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Review

Caveolins and the regulation of endothelial nitric oxide synthase in the heart

Olivier Feron, Jean-Luc Balligand *

Unit of Pharmacology and Therapeutics, FATH 5349, Department of Medicine, Université catholique de Louvain, 53 avenue Mounier, 1200 Brussels, Belgium

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Abstract

Virtually all cell types within the myocardium express caveolae, where cell-specific isoforms of caveolin both maintain the structural organisation of these cholesterol-rich of the plasmalemma and serve as scaffolds for the dynamic constitution of "signalosomes", or hubs concentrating numerous transmembrane signaling proteins and their effectors. Analysis of the phenotype of mice with genetic deletion or overexpression of specific caveolin isoforms has provided key evidence for the importance of caveolins and caveolae in several aspects of the cardiovascular biology, including vascular contractility, lipid metabolism, angiogenesis, or the control of cardiac hypertrophy. Among specific protein–protein interactions involving caveolins in cardiac tissue, these genetic models unequivocally confirmed the functional importance of the dynamic association of the endothelial isoform of nitric oxide synthase (eNOS) for its post-translational regulation in endothelial cells and cardiac myocytes, which bears on the enzyme's capacity to modulate nitric oxide (NO)-dependent endothelial function, angiogenesis, and excitation–contraction coupling. We will review the current understanding of this regulation of eNOS (and potentially other NOS isoforms) through protein–protein interactions involving several G-protein-coupled receptors and other allosteric modulators in the context of emerging paradigms on the regulation of cardiac function by NO.

Keywords: Nitric oxide synthase; Caveolin; Endothelium; Cardiac myocytes; Angiogenesis; Contractility

1. Introduction

Nitric oxide (NO) is synthesized by a family of calmodulin-activated enzymes collectively termed NO synthases (NOS) [1]. The endothelial isoform of NOS (eNOS) is involved in many aspects of cardiovascular homeostasis including regulation of blood pressure and cardiac contractility [2]. eNOS activation relays the signals from multiple extracellular stimuli to a variety of NO-mediated autocrine and paracrine responses. For example, although the NO/cGMP signaling pathway is critical to regulate smooth muscle cell contractility, a large part of the biological influence of NO in this tissue comes from its production in the adjacent endothelial cell layer. Alternatively, autocrine NO production following activation of eNOS in endothelial cells promotes cell migration and organization in tubes, a phenomenon participating in the first steps of angiogenesis. Likewise, eNOS expressed in cardiac myocytes participates to the autocrine regulation of excitation–contraction coupling. In all these situations, the subcellular site of NO synthesis has a major influence on its biological activity. The initial identification of eNOS in plasmalemmal caveolae in the mid-90s provided a structural basis for such signal compartmentation [3,4]. The subsequent observation that eNOS directly interacts with the structural proteins of caveolae, or caveolins, provided a biochemical rationale for the compartmentation of eNOS to caveolae and its interaction with numerous signalling molecules concentrated there. It also added another layer

* Corresponding author. Tel.: +32 2 764 9326; fax: +32 2 764 9322.
E-mail address: Balligand@mint.ucl.ac.be (J.-L. Balligand).

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of complexity on the mechanisms of post-translational regulation of eNOS, that now must take into account both the stoichiometric binding of the two proteins but also the independent impact of caveolin on the formation of caveolae and assembly of other signalling modules up- and downstream of eNOS. This is exemplified in the phenotype of mice with genetic deletion or overexpression of caveolins, in which some functional responses are in agreement with current biochemical paradigms, but others are unexpected. In this review, we will summarize the current understanding of the molecular regulation of eNOS (e.g., through caveolin interaction) and, with this as a hindsight, interpret the phenotype of mouse models genetically modified for caveolins, with a special emphasis on their cardiovascular functions.

