NEFROLOGIA. Vol. XVIII. Suplemento 1. 1998

New paradigms in the post-translational regulation of the endothelial isoform of nitric oxide synthase (eNOS)

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SUMMARY

In this review, knowledge acummulated on the post-translational regulation of endothelial nitric oxide synthase (eNOS) since its molecular characterization is up - dated. The biochemical basis for its membrane association through myristilation and palmitoylation have been defined. A further effort established its presence wit - hin caveolae, its association with caveolin and interaction both structural and func - tional, with caveolin and calmodulin. A model for this interaction is proposed.

Key words: ***Caveolae, calmodulin, nitric oxide synthase (eNOS).***

NUEVOS PARADIGMAS EN LA REGULACION POST-TRADUCCIONAL DE LA ISOFORMA ENDOTELIAL DEL OXIDO NITRICO SINT ASA ENDOTELIAL (eNOS)

RESUMEN

En esta revisión se actualizan los conocimientos sobre la regulación post-tra - ducccional de la óxido nítrico sintasa endotelial (eNOS). Esta enzima, desde el mo - mento de su caracterización molecular, ha sido objeto de intenso estudio, ha - biéndose definido las bases bioquímicas de su anclaje a membranas a través de miristilación y palmitoilación. Un paso ulterior estableció la presencia de este en - zima en las caveolas, su asociación con la caveolina y la interacción estructural y funcional de la eNOS, con la caveolina y la calmodulina, proponiéndose un modelo para esta última.

Palabras clave: ***Caveolina, calmodulina, óxido nítrico sintasa (eNOS).***

Nitric oxide is synthesized in mammalian cells by a family of three nitric oxide synthases: nNOS, iNOS and eNOS, a nomenclature reflecting the tissues of origin for the first cDNA isolates, respectively neurons, immunoactivated macrophages and endothelial cells. The endothelial isoform of nitric oxide synthase (eNOS), originally described in large vessel endothelium, is now known to be expressed in numerous cell types including platelets, cardiac myocytes, microvasculature endothelial cells and hippocampal cells. Added to this tissue diversity of eNOS expression is a broad palette of effects for L-arginine-derived nitric oxide (NO), such as anti-atherogenic and anti-thrombotic properties, autonomic regulation of cardiac function and long-term memory modulation. Although eNOS may subserve these very distinct biological roles when expressed in different tissues, changes in intracellular calcium concentration ($[Ca^{2+}]_i$) is considered to be the major trigger responsible for eNOS activation in a given cell. The presence of a calmodulin (CaM) binding site within the eNOS sequence makes, indeed, this 135 kDa-protein, a classical Ca^{2+}-sensitive enzyme in which the active conformation is induced by the interaction

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with Ca^{2+}-bound CaM. The rate of nitric oxide (NO) production therefore reflects changes in $[Ca^{2+}]_i$, increasing as Ca^{2+} rises and decreasing as Ca^{2+} falls. This simplistic bimodal mechanism of Ca^{2+}-dependent NO production regulation has recently been refined by our finding that the binding of Ca^{2+}/CaM to eNOS involves the disruption of the association of eNOS from the integral membrane protein caveolin and the subsequent translocation of eNOS from caveolae.

eNOS, CAVEOLIN AND CAVEOLAE

For several years, determining the specific particulate subcellular fraction to which the eNOS is targeted was an elusive goal; mutually contradictory reports have only recently been resolved by the study of Shaul and colleagues documenting the localization of eNOS in plasmalemmal caveolae[1]. The term *caveola intracellularis*[2] had been introduced more than 40 years ago to describe plasma membrane invaginations identified by electron microscopy in a wide variety of cell types including epithelial and endothelial cells, adipocytes and myocytes. Functionally, these 50-100 nm plasmalemmal vesicles were first shown to participate in the transcellular transport of macromolecules (transcytosis) and in the upake of small molecules (pinocytosis)[3]. More recently, however, the discovery of caveolin[4-5], the structural coat component of caveolae (and its use as a biochemical marker of these unusual organelles) has provided the impetus for a new wave of studies suggesting that caveolae also participate in signal transduction by assuring the compartmentalisation of signaling molecules such as growth-factor and hormonal receptors, G proteins, protein kinases, as well as eNOS[6].

