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CAVEOLAE, CAVEOLIN AND CONTROL OF VASCULAR TONE: NITRIC OXIDE (NO) AND ENDOTHELIUM DERIVED HYPERPOLARIZING FACTOR (EDHF) REGULATION

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Endothelium plays a crucial role in the regulation of cardiovascular homeostasis through the release of vasoactive factors. Nitric oxide (NO) and endothelium-derived hyperpolarizing factors (EDHF) are the two major actors controlling the vasomotor tone. The endothelial nitric oxide synthase (eNOS) was reported in the mid 90^{ies} to be under the control of caveolin, the structural protein of caveolae. Nowadays, a large body of evidence has confirmed that the caveolin/eNOS interaction was needed to prevent inadequate NO production under basal conditions but also to facilitate the integration of extracellular stimuli to intracellular NO signals. Compartmentation of key actors in the EDHF signaling pathway is now also proposed to take place into caveolae. Accordingly, caveolin-deficient animals revealed both an unopposed NO production promoting vessel dilation and a lack of EDHF-driven vasorelaxation. The transient receptor potential (TRP) channels are the link between caveolae and EDHF. Different TRP channels involved in the capacitative calcium entry were found to directly interact with caveolin-1 in endothelial cells. TRPC1 and TRPC4 form a complex with the endoplasmic reticulum IP3 receptor thereby optimizing calcium signaling. EDHF-driven vasodilation was documented to be altered in a TRPV4-deficient mouse model. The close vicinity between TRPV4 and SKCa channels in caveolae together with the gap-junctions subunits connexins support a role of these microdomains in the generation and propagation of EDHF to vascular smooth muscle cells. In conclusion, caveolae and caveolin are important control points in the control of blood pressure by the endothelium. This also highlights how any alteration in the caveolae integrity or caveolin abundance may lead to and/or exacerbate endothelial dysfunction and associated cardiovascular diseases.

Key words: *caveolae, caveolin, endothelium, nitric oxide, NO, endothelium derived hyperpolarizing factor, EDHF, transient receptor potential (TRP) channel, TRPC1, TRPV4, connexin, gap junction*

INTRODUCTION

Caveolae are a subset of invaginated plasmalemmal rafts in which key transduction proteins are located. Cholesterol and glycosphingolipids are sequestrated in caveolae together with self-associating molecules named caveolins acting as regulatory scaffolds for numerous signaling proteins (1). Receptor tyrosine kinases and G-protein coupled receptors are found in caveolae as well as all the downstream machinery necessary for the transmission of the extracellular signal to intracellular targets (2). During the last decade, an increasing amount of literature highlights the importance of this compartmentalization in physiology and pathophysiology (3). Caveolae are present in most cell types of the cardiovascular system and it is therefore not surprising that the roles of caveolin in cardiac and vascular cells attract so much interest (4, 5). The caveolin-dependent regulation of cardiac myocyte key functions has been extensively reviewed elsewhere (6). Here, we will focus on the specific role of caveolae and caveolin-1 in the control of vascular tone, in particular through the regulation of the nitric oxide (NO) and endothelium-derived hyperpolarizing factor (EDHF) pathways.

NITRIC OXIDE PATHWAY

The endothelial nitric oxide synthase (eNOS) was one of the very first non-receptor proteins found to be located in caveolae and interacting with caveolin (7-9). Since then, many studies have characterized *in vitro*, the interaction between eNOS and caveolin-1 which tonically clamps the enzyme in a repressed configuration (10). In particular, changes in the abundance of caveolin were documented to directly influence the basal and agonist-stimulated NO production in endothelial cells. We showed that the exposure of endothelial cells to excess low-density lipoprotein cholesterol leads to an increase in caveolin abundance (11). The consecutively reinforced caveolin/eNOS complex is then less easily disrupted by calcium-calmodulin, thereby preventing NO release. Conversely, statins by reducing the caveolin abundance in endothelial cells displaces the equilibrium towards caveolin-free eNOS and thus promotes NO formation (12). Different NO-driven functions in endothelial cells were documented to be influenced by changes in the caveolin abundance and/or in the extent of the interaction of eNOS with native caveolin. Angiogenesis and vascular permeability may for instance be blocked both *in vitro* and *in*

vivo by overexpressing caveolin or by transducing cells with a the caveolin scaffolding domain (CSD) peptide (*i.e.* a peptide corresponding to the caveolin sequence involved in the interaction with eNOS) (13-15).