2. The reversible caveolin–eNOS interaction

eNOS is the only NOS isoform dually acylated by the fatty acids myristate and palmitate [1], thereby strongly supporting a preferential membrane location of the enzyme. In fact, each acylation process (i.e., myristoylation on glycine at position 2 and palmitoylation on cysteines −15 and −26) was documented to enhance the caveolar enrichment (versus the rest of the plasma membrane) by 10-fold [3]. Shortly thereafter, eNOS was reported to be quantitatively associated with caveolin-1 and caveolin-3 isoforms in endothelial cells and cardiac myocytes, respectively [5]. The subsequent dissection of this interaction led to the identification of multiple binding sites within both proteins [6–11]. Two cytoplasmic domains of caveolin-1 were documented to interact with eNOS: the N-terminal oligomerization domain (mapping to residues 61–101 in human caveolin-1 sequence) and to a lesser extent the C-terminal tail (amino acids 135–178). Indeed, within the oligomerization domain, a sequence called the Caveolin Scaffolding Domain (CSD) (amino acids 81–101) [12] appeared to be critical to bind a series of caveolar residents. Using a GST-CSD fusion protein as a bait to select peptide ligands from a bacteriophage display library, Couet et al. identified a "Caveolin Binding Motif (CBM)" that appeared to be present in whole or in part in many proteins located in caveolae (ΦXΦXXXXΦXXΦ, where Φ represents an aromatic amino acid) [13], including non-receptor tyrosine kinases, G-proteins and eNOS [6,7,12,14]. In the eNOS sequence, the caveolin binding motif (CBM) corresponds to the consensus sequence FPAAPFSGW located within the oxygenase domain (350–358 in the human eNOS sequence). Still, sites of caveolin binding were also localized in the reductase domain of eNOS and could act synergistically with the caveolin binding motif to modulate the catalytic activity of eNOS (see below).

The identification of the interaction between caveolin and eNOS and its impact on eNOS activity is now regarded as a major breakthrough in the understanding of the post-translational regulations of this enzyme. Accordingly, the paradigm of eNOS activation through calmodulin (CaM) binding in response to an increase in intracellular calcium has now been modified to take into account the initial, obligatory disruption, by calcium-bound CaM, of the heteromeric complex formed between eNOS and caveolin [1,7–9]. Specifically, when interacting with caveolin, eNOS is in an inactive form and CaM acts as a direct allosteric competitor (vs. caveolin) to promote the Ca^{2+}-dependent activation of eNOS [6,9]. Both the Caveolin Binding Domain and Caveolin Scaffolding Domain sequences within eNOS and caveolin proteins, respectively, were identified as key regulators of the close interaction between these two proteins [6,7]. Mechanistically, a model can be proposed where caveolin interaction with the oxygenase domain helps targeting the eNOS–caveolin complex to caveolae whereas caveolin interaction with the reductase domain is primarily responsible for antagonizing CaM binding to its consensus sequence in the "hinge" region and for slowing electron transfer from the reductase, thus inhibiting heme iron reduction and NO synthesis [15]. Also, the caveolin binding motif in eNOS lies between the heme and the calmodulin binding domains adjacent to a glutamate residue (Glu-361) necessary for the binding of L-arginine, suggesting that caveolin may interfere with heme iron reduction [15], similarly to L-arginine-based NOS inhibitors. Of note, incubation of pure eNOS with Caveolin Scaffolding Domain peptides resulted in inhibition of eNOS, inducible NOS (iNOS) and neuronal NOS (nNOS) activities, suggesting at least some common mechanism(s) and site(s) of inhibition for all the NOS isoforms.

3. eNOS "trafficking"

A dynamic cycle of eNOS–caveolin interactions regulating eNOS activation in response to agonist stimulation (and shear stress) has been proposed to occur based on a series of independent observations [1,10]; (i) after agonist stimulation, eNOS is de-palmitoylated by the acyl-protein thioesterase and is no longer selectively sequestered in the caveolae [16,17]; (ii) the long chain fatty acyl coA synthetase was identified as a key modulator of eNOS re-palmitoylation [18], suggesting that after the decline in $[Ca^{2+}]_i$ to basal levels, the affinity of eNOS for the caveolar membrane is restored; (iii) the arginine transporter CAT1 has been located in the caveolae [19], and the dissociation of the enzyme from its proximity as well as from several receptors/effectors localized in the caveolae [20,21] is likely to serve as a feedback mechanism for eNOS activation; (iv) by using either cell lines expressing caveolin but lacking caveolae [22] or siRNA to knockdown caveolin-1 in cells normally expressing caveolin [23], two groups recently reported that caveolin-1 itself was not required for the targeting of eNOS to caveolae/lipid rafts, underlying a critical role for other partners; (v) the group of Muller-Esterl