The development of antibodies directed against different tissue-specific isoforms of caveolin (caveolin-1, -2 and -3) has permitted a better characterization of caveolar microdomains and facilitated the identification of caveolar residents. Using these antibodies in immunoprecipitation experiments, we have reported that eNOS is quantitatively associated with caveolin-1 in endothelial cells and with caveolin-3 in ventricular myocytes[7]. Experiments performed in transfected NOS cells revealed that this association leads to the inhibition of the enzyme activity[8, 9], suggesting that a stable protein-protein interaction takes place between both proteins. We have also documented that the co-immunoprecipitation of caveolin and eNOS is completely and specifically blocked by an oligopeptide corresponding to the scaffolding domain of caveolin[10], a region of 20 amino acids (82 to 101 in caveolin-1 sequence) known to interact with other signaling proteins such as G proteins and non-receptor tyrosine kinases[11, 12]. The same peptide also appears to block the enzymatic activity of purified recombinant eNOS as well as of eNOS endogenously expressed in endothelial membranes[10]. Finally, using eNOS truncation and deletion mutants in transfection experiments, we have identified an obligatory role for the N-terminal half of eNOS (residues 85 to 528) in stabilizing its association with caveolin[9]. Together with a recent study of Ju y cols.[13] identifying *in vitro* the oxygenase domain of eNOS and two regions of caveolin (including the scaffolding domain) as the sites of mutual interaction of the two proteins, our data demonstrate the existence of a stable, cell-specific protein-protein interaction between eNOS and caveolin isoforms.

Formation of an inhibitory eNOS/caveolin heteromeric complex may serve to ensure the latency of the NO signal until calcium-mobilizing extracellular stimuli destabilizes this complex and activates the enzyme (see below). However, it is unclear how caveolin inhibits such diverse enzyme activity as GTPase, tyrosine kinase and nitric oxide synthase. Interestingly, Lisanti and colleagues[11, 12, 14] have recently identified, from phage display libraries, consensus peptide sequences rich in aromatic residues responsible for the specific interaction with caveolin. This consensus sequence exists within the proteins to have been shown, to date, to specifically interact with the scaffolding domain of caveolin (H-Ras, Gas, Src, eNOS, EGF receptor and PKC). This short sequence (amino acids 350-358 in bovine eNOS) was recently shown to be required for the binding of eNOS to caveolin[15]. Interestingly, this sequence is also present in nNOS and we have recently documented the *in vitro* interaction of purified recombinant nNOS with the caveolin scaffolding domain peptide (unpublished observations). This was confirmed by Venema and colleagues[16] who have reported that in skeletal muscle, nNOS and caveolin-3 interact together, probably within the dystrophin complexes.

eNOS, CAVEOLIN AND CALMODULIN

In exploring the factors governing eNOS/caveolin association, we have discovered that CaM disrupts the heteromeric complex formed between eNOS and caveolin in a Ca^{2+}-dependent fashion[8-10]. We have also shown that Ca^{2+}/CaM completely reversed the inhibitory effect of caveolin on eNOS activity in transfected cells as well as in a soluble system involving purified components[8-10]. In this re-