As anticipated from the accumulation of data related to the exquisite interaction between eNOS and caveolin, generation of caveolin-1 deficient mice led to reproducible vascular phenotypes. The study by Drab and colleagues originally reported that aorta rings derived from caveolin-deficient mice failed to establish a steady contractile tone and were characterized by a marked relaxation in response to acetylcholine (16). These authors further identified that the basal release of NO and cGMP production were significantly higher in caveolin-deficient mice than in wild-type mice, confirming the hyperactivation of eNOS in the absence of caveolin. In the concomitant study by Razzani and colleagues, a greater relaxation in response to acetylcholine and a lower L-NAME-sensitive steady-state maximal tension in response to phenylephrine were observed in caveolin null aortic rings *vs.* wild-type aortic vessels (17).

More recently, investigators used caveolin-1-deficient mice reconstituted with a transgene expressing Cav-1 specifically in endothelial cells and showed that the flow-mediated dilation which was markedly reduced in Cav-1-deficient arteries was completely rescued (18). In the same study, these authors reported that in the reconstituted mice, external left carotid ligation for two weeks reduced the lumen diameter of carotid arteries whereas in Cav-1-deficient mice, the post-ligature decrease in blood flow increased the wall thickness but did not reduce the lumen diameter. Endothelial re-expression of caveolin in caveolin-deficient mice also restored the smooth muscle contractility in pulmonary arteries and corrected the pulmonary hypertension observed in caveolin-deficient mice (19).

Although these data confirmed the importance of the caveolae platform in both rapid and long-term mechanotransduction in intact blood vessels, it is only recently that the allosteric regulation of vascular NOS by caveolin-1 was demonstrated to support these effects (20). These more definitive insights originated from the use of implanted telemetry in awake caveolin-deficient mice to evaluate hemodynamics together with direct measurements of blood nytrosyl-hemoglobin (Hb-NO) and NO by EPR spin-trapping in isolated aortas. Indeed, the almost doubling of the whole blood Hb-NO in caveolin-1 deficient mice (*vs.* wild-type mice) was shown to be prevented by the administration of L-NAME but also of the caveolin scaffolding domain (CSD) peptide. These same treatments concomitantly increased the very low frequency (VLF) variability of systolic blood pressure which was significantly reduced in caveolin-1-deficient mice (*vs.* wild-type mice). The extent of VLF variability was documented in other studies to reflect the NO bioactivity (21) and more generally, an increase in general blood pressure variability is associated with adverse cardiovascular outcomes (22). The observed changes in VLF therefore not only confirm the influence of caveolin on the NO component of the regulation of blood pressure but also underscores the role of an adequate expression of caveolin to prevent cardiovascular diseases.

EDHF PATHWAY

In the study described above using telemetry implant, systolic blood pressure was found to be unaltered in caveolin-deficient animals raising questions about the existence of compensatory mechanism in this genetic model (20). The absence of hypotension, despite increased NO signaling could arise from chronic adaptations in response to the increased NO production itself. Also, other forms of defective caveolae-dependent functions could account for the lack of overall changes in animal blood pressure. Interestingly, a complete lack of EDHF-mediated hyperpolarization and relaxation was actually observed from mesenteric arteries of caveolin-1 deficient mice (23). This study confirmed the suspected implication of caveolae in this other vasodilating pathway, particularly in the resistance arteries. The deficit in EDHF-dependent relaxation in caveolin-1 deficient mice actually echoed previous reports about the caveolin-1 interaction or caveolae localization of some putative EDHF mediators (24). Indeed, numerous recent studies concur to validate this hypothesis.

A hallmark of the EDHF-mediated response is its initiation by an elevation of intracellular calcium ($[Ca^{2+}]i$) (25). Interestingly in several cell types, including endothelial and smooth muscle cells, calcium handling proteins are located in caveolae. Direct measurement of Ca^{2+} waves in endothelial cells have also suggested that caveolae could be the sites that initiates Ca^{2+} entry and Ca^{2+} dependent signal transduction (26).

Activation of calcium-signaling cascade is complex and may depend on cell and channel types. Several studies evidenced that members of the transient receptor potential cation (TRPC) channel family are involved in capacitative calcium entry (27). TRPC1 is associated with caveolar lipid raft domains where it is assembled into a signaling protein complex that includes caveolin-1, IP3 receptor (type III) and $G_{\alpha q/11}$ (28, 29). Importantly, direct interactions between caveolin-1 and the N- and C-termini of TRPC1 are essential for both the membrane location of TRPC1 and Ca^{2+} influx regulation (30, 31). Murata and colleagues showed that abnormal Ca^{2+} handling in caveolin-deficient mice could be restored to physiological level by genetic reconstruction of caveolin-1 back into endothelial cells. They evidenced that caveolin-1 is essential for calcium entry into endothelial cells and for the assembly of a multiprotein channel complex consisting of TRPC1 and TRPC4 channels with IP_3 receptor upon agonist stimulation (19). In the above studies, the IP3R located in the endoplasmic reticulum is proposed to interact with caveolar proteins through a putative N-terminal spanning domain. Agonist-induced hydrolysis of PIP2 (particularly enriched in caveolae) would then activate IP3R and coordinate calcium signaling

