Regulation of endothelial derived nitric oxide in health and disease

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Endothelial nitric oxide synthase (eNOS) is the primary physiological source of nitric oxide (NO) that regulates cardiovascular homeostasis. Historically eNOS has been thought to be a constitutively expressed enzyme regulated by calcium and calmodulin. However, in the last five years it is clear that eNOS activity and NO release can be regulated by post-translational control mechanisms (fatty acid modification and phosphorylation) and protein-protein interactions (with caveolin-1 and heat shock protein 90) that direct impinge upon the duration and magnitude of NO release. This review will summarize this information and apply the post-translational control mechanisms to disease states.

Key words: nitric oxide - endothelium - caveolin-1 - heat shock protein 90 - atherosclerosis - inflammation

In the past decade the importance of the vascular endothelium as a multifunctional regulator of vascular smooth muscle physiology and pathophysiology has been appreciated. Indeed, the endothelium responds to hemodynamic stimuli (pressure, shear stress, and wall strain) and locally manufactured mediators (such as bradykinin, prostaglandins, and angiotensin) and in turn can release factors that can influence the adhesion and aggregation of circulating cells to the endothelium and the tone of vascular smooth muscle. In many diseases, including atherosclerosis, diabetes or cirrhosis endothelial dysfunction manifested as an impairment nitric oxide (NO) production or bioactivity may be an early hallmark of disease and a treatable entity. In this chapter, the importance of NO as a mediator of vascular function and potential mechanisms of endothelial NO synthase (eNOS) activation in disease will be discussed.

Regulation of vascular tone by endogenous NO

eNOS is the NOS isoform responsible for producing the classical endothelium-derived relaxing factor as originally described by (Furchgott & Zawadski 1981). Evidence for the importance of eNOS derived NO in the regulation of vascular tone is based on experiments in animals and in humans demonstrating that L-arginine based inhibitors of NO increase blood or perfusion pressure and vascular resistance and reduce blood flow in vivo and in vitro. More recently, this has been unequivocally confirmed using mice with targeted disruption of the eNOS gene locus. eNOS knockout mice (-/-) are mildly hypertensive relative to wild-type littermate control mice (+/+) of the same generation. Importantly, the pressor effect of nitro-L-arginine, a NOS inhibitor, is attenuated in the +/- mice and endothelium-dependent relaxation in response to acetylcholine is abrogated in isolated vessels (Huang et al. 1995, Shesely et al. 1996). This fundamental finding is direct “proof of-principal” for the major contribution of NO in vasomotor control in large blood vessels.

Physiological activation of eNOS and NO release

Typically, endothelial cells release NO in response to autacoids that mobilize intracellular calcium such as thrombin, VEGF or ADP. The proposed mechanism for eNOS activation is that the released calcium will bind to calmodulin (CaM) and the calcium/CaM complex will bind to the CaM site in the enzyme promote NO synthesis. However, the most physiological agonist for NO release is fluid shear stress. Shear stress in vitro or shear rate in vivo, is the tangential vector of force elicited by the flow of blood over the endothelial cell surface. Exposure of endothelial cells to shear stress results in a burst of NO release, followed by a sustained phase. In vivo, increasing shear rate due to high blood flow will cause flow dependent dilations of certain vascular beds while decreasing shear rate will promote vasoconstriction. Shear induced NO release in vitro and flow-dependent vasodilatation in vivo can be blocked with NOS inhibitors. Interestingly, shear-induced NO release appears to “independent” of fluctuations of calcium since shear causes a rapid burst of calcium release that does not parallel the sustained release of NO; chelation of intracellular calcium does not influence the rate of NO production elicited by shear and calmodulin antagonists can block bradykinin induced NO release but not shear induced release (Kuchan & Frangos 1994, Fleming et al. 1998). These data collectively suggest a fundamental difference in the signal transduction mechanisms for agonist versus shear- or growth factor-induced activation of eNOS.
positions 15 and 26 (Fulton et al. 2001) and these fatty acids are important for its targeting in the Golgi region and plasmalemmal caveolae. The proper localization of eNOS is necessary for its interactions with other regulatory proteins (scaffolds, chaperones, kinases) that fine tune the cycles of eNOS activation and inactivation.

The major negative regulatory protein for eNOS is caveolin-1. Caveolin-1 is the major coat protein of caveolae, and has several faces that may influence the biology of proteins that localize to cholesterol rich plasmalemma caveolae. Indeed caveolin-1 is necessary for the biogenesis of caveolae through an unknown mechanism (Smart et al. 1999). In addition, caveolin-1 can serve as a cholesterol binding protein and traffic cholesterol from the endoplasmic reticulum through the Golgi to the plasma membrane. Finally, caveolin has the capacity to directly interact with other intracellular proteins such as c-Src and H-Ras through amino acids 82-101, the putative scaffolding domain. Indeed, three groups independently demonstrated that eNOS could directly interact with caveolin-1 or caveolin-3 (Feron et al. 1996, García-Cardeña et al. 1996, Ju et al. 1997). The primary binding region of caveolin-1 for eNOS is within amino acids 60-101 and to lesser extent amino acids 135-178. Furthermore, the caveolin-eNOS immunocomplex is disrupted in presence of caveolin scaffolding peptides (amino acids 82-101).