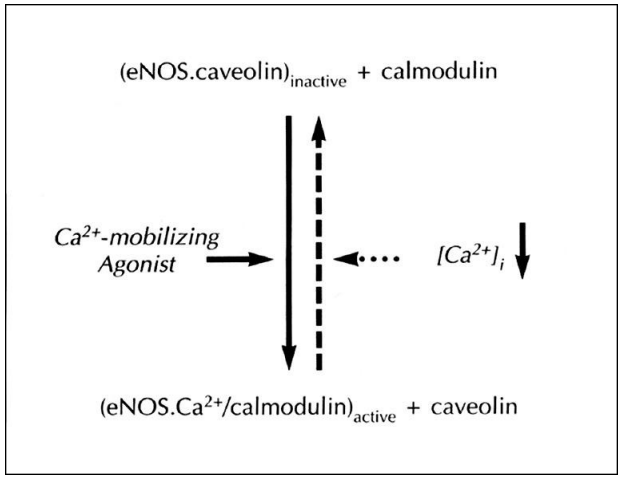

Fig. 1.–Reciprocal regulation of eNOS by caveolin and Ca^{2+}-calmodulin. *At the low intracellular concentrations of Ca^{2+} characteristic of unactivated cells, the stable interaction of eNOS with caveolin locks the enzyme in its inactive state and NO production is blocked. Following agonist stimulation, local increases in Ca^{2+} lead to the competitive displacement of caveolin from eNOS by Ca^{2+}-liganded calmodulin, which binds to the enzyme, leading thereby to enzyme activation and NO production.*

constructed system, enzyme kinetic analyses revealed that caveolin serves as a competitive inhibitor of CaM-dependent eNOS activation. There are here striking parallels with the recently identified regulation by CaM of the interaction between PKC and the A kinase anchoring protein AKAP79, known to coordinate the location of calcineurin, PKA and PKC at the postsynaptic densities in neurons[17]. Indeed, in presence of calcium, CaM appears to compete with PKC for binding to AKAP79, releasing the inhibited kinase from its association with the anchoring protein. Thus, the interactions of eNOS with CaM *versus* caveolin provide a novel example of the reciprocal regulation of enzyme activity by competing allosteric protein-protein interactions (see figure 1). This close control of enzyme activity may be particularly important for eNOS in caveolae, where CaM is also largely enriched[1] and where even subtle increases in intracellular calcium could thus lead to enzyme activation if the interaction of caveolin with eNOS were not keeping the system in check. Because NO has cytotoxic as well as signaling functions, attenuation of basal enzyme «leakiness» by caveolin may be of particular importance. However, aside from eNOS, the other caveolin-modulated signaling proteins are not known to be CaM-dependent and therefore it is plausible that elements of higher order structure, as yet unidentified, may, in fact, serve as the basis for caveolin inhibition of NOS activity.

eNOS ACYLATION AND REVERSIBLE CAVEOLAR TARGETING

An obvious question raised by the findings of the counterbalancing modulation of eNOS by caveolin and CaM is related to the cellular regulation of eNOS/caveolin interaction in the context of enzyme acylation. eNOS is unique among the NOS isoforms in its being dually acylated by myristate and palmitate, 14- and 16-carbon saturated fatty acids respectively, and importantly, both modifications are required for an efficient targeting of the enzyme to caveolae.

Myristoylation occurs co-translationally on a N-terminal glycine residue within a specific consensus sequence (not present in nNOS and iNOS). This modification is required for eNOS targeting to the endothelial cell membrane (caveolae)[1, 9] and is essentially irreversible, precluding its dynamic regulation by agonists or other stimuli. Reversible post-translational modifications may, however, determine the subcellular localization of myristoylated proteins and provide a mechanism for their regulation. The stable membrane association of myristoylated proteins often requires hydrophobic or electrostatic interactions in addition to those between myristate and membrane lipids. Several lines of evidence from our laboratory[18, 19] suggest that, for eNOS, this double role of modulator and stabilizer of the interaction of the enzyme with the membrane is in part subserved by palmitoylation.

Palmitoylation (for which no unique consensus sequence exists) takes place on two cysteine residues near the eNOS N-terminus (Cys-15 and Cys-26)[18], and has not been reported for the other NOS isoforms. Pulse-chase experiments in endothelial cells biosynthetically labeled with [^{3}H]palmitate showed that bradykinin treatment may promote eNOS depalmitoylation[19]. Loss of the fatty acid palmitate (and its hydrophobic interactions with cell membranes) is not limited to eNOS since it has been shown that agonists activating (receptors coupled to) $G\alpha_s$ appear to stimulate the palmitate turnover of the α_s subunit, specifically accelerating de-palmitoylation[20]. Depalmitoylation could therefore be part of a cellular mechanism for the release of signaling proteins from the membrane and translocation to the cytosol in response to agonist stimulation.