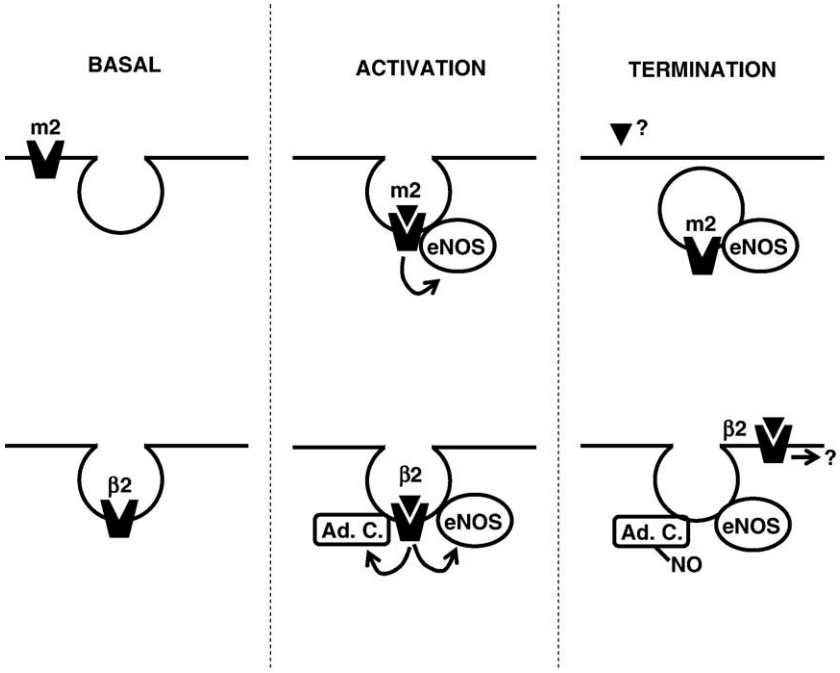

Fig. 1. Diversity of GPCR regulation in the caveolae. The proposed preferential locations of the activatable pools of the muscarinic cholinergic m2 and β2-adrenergic receptors in basal conditions are represented (left) as well as their translocation in or out of caveolae upon agonist stimulation (middle) or for signal termination (right). Note the diversity in the pathways involving caveolae to terminate agonist-induced signaling: the m2 receptor is made inaccessible to agonist following its sequestration in budded caveolae; the β2 adrenergic receptor is translocated out of caveolae and consequently, segregated from effectors such as adenylate cyclase and eNOS.

and co-workers precisely identified two new proteins named NOSIP ('eNOS interacting protein') and NOSTRIN ('eNOS traffic inducer') that specifically modulate the caveolin/eNOS interaction [24,25]. Overexpression of either protein in eNOS-expressing cells has similar effects, i.e., the translocation of eNOS away from the plasma membrane and an inhibition of agonist-induced NO production. Uncoupling of eNOS from the signaling platforms concentrated in plasma membrane caveolae is thought to account for the inhibitory effect of both proteins.

Although more work is needed to further characterize the determinants of eNOS trafficking between plasmalemmal caveolae and intracellular compartments, the consensual view today is that eNOS is part of multiprotein complexes continuously forming and dissociating in basal conditions and modulated by agonists (or shear stress), as other signaling proteins assembled in dynamic "signalosomes" in many organisms.

4. Caveolin, eNOS and G-protein-coupled receptors in the heart

Different GPCR were found to be enriched in cardiomyocyte caveolae, either under basal condition or upon agonist stimulation, including the muscarinic cholinergic (m2), the beta-adrenergic (β1 and β2) and the adenosine (A1) receptors. Stimulation of these receptors either directly activates eNOS or, reciprocally, NO modulates the signaling triggered through these receptors; the co-localization of eNOS with these GPCR supports such interaction.

4.1. Muscarinic cholinergic receptors

The muscarinic cholinergic m2 receptor was the first GPCR found to mediate an agonist-induced disruption of the caveolin–eNOS heterocomplex [10]. This finding followed our initial discovery that in adult rat ventricular myocytes, a muscarinic cholinergic agonist (carbachol) could promote the translocation of the m2 receptor into caveolin-3-enriched low density gradient fractions [26] (Fig. 1). We also documented that the coupling of the muscarinic cholinergic receptor stimulation to eNOS activation required the location of eNOS in caveolae [20]. In this study, neonatal cardiac myocytes from eNOS-null mice were transfected back with wild-type (WT) eNOS or myristoylation-deficient (myr^-) eNOS mutant cDNA. In myocytes expressing recombinant WT eNOS (that we verified to be located in caveolae), carbachol induced a 4-fold elevation in cGMP levels and completely abrogated the spontaneous beating rate. By contrast, in myocytes expressing the myr^- eNOS (found exclusively in the cytosol), the m2 agonist failed to exert its negative chronotropic effect and to increase cGMP levels. In the same study, a Caveolin Scaffolding Domain peptide (derived from the caveolin-3 sequence) introduced in neonatal rat myocytes by reversible permeabilization, was shown to lead to the complete and specific inhibition of the carbachol-induced negative chro-