Another member of the TRP channel family, TRPV4, recently raised in interest as a major regulator of vascular tone. TRPV4 channels provide a significant Ca^{2+} entry pathway in endothelial cells because of their high Ca^{2+} permeability (32, 33). These channels can be activated by diverse physical and chemical stimuli such as cell swelling (34), shear stress (35), moderate warmth (36), low pH (37) and in response to the non-PKC activating phorbol ester 4αPDD (36). Noteworthy, some of the proposed EDHF, including the arachidonic acid metabolites EETs, also modulate the activity of TRPV4 channels (38, 39). Interestingly, we could recently identify TRPV4 as the link between the impairment of calcium entry and the defect in EDHF-induced relaxation observed in caveolin-1 deficient mice (23). We first evidenced that 4αPDD-evoked $[Ca^{2+}]_i$ increase is blocked by endothelial cell transduction with caveolin-1-siRNA and further documented altered EDHF-mediated dilation in TRPV4-/- mice. Moreover, as already demonstrated for TRPC channels, we could evidence a physical interaction between caveolin-1 and TRPV4.

Other studies have also addressed the role of caveolin-1 in EDHF-mediated relaxation involving EETs and calcium activated potassium channels (K_{Ca}). Caveolae disruption by Methyl-βcyclodextrin (MβCD) cholesterol depletion was reported to inhibit EDHF-mediated relaxation in pig coronary arteries through a defect in cPLA2 translocation and thus in