The targeting of eNOS to plasmalemmal caveolae is dependent upon palmitoylation of the protein: a palmitoylation-deficient eNOS mutant, which shows a reduced affinity for the membrane fraction overall, fails entirely to be targeted to caveolae[1]. It is therefore plausible that agonist-induced depalmitoylation of eNOS promotes the dissociation of the enzyme

from proximity to activating molecules (or substrate or cofactors?) localized in caveolae, and may serve as a feedback mechanism for eNOS activation. However, as discussed above, the inhibitory caveolin/eNOS interaction constitutes the biological framework in which the regulation of the catalytic activity of the enzyme has to be interpreted and therefore, translocation appears more likely to be a consequence of, or at the best, as a process favored by the dissociation of eNOS from the caveolin complex. Finally, in addition to their distinctive protein content, it is also important to note that caveolae have a characteristic lipid composition and contain virtually no phospholipids. In this context, one may re-interpret the *in vitro* observations that membrane phospholipids attenuate eNOS enzyme activity[21] and speculate that the re-targeting of depalmitoylated eNOS from a phospholipid-free environment, characteristic of caveolae, to the non-caveolar plasmalemma (which contains abundant phosholipids) might also serve as a feedback mechanism leading to enzyme de-activation. Furthermore, since NO activates molecular targets outside the endothelial cell, such as the guanylate cyclase in platelets or in vascular smooth muscle, it further seems likely that the intracellular localization of the eNOS could affect the signaling roles of its product, and thereby modulate the response to extracellular signals.

CAVEOLIN AS AN eNOS CHAPERONE. COMPLEMENTARY ROLE OF ENZYME ACYLATION

By exploring the differential effects of detergents used for cell lysis, we have recently documented that CHAPS does not allow the discrimination between direct caveolin association and acylation-driven interaction of eNOS with caveolin complexes contrary to octylglucoside which abrogates «caveolae interaction» but not «caveolin association» [9].

The exploitation of the differential effects of these detergents allowed us to show that the direct interaction between eNOS and caveolin is facilitated by, but does not require eNOS acylation, and importantly that treatment of intact aortic endothelial cells with a calcium ionophore leads to the rapid disruption of the caveolin/eNOS complex[9]. We have also observed that the myristoylation- and palmitoylation-deficient eNOS mutants may both interact with caveolin in the cytosol; this association also leads to a marked inhibition of enzyme activity, which is completely reversed by addition of CaM[9]. The regulatory caveolin/eNOS association appears therefore independent of the state of eNOS acylation, indicating that agonist-evoked Ca^{2+}/CaM-dependent disruption of caveolin-eNOS complex, rather than agonist-promoted depalmitoylation of eNOS relieve caveolin's tonic