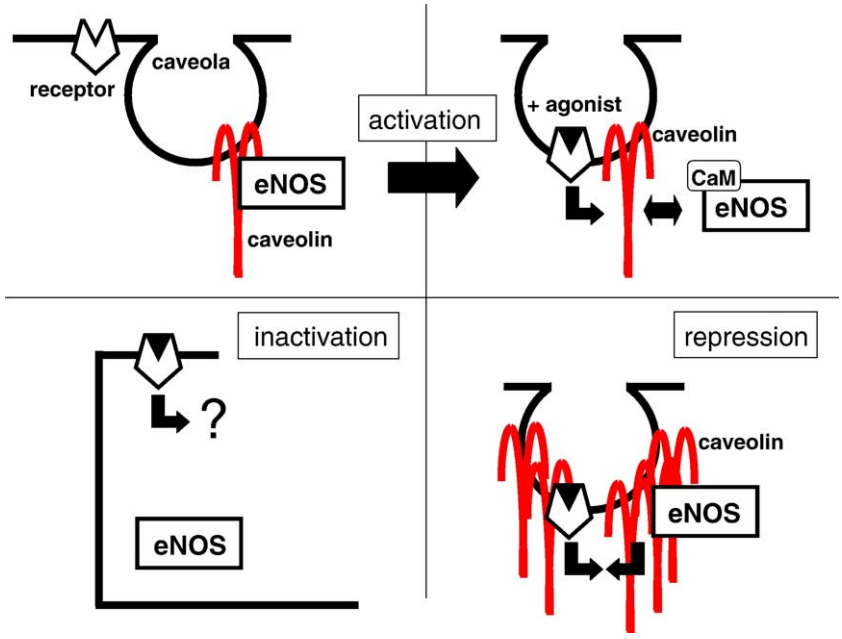

Fig. 2. The key roles of caveolin-1 and caveolae in receptor-dependent regulation of eNOS. (Top:) Left, In basal conditions, caveolin (cav-1 and cav-3) maintains eNOS in its inactivated state and thereby limits the production of NO. Right, receptor targeting to caveolae upon agonist stimulation leads to the activation of eNOS through the local increase in intracellular calcium (the sarcoplasmic reticulum is in close vicinity to caveolae) and the consecutive disruption of the caveolin/eNOS heterocomplex. (Bottom:) Left, in cells lacking caveolae (or with a reduced amount of caveolin), the coupling between agonist-bound receptor and cytosolic eNOS is lost and the production of NO is decreased; note however that in basal condition, the NO release is slightly but significantly *increased*. Right, when the abundance of caveolin is increased, eNOS is inactivated by this excess inhibitory clamping, preventing activation of the receptor signaling cascade leading to NO production.

notropic effect. These data led to propose the so-called "caveolae paradox" [27], whereby caveolin reduces eNOS activity in basal conditions but facilitates its activation upon agonist stimulation. Further analysis of the caveolar targeting of the m2 receptor in the context of eNOS activation led us to another important finding. The use of differential binding of membrane-permeant and -impermeant mAchR radioligands revealed that carbachol stimulation of cardiac myocytes induced sequestration of m2 through caveolae fission [21]. Accordingly, GTP but not its non-hydrolyzable analog GTP-gamma-S, and recombinant GTPase dynamin (but not the dominant-negative K44A dynamin), induced the m2 receptor sequestration in budded caveolae. Also, repeated carbachol stimulations led to a progressive increase in m2 sequestration and a concurrent stabilization of the inhibitory eNOS–caveolin complex. These findings emphasize the role of caveolae in muscarinic cholinergic receptors trafficking and NO signaling, and suggest that caveolae fission may contribute to G-protein-coupled receptor desensitization (Fig. 1).

