Calcium/calmodulin-dependent protein kinase II (CaM Kinase II) is a known modulator of cardiac pathophysiology. The present review uniquely focuses on novel CaM Kinase II-mediated endothelial cell signalling which, under pathophysiological conditions, may indirectly modulate cardiac functions via alterations in endothelial or endocardial responses. CaM Kinase II has four different isoforms and various splicing variants for each isoform. The endothelial cell CaM Kinase II isoforms are sensitive to KN93 and a threonine 286-mutated inhibitory peptide. In macrovascular endothelial cells derived from aortas, CaM Kinase II mediates redox-sensitive upregulation of endothelial nitric oxide synthase (eNOS) gene expression by hydrogen peroxide (H₂O₂) and oscillatory shear stress, and a rapid activation of eNOS in response to bradykinin. In endothelial cells derived from lung microvessels, CaM Kinase II mediates barrier dysfunction, particularly when activated by thrombin. In brain capillary endothelial cells, CaM Kinase II lies upstream of voltage-gated potassium channels and hypoxia-induced cell swelling. In both macrovascular and microvascular endothelial cells, CaM Kinase II mediates actin cytoskeleton reorganization via distinct p38 MAPK/HSP27 and ERK1/2/MLCK signalling pathways, respectively. Although understanding of endothelium-specific CaM Kinase II signalling is nascent, data accumulated so far have demonstrated a potentially significant role of CaM Kinase II in endothelial cell pathophysiology.

KEYWORDS
CaM Kinase II; Endothelial nitric oxide synthase (eNOS); Hydrogen peroxide; Shear stress; Actin cytoskeleton; Barrier function; Thrombin; Bradykinin

Calcium/calmodulin-dependent protein kinase II (CaM Kinase II) is a ubiquitously expressed serine/threonine protein kinase. When first discovered, its function was primarily studied in the neurons where CaM Kinase II has the highest expression levels. Subsequently CaM Kinase II-dependent signal transduction was found critical for long-term memory.

CaM Kinase II was named by its sensitivity to calcium/calmodulin (Ca²⁺/CaM). However, it is not a simple sensor to changes in intracellular calcium. Auto-phosphorylation of threonine 286 occurs upon an initial increase in Ca²⁺ and Ca²⁺/CaM binding, resulting in structural changes of the regulatory domain of the CaM Kinase II. This releases the catalytic domain from auto-inhibition, resulting in an active enzyme that remains active for a long-term even after disassociation of Ca²⁺/CaM. This unique molecular mechanism of activation seems to explain why CaM Kinase II mediates long-term memory, as it prolongs or ‘remembers’ an initial calcium signal, which is further passed on via CaM Kinase II-dependent phosphorylation of downstream effectors. Similarly in the endothelial cells, CaM Kinase II activation has been shown to precede activations of downstream tyrosine kinase and serine/threonine kinase, resulting in changes in gene expression, enzyme activity, channel openings, and additional events that are eventually translated into pathophysiological phenotypes of endothelial dysfunction.

1. Endothelial isoforms of CaM Kinase II
CaM Kinase II is encoded by four different genes (α, β, δ, and γ) with each isoform exhibiting various splicing variants. The α and β isoforms are most abundant in, and largely restricted to, the neurons, whereas δ and γ isoforms express in most tissues. For cardiomyocytes, δ is the most...
prominent isoform. It has remained unclear which isoform dominants in the endothelial cells, but δ and γ isoforms have been found in both vascular smooth muscles\(^1\) \(^4\) and endothelial cells.\(^5\) Earlier study by Deli et al.\(^6\) and Krizbai et al.\(^7\) also documented an ischaemia-induced activation of α isoform in cerebral endothelial cells. Various studies from different groups have shown that the endothelial CaM Kinase II is KN93-sensitive (pharmacologic inhibitor). It also shares with the neuronal α isoform the same conservative kinase domain, which can be competitively inhibited by a peptide targeting at threonine 286.\(^8\) \(^12\)