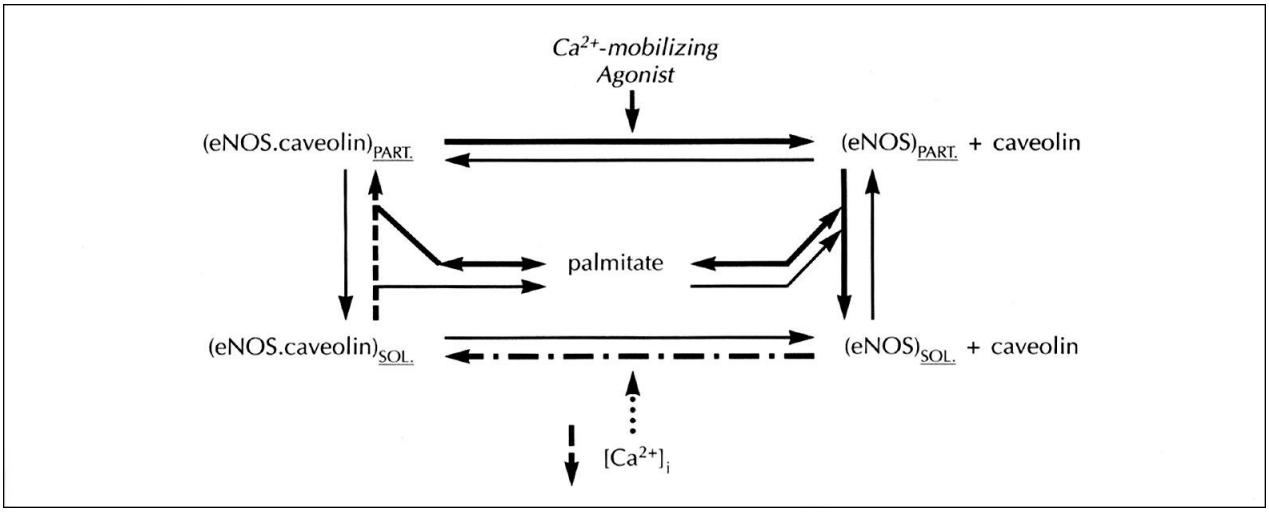

Fig. 2.–The eNOS/caveolin cycle. *The binding sites of Ca^{2+}/calmodulin and caveolin on eNOS are both located in the oxygenase domain of eNOS and are allosterically dependent. Following disruption of the caveolin/eNOS complex by Ca^{2+}/calmodulin within caveolae (with consequent enzyme activation), depalmitoylation of eNOS combined with the loss of anchoring of eNOS to caveolin leads to (i.e. displaces the equilibrium towards) the translocation of the enzyme from the caveolae. Caveolin-free, depalmitoylated eNOS is found in the soluble (SOL) and particulate (PART) cellular compartments, corresponding very likely the cytosol and the non-caveolar plasma membrane. The translocation of eNOS parallels the decrease of the intracellular Ca^{2+} concentration to the basal level, and thus the progressive restoration of the caveolin/eNOS interaction following «deactivation» of CaM. The re-assotiation of eNOS with caveolin complexes shuttle between caveolae and the trans-Golgi network. Note that during all the recycling pathway, eNOS is still myristoylated and this modification probably governs the re-targeting to caveolin/eNOS to caveolae, where palmitoylation may then take place.*

inhibition of enzyme activity. Thus, we propose that caveolin may serve as an eNOS chaperone regulating NO production independently of the enzyme's residence within caveolae or its state of acylation.

CONCLUSIONS

We postulate the existence of a dynamic cycle of eNOS-caveolin interactions[23] initiated by agonist-promoted increases in $[Ca^{2+}]_i$ that disrupt the caveolin-eNOS complex, leading to enzyme activation (see figure 1 and 2). Following more prolonged agonist stimulation, eNOS is de-palmitoylated[19], and is no longer selectively sequestered in caveolae. The translocated enzyme probably partitions both into non-caveolar plasma membrane and into more hydrophilic regions of the cell, the precise identity of which has not been established. Subsequent to the enzyme's translocation into this more «soluble» cell compartment, and following the decline in $[Ca^{2+}]_i$ to basal levels, caveolin may once again interact with eNOS (see figure 2). The re-association of eNOS with caveolin may occur either at the membrane level or in the cytosol through which caveolin complexes may shuttle between caveolae and an internalized caveolar vesicle/trans-Golgi network[24]. The reassociation and re-targeting of the heteromeric eNOS-caveolin complex appears to be accelerated (or stabilized) by enzyme palmitoylation, this interaction taking place either within caveolae or en route to this organelle (see figure 2). The re-palmitoylation of eNOS facilitates rapid and efficient stabilization of the inactivated enzyme in the caveolar environment ready for another cycle of stimulation by agonists. This dynamic cycle of eNOS intracellular regulation adds another level of complexity to the post-translational life history of this vital signaling protein, and may represent an important control point for the modulation of NO-dependent signaling in the vascular wall.

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