4.2. Beta-adrenergic receptors

Xiang et al. used a variety of techniques, including confocal imaging and co-immunoprecipitation, to document that the β2 but not the β1 adrenergic receptors (AR) were located in caveolae (directly associated with caveolin-3) [28]. They further reported that part of the discrepancies in the regulation of the contraction rate occurring through the β1- and β2AR were dependent on this caveolar localization. The β2AR-stimulated myocyte contraction rate was increased by 2-fold caveolin-3 (and the compartmentation within caveolae) was altered by filipin, a raft-disrupting drug; filipin had no effect on β1AR signaling. Rybin et al. reported a similar observation with cyclodextrin, an agent used to deplete membranes from cholesterol and thereby to alter caveolae organization [29]. Interestingly, Rybin et al. [29] and Ostrom et al. [30] independently reported that both β1 and β2 receptors were present in cardiac myocyte caveolae (although to a lesser extent for the former) but that agonist stimulation led to the translocation of the β2AR but not of the β1AR. This translocation out of caveolae caused the sequestration of the β2AR away from adenylate cyclase and thereby the lower efficacy of this receptor-effector coupling (Fig. 1). Therefore, although there is still uncertainty about the caveolar location of the β1AR, it is clear that differential caveolar compartmentation supports the negative regulation of cAMP accumulation specifically by β2AR stimulation. In two functional studies, the co-localization of eNOS and the β-AR in caveolae was proposed to account for the NO-mediated attenuation of the beta-adrenergic-induced inotropy [31,32], as initially described more than a decade ago [33]. The precise mechanism by which NO attenuates β-AR signaling could involve the S-nitrosylation of adenylate cyclases (types 5 and 6), the predominant AC isoforms expressed in the heart. NO was indeed shown to decrease the Vmax of adenylate cyclase (without altering the Km for ATP) and this

inhibition was reversed by reducing agents, implicating cysteine residue(s) as the target for nitric oxide [34]. Recent data by Ostrom et al. confirmed that NO inhibits AC activity, attenuating signaling via GPCR that are co-localized with eNOS and AC in caveolae [30] (Fig. 2). In contrast, AC activity stimulated by GPCR not localized in lipid raft domains is unaffected by NO or raft disruption unless eNOS is overexpressed and lipid rafts are disrupted.

4.3. Adenosine receptor

The adenosine A(1) receptor exemplifies the reverse translocation of GPCR out of caveolae upon agonist stimulation [35]. Such translocation may explain why the activation of ventricular myocyte A(1) receptors is associated with few direct effects (e.g., on contractility), although activation of PKC and downstream eNOS by adenosine were found to be critical to mediate adenosine-triggered cardioprotection in the context of ischemic preconditioning [36].

In another cell type (i.e., smooth muscle cells), however, the A(1) adenosine receptor was shown to be internalized through caveolae before being recycled to the cell surface [37].

5. Caveolin, eNOS and cardiovascular biology

Recent studies using caveolin-deficient mouse models revealed that the lack of caveolae leads to various pathological conditions, some of which are related to the cardiovascular system, e.g., defective angiogenesis, pulmonary hypertension, and cardiac hypertrophy. Amazingly, some of the same cardiovascular diseases have otherwise been associated with an *upregulation* of caveolin abundance. In the next paragraphs, we will expose the current knowledge on some important (NO-related) roles of caveolins on myocardial tissue biology in order to illustrate that, as mentioned above, such paradox arises from the complex balance between the allosteric inhibitory role of caveolin and the facilitating effects of caveolar compartmentation on signaling.

5.1. Angiogenesis

A decrease in caveolin abundance induced an increase in NO production [38], but also stimulated tube formation from macrovascular endothelial cells cultured on Matrigel [39]. Using the calcium chelator BAPTA to prevent the calcium-CaM-mediated disruption of the caveolin/eNOS complex, Brouet et al. confirmed that caveolin was an important control point for angiogenesis regulation [39]. Accordingly, the following sequence of events leading to eNOS activation was identified: (i) disruption of the caveolin/eNOS interaction in response to calcium transient, (ii) recruitment of the chaperone protein hsp90 and consecutively of Akt (that interacts with hsp90) and (iii) phosphorylation of eNOS on Serine 1177. This signalling cascade was confirmed for the pro-angiogenic effect of VEGF [40]. More recently, the recruitment of the phosphatase calcineurin to hsp90 and the consecutive dephosphorylation of eNOS on threonine 495 were also identified [41,42]. Of note, an intriguing observation was recently reported showing that pathological alteration in eNOS dimerization could impact NO-dependent endothelial function, independently of the eNOS phosphorylation status [43]. Whether caveolin plays a role in facilitating or preventing eNOS dimerization requires further investigation.