2. Role of CaM Kinase II in redox-sensitive regulation of eNOS gene expression

Oxidant stress has been shown to contribute to endothelial dysfunction and cardiomyopathy.\(^13\) \(^15\) Among biologically relevant reactive oxygen species (ROS), hydrogen peroxide (H\(_2\)O\(_2\)), one of the dismutation/disproportionation products of superoxide anion (O\(_2^\cdot\)\(^\prime\)), often mediates important signalling events, for example, regulation of endothelial nitric oxide synthase (eNOS) expression and function.\(^8\) \(^16\) \(^19\) By producing nitric oxide (NO\(^\prime\)) to inactivate O\(_2^\cdot\)\(^\prime\) and its derivatives, eNOS may also contribute to the anti-oxidative activities of endothelial cells. However, the resulting peroxynitrite is still more reactive than NO\(^\prime\) or O\(_2^\cdot\)\(^\prime\). Recent studies have shown that it can also become ‘uncoupled’ to produce O\(_2^\cdot\)\(^\prime\) rather than NO\(^\prime\). This phenomenon occurs in atherosclerosis or hypertension, representing a mechanism whereby oxidant stress sustains.\(^14\) \(^16\) \(^17\) \(^20\) \(^22\) On the other hand, it also seems to suggest that a potentially compensatory upregulation of eNOS, observed in some hypertensive or diabetic animals,\(^23\) \(^25\) may not be beneficial anymore.

Indeed, H\(_2\)O\(_2\) has been found to upregulate eNOS gene expression\(^8\) \(^26\) and mediate angiotensin II-induced uncoupling of eNOS.\(^22\) Interestingly, the critical mediator of H\(_2\)O\(_2\)-dependent upregulation of eNOS mRNA is CaM Kinase II.\(^8\) It turns out that CaM Kinase II can be rapidly phosphorylated upon exposure to exogenous H\(_2\)O\(_2\), resulting in tyrosine kinase activation, and an increase in eNOS gene transcription.\(^8\) These observations demonstrated that CaM Kinase II is indeed redox-sensitive and can unusually lie upstream of a tyrosine kinase janus kinase 2 to result in changes in gene transcription.\(^8\) Although the downstream transcriptional factors involved are yet revealed, cAMP response element binding protein (CREB) is excluded. H\(_2\)O\(_2\) induced a potent activation of CREB and ATF-1, but none of their phosphorylations was affected by inhibition of CaM Kinase II with KN93, or scavenging intracellular calcium with BAPTA/AM (unpublished results). Of note, earlier work by Marsen et al.\(^27\) demonstrated that thrombin induction of endothelin-1 mRNA expression is dependent on CaM Kinase II and its downstream transcriptional factor calcineurin. In human umbilical vein endothelial cells, histamine upregulation of eNOS mRNA and protein expression was also found dependent on CaM Kinase II.\(^28\)

In addition to exogenously applied H\(_2\)O\(_2\), oscillatory shear stress (OSS) activates endothelial CaM Kinase II via an increase in intracellular production of H\(_2\)O\(_2\).\(^11\) OSS occurs at bifurcations and branching points of the vascular tree, where atherosclerotic lesions are more frequent and severe.\(^29\) \(^32\) On the other hand, unidirectional laminar shear stress, which occurs in the plain area of the vasculature representing unidirectional, tangential force evenly applied to the endothelial surface, is protective against atherosclerosis.\(^33\) Interestingly, both shear forces upregulate eNOS gene expression but via distinctive signalling mechanisms.\(^8\) \(^11\) \(^34\) CaM Kinase II is activated by oscillatory shear but inhibited by laminar shear. Inhibition of CaM Kinase II with KN93 blunted eNOS mRNA upregulation by oscillatory shear, so did scavenging intracellular H\(_2\)O\(_2\) by cell-permeable catalase.\(^11\) Different from laminar shear, oscillatory shear induces sustained increase in ROS production, which could potentially induce eNOS uncoupling. Thus, an upregulation of eNOS probably just turns the enzyme into a more efficient ‘peroxynitrite’ generator (peroxynitrite is formed by NO\(^\prime\) reaction with O\(_2^\cdot\)\(^\prime\)), when eNOS is partially uncoupled. Also these data would suggest that similar to a potential causal role of CaM Kinase II in cardiac hypertrophy and post myocardial infarction remodelling,\(^35\) \(^40\) CaM Kinase II activation may contribute to chronic endothelial dysfunction by mediating eNOS regulations in response to pathological agonists.

