonary hypertension,[133] chronic heart failure,[134] and cardiovascular diseases in general[135] although no correlation is noted with the level of high-sensitivity C-reactive protein.[134] Thus, the Rho-kinase activity may represent not only the extent of vasospastic disorder but also a new aspect of systemic inflammation. Taken together, the role of the Rho-kinase pathway in the pathogenesis of cardiovascular diseases has been emerging and the possible indications of Rho-kinase inhibitors have been expanding in cardiovascular medicine (*Figure 8*).[91,123,124]

Japanese coronary spasm association

In order to develop evidence-based medicine on coronary artery spasm, we established the Japanese Coronary Spasm Association (JCSA) in 2006, in which 72 leading institutes in Japan participated (see Supplementary material online, *Figure S1*). We first performed the retrospective registry study of Japanese VSA patients, demonstrating that those who had survived out-of-hospital cardiac arrest are at high risk,[136] that the spasm provocation tests have an acceptable level of safety,[137] that there are gender differences in the characteristics and outcomes of VSA patients,[138] that the novel JCSA risk score may provide the comprehensive risk assessment and prognostic stratification for VSA patients,[139] and that combination therapy with long-acting nitrates and CCBs may be associated with reduced long-term survival.[86] We are now conducting the prospective international registry study of vasospastic angina in six countries, which should elucidate the unknown aspects of the disorder (Supplementary material online, *Figure S2*). It is possible that the prevalence of coronary artery spasm in Western population is not so low as previously considered compared with Japanese population.[140,141]

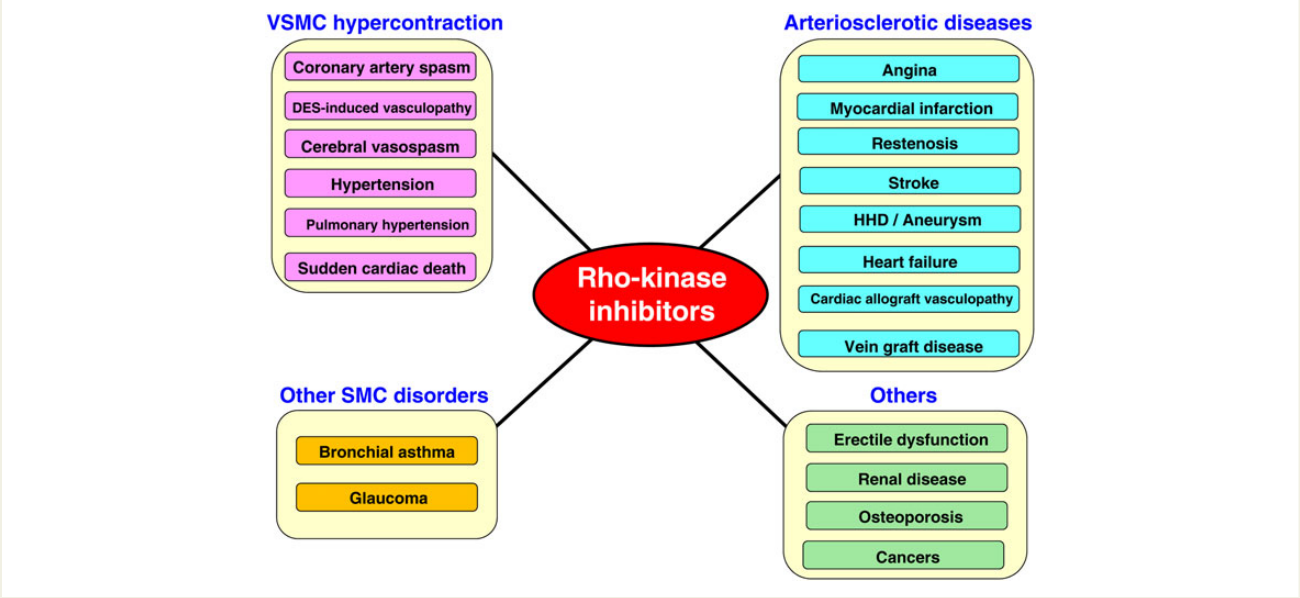

Figure 8 Possible indications of Rho-kinase inhibitors. Selective Rho-kinase inhibitors are expected to exert therapeutic effects for a number of indications.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