The inhibitory role of caveolin in angiogenesis was also validated in vivo. By exploiting the propensity of cationic lipids to target endothelial cells lining tumor blood vessels, the delivery of a caveolin plasmid was found to be associated with a dramatic inhibition of angiogenesis and vascular function in solid tumors [44]. Inversely, a decrease in endothelial caveolin abundance was observed in tumor blood vessels following exposure to ionizing radiations [45]. This was further associated with the triggering of NO-mediated neoangiogenesis and the consecutive tumor regrowth after treatment [46].

Cav-1$^{-/-}$ mice, however, appeared to have a deficient angiogenesis. A dramatic reduction in vascular density in tumors or implanted Matrigel plugs was initially reported in Cav-1$^{-/-}$ mice when compared with wild-type Cav-1$^{+/+}$ mice [47]. Also, in a model of adaptive angiogenesis obtained after femoral artery resection, Cav-1$^{-/-}$ mice failed to recover a functional vasculature and even lost part of the operated limbs [48]. In endothelial cells isolated from Cav-1$^{-/-}$ vessels, VEGF failed to induce NO production and endothelial tube formation when compared with Cav-1$^{+/+}$ endothelial cells. Dissection of the VEGF/eNOS pathway in Cav-1$^{-/-}$ endothelial cells led to the finding that the VEGFR-2 was absent from low-density membranes (e.g., caveolae) and that consecutively, Ser1177 phosphorylation and Thr495 dephosphorylation of eNOS were both impaired. Interestingly, caveolin re-expression in Cav-1$^{-/-}$ endothelial cells redirected the VEGFR-2 in caveolar membranes and restored the VEGF/eNOS coupling. Still, when high levels of recombinant caveolin were reached in these knock-in experiments, activation of eNOS by VEGF was prevented. This observation perfectly illustrated the caveolar "paradox" with the co-existence of the "signaling inhibitory" paradigm (e.g., allosteric inhibition of eNOS proportional to caveolin levels) and the "signaling promoting" effect of caveolin through compartmentation (e.g., receptor/effector coupling is promoted when/where caveolin is up-regulated, but abrogated when caveolin is down-regulated below a threshold level; see Fig. 2).

5.2. Vasodilation

Shear stress is known as the major trigger of eNOS activation in the vascular endothelium. Similar to the

downstream events following agonist stimulation, acute changes in pressure or shear stress can also dissociate eNOS from caveolin through a calcium-CaM-dependent pathway [49] and lead to vasodilation [50]. It is therefore not surprising that alterations in caveolin abundance were associated with changes in the ability of vessels to relax. Accordingly, increases in caveolin abundance were associated with portal hypertension [51]; conversely, a decrease in caveolin expression following estradiol treatment was shown to mediate the estradiol-dependent correction of the vasodilatory defects in ovariectomized rats [52]; similarly, downregulation of caveolin by ionizing radiations accounted for the improvement of tumor perfusion after irradiation [45]. The relaxation in response to acetylcholine was also much larger in aortic rings collected from Cav-1$^{-/-}$ mice than wild-type littermate [53,54]. Of note, isolated aortic rings from Cav-1$^{-/-}$ mice were unable to maintain a constant contractile tone, oscillating at a 1 Hz frequency. The frequency of spontaneous transient outward currents (STOCs) was also significantly reduced in the vascular smooth muscle cells of Cav-1$^{-/-}$ mice at physiologic membrane potential [53]. Altogether, these studies support the conclusions that in basal conditions, eNOS becomes hyperactivated in the absence of caveolin-1.

5.3. Cell proliferation and fibrosis

Besides the vascular phenotype, Cav-1$^{-/-}$ mice present abnormalities in the lung and cardiac tissues. The lungs of Cav-1$^{-/-}$ mice have constricted alveolar spaces, associated with a hypercellular thickening of the alveolar walls and hypertrophy of certain cells (i.e, type II pneumocytes) [53,54]. Immunohistochemical analyses further revealed intense hyperproliferation, probably involving non-differentiated Flk-1^{+} vWF^{-} angioblastic cells. The resulting hypercellularity is associated with an increase in extracellular collagen deposition associated with fibrosis, making the Cav-1$^{-/-}$ mice exercise-intolerant in swimming tests [53,54].