3. Role of CaM Kinase II in phosphorylation-dependent activation of eNOS and calcium-dependent vasorelaxation

In addition to chronic changes in gene expression, CaM Kinase II also mediates rapid activation of eNOS and calcium agonists-induced vasorelaxation. Bradykinin induced eNOS phosphorylation at serine 1179 was found inhibitable by KN93, which is accompanied by an attenuation of eNOS activity.\(^41\) \(^42\) Phosphorylation of eNOS often sensitizes eNOS to lower concentrations of calcium.\(^43\) \(^44\) Consistent with activation of eNOS, vasorelaxation induced by acetylcholine and calcium agonist A23187 was also attenuated by KN93.\(^42\) KN93, however, had no effects on vasorelaxation induced by NO\(^\prime\) donors.\(^44\) Thus, it seems that calcium dependent, physiological vasorelaxation is at least in part mediated by CaM Kinase II-dependent rapid enzymatic activation of eNOS. Interestingly, in human umbilical endothelial cells, thrombin activation of NO\(^\prime\) production was also found dependent on CaM Kinase II.\(^45\) However, whether CaM Kinase II is rapidly inactivated after initial increase in NO\(^\prime\) production, or cross-talks with other eNOS-activating protein kinase such as AKT/PKB, remains to be elucidated. It is also unclear that how CaM Kinase II activation by different agonists diverges to differentially mediate patho- or physiological responses of the endothelium.

4. CaM Kinase II and actin cytoskeleton regulation

Interestingly, translocation of filamin, an actin-binding protein, is dependent on CaM Kinase II activation in endothelial cells\(^46\) with direct phosphorylation of filamin by CaM Kinase II.\(^47\) H\(_2\)O\(_2\) induction of actin stress fiber formation is dependent on p38 MAPK phosphorylation of the actin-binding protein heat shock protein 27 (HSP27).\(^48\) CaM Kinase II not only lies upstream of p38 MAPK/HSP27, but also precedes ERK1/2 activation, with both parallel pathways contributing to formation of actin stress fibers in response to H\(_2\)O\(_2\).\(^12\)
Recent innovative studies indicate the ‘house-keeping’ actin to exhibit important regulatory roles including cytoskeletal rearrangement, control of cell shape and movement, and regulation of gene expression. By determining subcellular localizations of transcriptional factors, or regulating chromatin remodelling complexes, actin can modulate gene transcription.49 Moreover, via modulation of its actin binding proteins, actin can influence mRNA stability, for example, that of eNOS. Interestingly, eNOS mRNA stability is increased in proliferative endothelial cells comparing with confluent cells, contributing to higher protein abundance.50 This response is consequent to a reduced binding of an actin-containing complex to the 3’-untranslated region of eNOS.51

5. CaM Kinase II and regulation of microvascular barrier function

CaM Kinase II also appears to be involved in agonist-mediated endothelial cell contraction and barrier dysfunction.10 Using a well-established model of thrombin-induced endothelial cell barrier dysfunction involving myosin light chain kinase-regulated cytoskeletal rearrangement and contraction, and phosphorylation of the actin- and myosin-binding protein caldesmon, it was demonstrated that similar to thrombin, infection with a constitutively active adenoviral protein caldesmon, it was demonstrated that similar to thrombin infusion with a constitutively active adenoviral alpha-Cam Kinase II construct induced significant ERK activation, indicating that Cam Kinase II activation lies upstream of ERK1/2.10 Thrombin-induced ERK1/2-dependent caldesmon phosphorylation (Ser789) was inhibited by KN93, a specific CaM Kinase II inhibitor, or U0126, an inhibitor of MEK1/2 activation. Immunofluorescence microscopy studies revealed phosphocaldesmon colocalization within thrombin-induced actin stress fibers. Pretreatment with either U0126 or KN-93 attenuated thrombin-mediated cytoskeletal rearrangement and evoked declines in transendothelial electrical resistance while reversing thrombin-induced dissociation of myosin from nonadenating caldesmon immunoprecipitates. These results strongly suggest the involvement of CaM Kinase II and ERK1/2 enzymatic activities in thrombin-mediated caldesmon phosphorylation and both contractile and barrier regulation.10