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CARDIOVASCULAR FLASHLIGHT

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A rare case of lupus with multiple unusual cardiovascular complications

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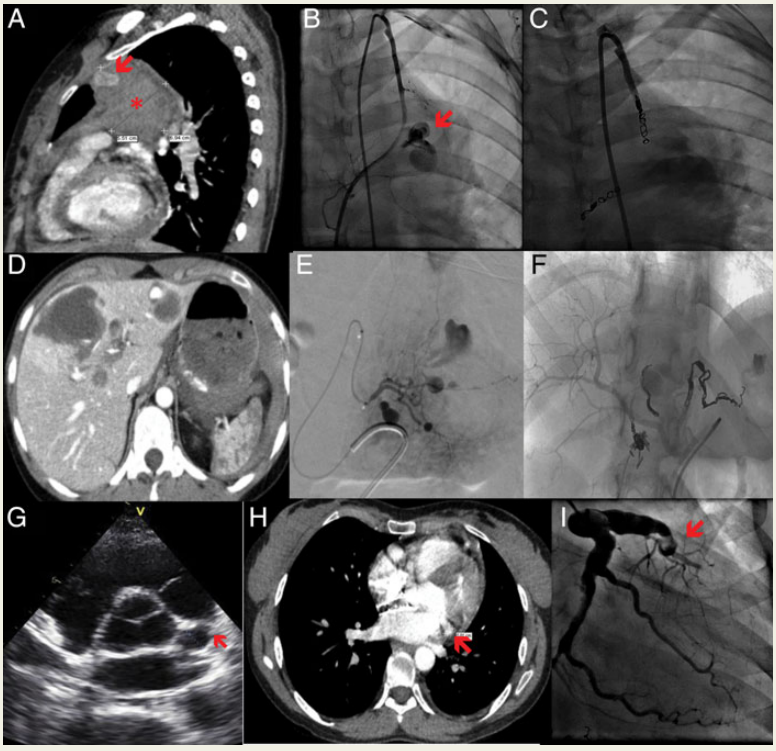

A 25-year-old woman presented with sudden severe left-sided chest pain. Her co-morbidities include systemic lupus erythematosus, antiphospholipid antibody syndrome, four prior strokes, patent foramen ovale (closed percutaneously), and an abdominal aortic aneurysm repair. Upon admission, her INR was 3 (on coumadin). A chest CT scan (*Panel A*) showed a large mediastinal haematoma (asterisk) and active extravasation (arrow) secondary to rupture of left internal mammary artery aneurysm (*Panel B*, Supplementary material online, *Video S1*). She underwent coil embolization (*Panel C*, Supplementary material online, *Video S2*). Six days later, the patient went into shock preceded by abdominal pain. Abdominal CT (*Panel D*) revealed intrahepatic haematomas secondary to ruptured aneurysms seen on angiography (*Panel E*, Supplementary material online, *Video S3*). This was treated successfully with selective coil embolization of right and left hepatic arteries (*Panel F*, Supplementary material online, *Video S4*). A week later, while anticoagulation was being withheld, the patient had vague chest pain. Electrocardiogram and cardiac biomarkers confirmed myocardial infarction. An echocardiogram showed new wall motion abnormalities (Supplementary material online, *Video S5*) and left main (LM) coronary artery aneurysm (*Panel G*), also seen on a CT scan (*Panel H*). Emergent coronary angiography (Supplementary material online, *Video S6*) confirmed a giant aneurysm involving LM, left anterior descending (LAD) and left circumflex arteries with a large thrombus in the LAD (*Panel I*). Despite thrombectomy, revascularization was incomplete (Supplementary material online, *Video S7*). Aneurysms of the coronary, hepatic, and internal mammary arteries with or without thrombotic or bleeding complications are rare in lupus patients. No case has been reported in the literature in which all these findings were seen simultaneously in the same patient.

Supplementary material is available at *European Heart Journal* online.