Although caveolin-1-deficient mice retain their caveolae in striated muscle cells (i.e., cardiac and skeletal), abnormal cardiac function was detectable in these mice. The major structural defects were the enlarged right ventricular cavity and the thickening of the left ventricular wall with a decrease in systolic function. Histological analysis revealed myocyte hypertrophy with interstitial/perivascular fibrosis. The latter could be attributed to the hyperactivation of the p42/44 MAPK observed in both cardiac tissue and isolated fibroblasts of caveolin-1 null mice. Mora et al. also excluded that this cardiac hypertrophic phenotype was secondary to the development of pulmonary hypertension and the consecutive dilation of the right ventricle. Indeed, the pulmonary phenotype (due to local cellular hyperproliferation) appeared to be due to the defect in caveolin-2 expression that is present in caveolin-1 null mice (because of the instability of cav-2 when heteroligomers of cav-1 and -2 cannot form [55]). Using caveolin-2 null mice, the authors showed that a similar pulmonary pathology was present but did not lead to RV dilatation [56].

5.4. Cardiac muscle structure and contractility

As mentioned above, the specific isoform of caveolin expressed in striated (including cardiac) muscle is caveolin-3, and the phenotype of cav-3 KO mice provided initial insights into the role of this isoform in the maintenance of muscle integrity and contraction. Although genetic deletion of caveolin-3 does not affect the expression of the other isoforms or the formation of caveolae in non-muscle cells, the cardiac muscle fibres of cav-3 KO totally lack caveolae [57]. This is associated with the development of an eccentric hypertrophic cardiomyopathy, which also has been attributed to the loss of caveolin-3-mediated inhibition of the pro-hypertrophic p42/44 MAPK cascade [58]. Double knockout of caveolin-1 and caveolin-3 (resulting in total absence of caveolae in all cell types) produces an even more severe cardiomyopathy in mice [59]. Notably, caveolin-3 transgenics also develop cardiomyopathy at ages later than 6 months, but without cardiac hypertrophy [60]. Rather, the cardiomyopathy may be degenerative and the consequence of an age-dependent downregulation of the dystrophin–glycoprotein complex that is essential to maintain the integrity of muscle cell membranes. This has been proposed to result from a competitive binding by overexpressed caveolin-3 through its WW-like domain on a PPXY target motif of β-dystroglycan, preventing the recruitment of dystrophin (that uses a similar WW domain) in the membrane complex [61]. Whereas overexpression of caveolin-3 increases caveolar density in skeletal muscle (with the development of a Duchenne-like dystrophy [62]), this parameter was not specifically measured in cardiac muscle. However, caveolin-3 overexpressors also have a reduced activity of both eNOS and nNOS in cardiac muscle [60], again confirming the initial paradigm of negative allosteric modulation of NOS by caveolin in the cardiomyocytes. Such reduced NOS activity may have precipitated the development of cardiomyopathy. Conversely, transgenic overexpression of a caveolin-3 mutant (P104L) that results in a severe downregulation of endogenous caveolin-3 in cardiomyocytes induced an increase in cardiac eNOS activity, consistent with the above paradigm; these transgenic mice also exhibited significant cardiac hypertrophy perhaps attributable to the loss of p42/44 MAPK repression, as proposed above, although this was not specifically studied in these animals [63].

Although these transgenic models establish a proof of principle for the regulation of eNOS (and possibly nNOS) by caveolin-3, they represent extreme situations and may not reliably predict the functional impact of smaller expressional changes of caveolin-3 in pathophysiologic situations. Evidence is now accumulating on the regulation of caveolin-3 abundance in cardiac diseases, e.g., total