Additionally, in brain capillary endothelial cells, activation of CaM Kinase II δ and γ isoforms was found upstream of voltage-gated potassium channels, resulting in hypoxia-induced cell swelling that likely precedes barrier dysfunction.5 In this study, antibodies recognizing δα and γα isoforms were used to specifically characterize expression of these endothelial CaM Kinase II isoforms.5 Interestingly, calcium ion handlings were also recently found regulated by CaM Kinase II in bovine pulmonary artery endothelial cells (marco-).52,53 Differential and common roles of CaM Kinase II in macro- and microvascular endothelial cell signalling are summarized schematically in Figure 1.

6. Perspectives of endothelial-specific regulation of CaM Kinase II

In cardiomyocytes, calcineurin,36,54 ERK1/2,55 histone deacetylase,49 apoptosis signal regulating kinase 1, and NFkB57 have been found downstream of CaM Kinase II-dependent cardiac hypertrophy. In the endothelial cells, however, the major transcriptional targets or immediate kinase substrates are yet identified. Although eNOS has putative phosphorylation sites for CaM Kinase II, there has been no direct evidence as to whether CaM Kinase II directly phosphorylates eNOS. It remains unclear whether enhanced eNOS phosphorylation upon bradykinin stimulation is a result of CaM Kinase II activation of an intermediate kinase. CaM Kinase II activates ERK1/2 in both endothelial cells and vascular smooth muscle, but it is also unclear whether ERK1/2 can be directly phosphorylated by CaM Kinase II. Whether chronic, endothelium-specific inhibition of CaM Kinase II improves endothelial function or endothelial barrier function in vivo has not been studied. Therefore, much remain to be learned regarding mechanistic insights of CaM Kinase II-mediated endothelial cell signalling.

In summary, despite that endothelial-specific regulations of CaM Kinase II are now only beginning to better understood, data accumulated so far have demonstrated a potentially significant role of CaM Kinase II in endothelial cell pathophysiology. Original research articles focusing on CaM Kinase II signalling in endothelial cells are summarized in Table 1. The critical roles of CaM Kinase II in modulating eNOS expression and function may underlie its possible contribution to atherogenesis where eNOS dysfunction occurs. Activation of CaM Kinase II in microvessels results in barrier dysfunction. On the other hand, transient activation of CaM Kinase II in endothelial cells may have important physiological roles in modulating vascular homeostasis via nitric oxide production and handlings of potassium and calcium ions. Overall the possible connections among these functional roles of CaM Kinase II remain to be fully elucidated.

Figure 1 CaM Kinase II signalling in macro- and microvascular endothelial cells. In macrovascular endothelial cells, H2O2 induces CaM Kinase II/JAK2-dependent upregulation of eNOS mRNA expression. Bradykinin, via activation of its type 2 receptor (BKB2), rapidly activates eNOS. Whereas unidirectional laminar shear stress (LSS) inhibits CaM Kinase II activity, oscillatory shear stress (OSS) activates it via intracellular H2O2, resulting in upregulation of eNOS mRNA. In the microvascular endothelial cells, however, CaM Kinase II mediates thrombin-PAR-dependent activation of ERK1/2/MLCK pathway to induce actin cytoskeleton, whereas in macrovascular endothelial cells, CaM Kinase II mediates H2O2 induction of actin stress fiber formation predominantly via p38 MAPK/HP27 pathway. ERK1/2 is also transiently activated downstream of CaM Kinase II, likely playing a minor role in regulating actin cytoskeleton in macrovascular endothelial cells. In microvascular endothelial cells, CaM Kinase II is also involved in hypoxia-induced potassium channel openings and cell swellings, which likely precedes barrier dysfunction.
Table 1  Original articles focusing on CaM Kinase II signalling in endothelial cells

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