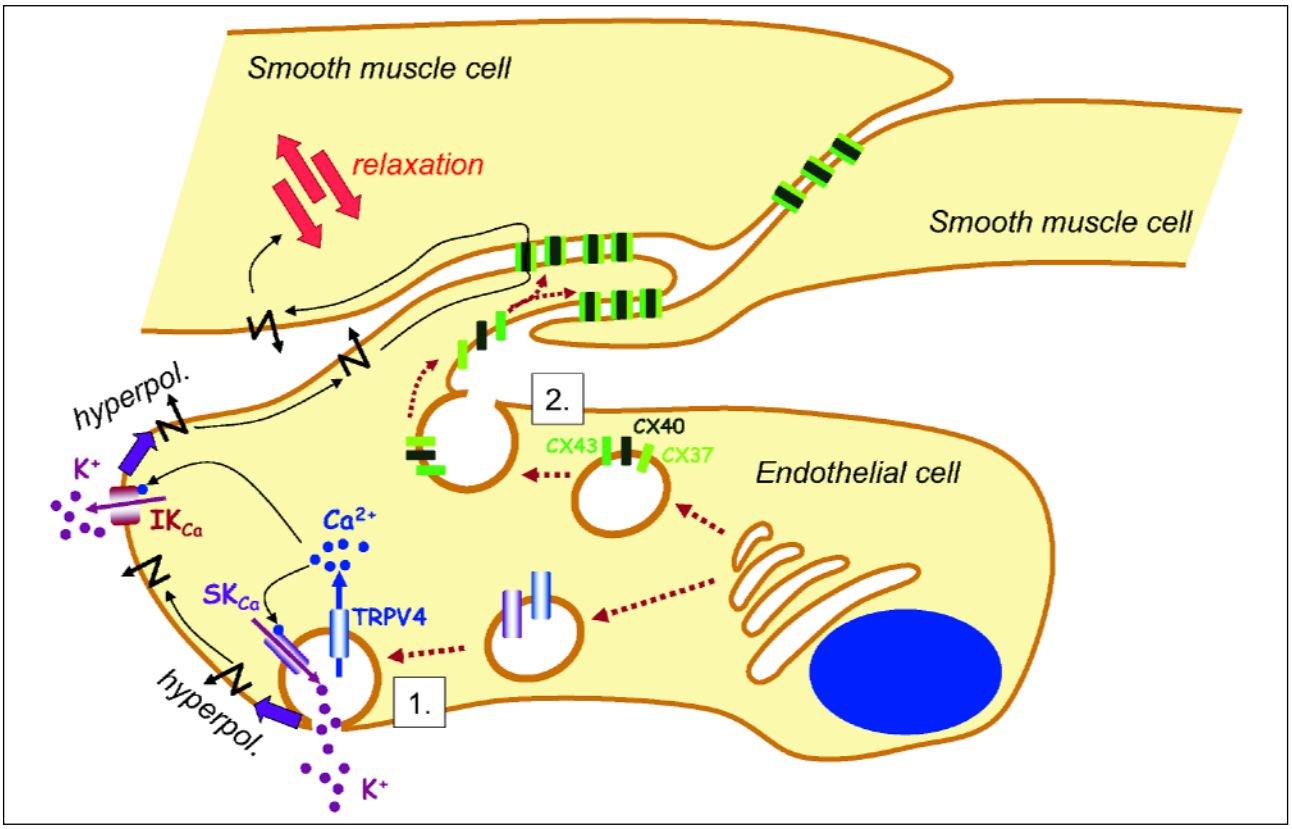

Fig. 1. Roles of caveolin-1 and caveolae in EDHF-mediated relaxation. Caveolin-1 and caveolae favor two major components of EDHF signaling. (1) Caveolin-1 facilitates the proper targeting of TRPV4 channels upon agonist stimulation and caveolae compartmentation promotes the coupling between Ca^{2+} (TRPV4) and K^+ (SK_{Ca}) channels facilitating the generation of membrane hyperpolarization; note that the hyperpolarization may also originate from potassium efflux through non-caveolae IK_{Ca} channels. (2) Caveolin-1 is involved in endothelial connexins shuttling to the plasma membrane and formation of gap-junctions. Cx37, connexin 37; Cx40, connexin 40; Cx43, connexin 43; TRPV4, transient receptor potential V4; SK_{Ca}, small conductance calcium-activated potassium channel; IK_{Ca}, intermediate conductance calcium-activated potassium channel.

EETs synthesis (40). Absi and colleagues also showed that in rat mesenteric and porcine coronary arteries, SK_{Ca} channel activity was selectively impaired by MβCD treatment (41). SK_{Ca} channels were actually shown to reside within caveolin-rich lipid domains whereas IK_{Ca} were present in cavelin-1-independent cellular fractions. It should however be stressed that EETs are not considered as ubiquitous participant in the EDHF-mediated vasorelaxation.

Altogether these results strongly support a model in which the caveolae microdomains offer a close proximity between mediators of the Ca^{2+} entry and K_{Ca} channels. TRPV4 channels are central in this model and promoting the Ca^{2+} entry to reach the Ca^{2+} threshold required to open K_{Ca} and support part of the EDHF-driven relaxation (*Fig. 1*).

Additional caveolae-associated effectors of the EDHF pathway have to be mentioned: namely, the gap junctions. In various microvessels the involvement of myoendothelial gap junctions in the spread of an electric current mediating the EDHF response has been established (42-45). Gap junctions are formed by docking of two connexons subunits each made up of six connexin proteins arranged around a central core (46). Four connexin subtypes, classified as Cx37, 40, 43 and 45 according to their molecular mass, are widely distributed in the vasculature (47, 48). Presence of Cx40 and Cx37 is consistently observed in endothelial cells whereas Cx 43 and 45 are commonly detected in smooth muscle cells. However, their location may vary with vessel size, vascular region and species (49, 50). The gap junction function may be regulated by association and interaction with a variety of proteins including interaction among connexins themselves (51, 52). Accumulating evidence show that connexins can be targeted to selected membrane microdomains such as lipid rafts and caveolae (53, 54). Schubert *et al.* have shown that Cx43 colocalizes with caveolin-1 in NIH 3T3 fibroblasts where both proteins are endogenously expressed. This colocalization is most prominent at junctional membranes in contacting cells. A recent study also showed that that Cx43 coimmunoprecipitates and colocalizes with caveolin-1 and -2 in rat epidermal keratinocytes. In our hands, immunohistochemistry and Western blot showed decreased expression of Cx37, -40, and -43 in caveolin-1 knockout arteries (*vs* wild-type vessels) (23). Additionally cell fractionation and immunoprecipitations demonstrated the caveolar location and caveolin-1 interaction of these connexins. Together with calcein-based functional assay, we could evidence that caveolin-1 is essential for proper plasma membrane location of connexins and consequently gap-junction formation and function, thereby affecting EDHF-related hyperpolarization and relaxation (23) (*Fig. 1*).

CONCLUSION

In conclusion, both NO and EDHF signaling are under the close control of the caveolae platform in endothelial cells. Compartmentation of the corresponding signaling cascades adds therefore a layer of complexity to the regulation of the vasomotor tone. The direct relationship between caveolin abundance and

caveolae structure and function therefore underscores how interference with the expression or subcellular location of caveolin may have dramatic impact on the regulation of blood pressure. Endothelial dysfunction as encountered in response to hypercholesterolemia or other pathological states was already associated with defects in the caveolae compartmentation or caveolin clamping of enzymes (4). The role of caveolae as mechanotransducer will likely lead to additional evidence of how caveolin may control vasomodulatory NO or EDHF pathways in response to shear stress. First insights on such regulation may be found in a genetic study focusing on eNOS gene polymorphism at Exon 7 (Glu298Asp). This study documented a lesser enrichment of the Asp eNOS variant in caveolae during static conditions and an impaired dissociation of eNOS from caveolin in response to shear stress (55).

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