In vivo evidence supporting the role of caveolin-1 as a negative regulator of eNOS is emerging. Recent work using the caveolin scaffolding domain as a surrogate for caveolin have demonstrated that eNOS can be regulated in situ. Exposure of permeabilized cardiac myocytes to the caveolin-3 scaffolding domain peptide (amino acids 55-74), but not a scrambled version, antagonized the negative chronotropic actions of carbachol (Feron et al. 1998). Our group recently used a membrane permeable form of the caveolin-1 scaffolding domain (amino acids 82-101) by fusing it to a cell permeable leader sequence (Bucci et al. 2000a, b). Exposure of the peptide to blood vessels blocked Ach induced relaxations, with no effect on relaxant responses to sodium nitroprusside or the release of prostacyclin showing that in an intact blood vessel, the caveolin peptide is a potent inhibitor of eNOS. In addition, the peptide also blocked inflammation in two different models by influencing vascular permeability suggesting that peptidomimetics may be useful therapeutically. Most recently, this peptide has been shown to block microvascular permeability in tumors and hyperpermeability of post-capillary venules (Gratton et al. 2003). Finally, genetic evidence from caveolin-1 knockout mice strongly supports the concept that eNOS is negatively regulated by caveolin-1 in vivo. Both basal and stimulated eNOS activation and relaxations are enhanced in vessels from caveolin-1 (-/-) mice (Drab et al. 2001, Razani et al. 2001). In addition, the vessels in the microcirculation are hyperpermeable from NO dependent vascular leakage (Razani et al. 2002).

With respect to disease mechanisms that may influence the caveolin/eNOS interaction, there is evidence that in atherosclerosis, diabetes, and cirrhosis, that there may be abnormalities in the eNOS-caveolin pathway. In the context of atherosclerosis, caveolin-1 is a cholesterol binding protein that can transport cholesterol from the endoplasmic reticulum to the plasma membrane (Murata et al. 1995), and major receptors for HDL, SR-B1, and a scavenger receptor for modified forms of LDL, CD36, can reside in and signal in caveolae type microdomains. In addition, oxidized LDL can extract caveolae cholesterol, mislocalize eNOS and impair NO release. Conversely, blockade of HMG CoA reductase with statin-based drugs reduces caveolin levels and promote eNOS activation. This concept has been validated in Apo E (-/-) mice where statin treatment decreases caveolin-1 expression and promotes NOS function in vivo. However, to date, there are no data showing changes in caveolin-1 levels in atherosclerotic lesions from humans.

Hyperglycaemia represents a major risk factor in the development of the endothelial impairment in diabetes. In non-obese diabetic mice, modest hyperglycemia triggers a selective reduction in the response to alpha1 and beta2 agonists but not to dopamine or serotonin (Bucci et al. 2004). When glycosuria is severe (500 to 1000 mg/dl), there is a complete ablation vasoconstrictor responses to the alpha1 receptor agonist stimulation and a marked reduced response to beta2 agonist stimulation. In the severe glycosuria model (500 to 1000 mg/dl), eNOS expression is unchanged, although caveolin-1 expression is significantly enhanced, indicating that high glucose levels cause an upregulation of the endogenous eNOS inhibitory clamp by caveolin. This is manifested by a significant reduction in acetycholine-induced vasodilatation. Thus, caveolin-1 could represent a new possible therapeutic target in vascular impairment associated with diabetes.

In a rat model of cirrhosis, caveolin-1 is over expressed, more caveolin-1 interacts with eNOS and the basal and stimulated production of NO is depressed (Shah et al. 1999a) suggesting that this interaction may increase portal pressures and contribute to the disease state. More recently in cholestatic models of disease, the upregulation of sinusoidal caveolin-1 and a decrease in eNOS activity is seen (Shah et al. 2001). Most importantly, elevated expression of caveolin-1 has been found in patients with hepatocellular carcinoma and hepatitis C related cirrhosis suggesting that the upregulation of caveolin-1 may be contribute to endothelial dysfunction in the liver (Yokomori et al. 2002, 2003).

Another protein interaction that regulates eNOS is via heat shock protein 90 (hsp90). Blockade of hsp90-mediated signaling with geldanamycin (GA), or more recently radicicol (RAD) attenuates histamine and VEGF stimulated cGMP production in cultured endothelial cells and blocked Ach-induced vasorelaxation of rat aortic rings, middle cerebral artery and flow-induced dilation indicating that hsp90 signaling was crucial for NO release and endothelial function (Garcia-Cardena et al. 1998, Ou et al. 2004). Further support for the relevance of hsp90/eNOS interactions in vivo was demonstrated in a model of portal vein ligation (PVL) in rats (Shah et al. 1999b) and in a model of inflammation (Bucci et al. 2000b). In the former study, the physical interaction of hsp90 with eNOS isolated from the mesenteric microcirculation was documented and GA attenuated Ach-dependent vasodilatation to the same extent as conventional NOS inhibi-
tors. In portal hypertensive rats, eNOS protein levels are not changed compared to control rats but NO activity is markedly enhanced in the mesenteric tissue of hypertensive rats. The enhanced activity correlated with hyporesponsiveness to the vasoconstrictor methoxamine (MTX) and GA potentiated the MTX-induced vasoconstriction after PVL, partially reversing the hyporeactivity to this agent, indicating that hsp90 can act as a signaling component leading to NO-dependent responses in the mesenteric microcirculation. In the latter study, GA dose-dependently inhibited inflammation, an effect as potent as a steroid. Since GA blocks NO release and NO inhibitors reduce edema formation, it is possible that drugs that specifically inhibit hsp90 will be good anti-inflammatory drugs.

**Future directions to correct endothelial dysfunction**

In many vascular based diseases endothelial dysfunction, characterized by an impairment of eNOS function or inactivation of NO by oxidative stress, will result in a deficit in bioavailable NO. Insights into how eNOS is regulated and the development of novel NO donors to supplant the “NO deficient state” will hopefully lead to improvements in blood flow in the intrahepatic circulation during cirrhosis.

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I apologize to colleagues whose references were omitted for the sake of brevity or whose contributions were cited in reviews.

**REFERENCES**