caveolin-3 proteins were shown to be upregulated in dog failing hearts [64] and post-infarcted human hearts [65]. Whether this is associated with increased functional signaling modules is less clear; in the failing dog heart, increased caveolin-3 levels were associated with a higher number of sarcolemmal caveolae [64], which could theoretically support an improved regulation of beta-adrenergic (and, possibly, other receptors-mediated) signaling by caveolae-associated eNOS. Similarly, mechanical unloading of the failing human heart increased sarcolemmal caveolin-3 staining (at unchanged total proteins level), along with an induction of CD36 expression [66]. Contrary to the dog heart, the expression of eNOS is decreased in the post-infarcted human heart, whereas nNOS increased. Its co-immunoprecipitation with caveolin-3 (also upregulated), expectedly, was increased and was taken as an indication of the integration of nNOS within functional signaling modules at the sarcolemma. However, the distribution of caveolin-3 (or the density of caveolae) was not examined in these human hearts [65]. In the aging and post-infarcted rat heart, the same group had shown that caveolin-3 was redistributed in cytosolic fractions [67] where eNOS or nNOS would be less amenable to/operative for receptor-mediated regulation. On the other hand, in two different models of hypertrophic cardiomyopathy (i.e., dogs with perinephritic hypertension [68] and 18 weeks spontaneously hypertensive rats [69]), caveolin-1 and caveolin-3 levels were downregulated; in the former study, the fraction of eNOS co-immunoprecipitated with caveolin-1 (normalized to total eNOS proteins) was decreased in hypertensive dogs and this was paralleled with enhanced NOS-dependent regulation of hemodynamics, suggesting increased NOS activity in situ. Overall, these observations emphasize that in addition to its direct correlation with the assembly of signaling modules (which may only result in functional alterations of signaling in extreme situations, such as illustrated in transgenic or knockout models), the abundance of caveolin (even over smaller changes) is also inversely correlated with NOS activity through caveolin's inhibitory allosteric binding (see above and Fig. 2). Therefore, an accurate interpretation of these changes should be based on a quantitative assessment of caveolin binding to NOS as well as morphologic assessment of caveolae density together with a measurement of NOS activity in situ, whenever possible.

One should also keep a critical eye on the interpretation of co-immunoprecipitation with caveolin in cardiac extracts as an index of plasmalemmal caveolar localization, because caveolin is distributed between different subcellular compartments and immunoprecipitation from a whole cell extract does not necessarily (or exclusively) reflect protein–protein interaction at the plasma membrane. Again, independent validation by immunogold staining and electron microscopy or subcellular fractionation techniques is warranted, as was done for cardiac eNOS [32]. Of note, a thorough analysis of caveolin-3 distribution in cardiac myocytes was recently provided and suggests the existence of several caveolar pools, each differentially associated with specific proteins involved in excitation–contraction coupling [70,71]. Although caveolin-3 co-localized with voltage-dependent (L-type) calcium channels, ryanodine receptors, the Na/Ca exchanger and Na channels at the cell surface, albeit in different caveolar pools, a distinct portion of caveolin-3 colocalized exclusively with ryanodine receptors in the cell interior, possibly in corbular SR [72]. Combined with the notion of colocalization of caveolin-3 with eNOS in caveolae, and the recent description of a direct interaction of the reductase domain of eNOS with the ryanodine receptor [73], this would support a functional modulation of ryanodine-dependent calcium currents and excitation–contraction coupling by eNOS, as previously suggested in response to stretch [74]. A more general role for caveolae in excitation–contraction coupling was suggested from the observation that caveolar disruption with cyclodextrin in neonatal cardiomyocytes abrogated the ignition of calcium sparks [75], which represent the localized intracellular calcium release events from the ryanodine receptors. If applicable to the adult heart (i.e., with fully mature T-tubules), this would extend the significance of changes in caveolin-3 abundance and caveolae density in cardiac diseases, as mentioned above, through their bearing on the structural integrity of the excitation–contraction coupling machinery with expected alterations of contractile properties.

6. Conclusion

The interaction between eNOS and caveolins provides a fascinating paradigm for the regulation of signaling through allosteric modulation of enzymatic activity, multi-proteins assembly, protein trafficking and internalization of protein complexes as part of signaling shut-down. The phenotype of caveolin transgenic or knock-out animals to a large extent confirmed the predictions of the mechanistic dissections in cultured cells. Some surprising findings (such as defective NO-dependent angiogenesis in cav-1 KO animals) have now been rationalized by taking into account the dual importance of caveolin both as a scaffold for the assembly of signaling modules and a repressor of eNOS (and possibly, nNOS) basal activity, with a balance between these functionally antagonistic influences according to the abundance of the protein. Importantly, the relationship between caveolin expression and caveolar assembly may not be linear in all cell types, and at higher caveolin levels, the promoting effect on signaling platform assembly may be offset by direct inhibition of caveolin binding partners, such as eNOS. This raises important caveats on the interpretation of expressional changes of caveolins and NOS in cardiovascular diseases, that should be confronted with the analysis of NOS activity and subcellular localization in situ, whenever possible. This would seem a pre-requisite for

the development of genetic or pharmacologic approaches of caveolin modulation for the treatment of specific cardiovascular disorders.

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