Regulation of endothelial derived nitric oxide in health and disease

William C Sessa

Department of Pharmacology and Program in Vascular Cell Signaling and Therapeutics, Boyer Center for Molecular Medicine, Yale University School of Medicine, 295 Congress Avenue, New Haven CT 06536-0812, US

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 NOS 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 vasodilation 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.

Regulation of NO production by protein-protein interactions and manifestations in disease

eNOS is a membrane associated NOS isoform that is modified by co-translational N-myristoylation at glycine 2 and post-translational cysteine palmitoylation at

Financial support: National Institutes of Health and the American Heart Association
+Corresponding author. E-mail: william.sessa@yale.edu
Received 8 November 2004
Accepted 30 December 2004

positions 15 and 26 (Fulton et al. 2001) and these fatty acids are important for its taragetting in the Golgi region and plasmalemmal caveolae. The proper localization of eNOS is necessary for its interactions with to 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 s 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 manesfested by a significant reduction in acetylcholine-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 radidicol (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 NOS 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 NOS 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 funtion 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 hope-fully lead to improvements in blood flow in the intrahepatic circulation during cirrhosis.

ACKNOWLEDGMENTS

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

REFERENCES

Bucci M, Gratton JP, Rudic RD, Acevedo L, Roviezzo F, Cirino G, Sessa WC 2000a. In vivo delivery of the caveolin-1 scaffolding domain inhibits nitric oxide synthesis and reduces inflammation. *Nat Med 6*: 1362-1367.

Bucci M, Roviezzo F, Brancaleone V, Lin MI, Di Lorenzo A, Cicala C, Pinto A, Sessa WC, Farneti S, Fiorucci S, Cirino G 2004. Diabetic mouse angiopathy is linked to progressive sympathetic receptor deletion coupled to an enhanced caveolin-1 expression. *Arterioscler Thromb Vasc Biol 24*: 721-726.

Bucci M, Roviezzo F, Cicala C, Sessa WC, Cirino G 2000b. Geldanamycin, an inhibitor of heat shock protein 90 (Hsp90) mediated signal transduction has anti-inflammatory effects and interacts with glucocorticoid receptor in vivo. *Br J Pharmacol 131*: 13-16.

Drab M, Verkade P, Elger M, Kasper M, Lohn M, Lauterbach B, Menne J, Lindschau C, Mende F, Luft FC, Schedl A, Haller H, Kurzchalia TV 2001. Loss of caveolae, vascular dysfunction, and pulmonary defects in caveolin-1 gene-disrupted mice. *Science 293*: 2449-2452.

Feron O, Belhassen L, Kobzik L, Smith TW, Kelly RA, Michel T 1996. Endothelial nitric oxide synthase targeting to caveolae. Specific interactions with caveolin isoforms in cardiac myocytes and endothelial cells. *J Biol Chem 271*: 22810-22814.

Feron O, Dessy C, Opel DJ, Arstall MA, Kelly RA, Michel T 1998. Modulation of the endothelial nitric-oxide synthase-caveolin interaction in cardiac myocytes. Implications for the autonomic regulation of heart rate. *J Biol Chem 273*: 30249-30254.

Fleming I, Bauersachs J, Fisslthaler B, Busse R 1998. Ca2+-independent activation of the endothelial nitric oxide synthase in response to tyrosine phosphatase inhibitors and fluid shear stress. *Circ Res 82*: 686-695.

Fulton D, Gratton JP, Sessa WC 2001. Post-translational control of endothelial nitric oxide synthase: why isn't calcium/calmodulin enough? *J Pharmacol Exp Ther 299*: 818-824.

Furchgott RF, Zawadski JV 1981. The obligatory role of the endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature 288*: 373-376.

Garcia-Cardena G, Fan R, Shah V, Sorrentino R, Cirino G, Papapetropoulos A, Sessa WC 1998. Dynamic activation of endothelial nitric oxide synthase by Hsp90. *Nature 392*: 821-824.

García-Cardeña G, Fan R, Stern DF, Liu J, Sessa WC 1996. Endothelial nitric oxide is regulated by tyrosine phosphorylation and interacts with Caveolin-1. *J Biol Chem 271*: 27237-27240.

Gratton JP, Lin MI, Yu J, Weiss ED, Jiang ZL, Fairchild TA, Iwakiri Y, Groszmann R, Claffey KP, Cheng YC, Sessa WC 2003. Selective inhibition of tumor microvascular permeability by cavtratin blocks tumor progression in mice. *Cancer Cell 4*: 31-39.

Huang PL, Huang Z, Mashimo H, Bloch KD, Moskowitz MA, Bevan JA, Fishman MC 1995. Hypertension in mice lacking the gene for endothelial nitric oxide synthase [see comments]. *Nature 377*: 239-242.

Ju H, Zou R, Venema VJ, Venema RC 1997. Direct interaction of endothelial nitric-oxide synthase and caveolin-1 inhibits synthase activity. *J Biol Chem 272*: 18522-18525.

Kuchan MJ, Frangos JA 1994. Role of calcium and calmodulin in flow-induced nitric oxide production in endothelial cells. *Am J Physiol 266*: C628-C636.

Murata M, Peranen J, Schreiner R, Wieland F, Kurzchalia TV, Simons K 1995. VIP21/caveolin is a cholesterol-binding protein. *Proc Natl Acad Sci USA 92*: 10339-10343.

Ou J, Fontana JT, Ou Z, Jones DW, Ackerman AW, Oldham KT, Yu J, Sessa WC, Pritchard Jr KA 2004. Heat shock protein 90 and tyrosine kinase regulate eNOS NO* generation but not NO* bioactivity. *Am J Physiol Heart Circ Physiol 286*: H561-H569.

Razani B, Engelman JA, Wang XB, Schubert W, Zhang XL, Marks CB, Macaluso F, Russell RG, Li M, Pestell RG, Di Vizio D, Hou Jr H, Kneitz B, Lagaud G, Christ GJ, Edelmann W, Lisanti MP 2001. Caveolin-1 null mice are viable but show evidence of hyperproliferative and vascular abnormalities. *J Biol Chem 276*: 38121-38138.

Razani B, Woodman SE, Lisanti MP 2002. Caveolae: from cell biology to animal physiology. *Pharmacol Rev 54*: 431-467.

Shah V, Cao S, Hendrickson H, Yao J, Katusic ZS 2001. Regulation of hepatic eNOS by caveolin and calmodulin after bile duct ligation in rats. *Am J Physiol Gastrointest Liver Physiol 280*: G1209-G1216.

Shah V, Toruner M, Haddad F, Cadelina G, Papapetropoulos A, Choo K, Sessa WC, Groszmann RJ 1999a. Impaired endothelial nitric oxide synthase activity associated with enhanced caveolin binding in experimental cirrhosis in the rat. *Gastroenterology 117*: 1222-1228.

Shah V, Wiest R, Garcia-Cardena G, Cadelina G, Groszmann RJ, Sessa WC 1999b. Hsp90 regulation of endothelial nitric oxide synthase contributes to vascular control in portal hypertension. *Am J Physiol 277*: G463-G468.

Shesely EG, Maeda N, Kim HS, Desai KM, Krege JH, Laubach VE, Sherman PA, Sessa WC, Smithies O 1996. Elevated blood pressures in mice lacking endothelial nitric oxide synthase. *Proc Natl Acad Sci USA 93*: 13176-13181.

Smart EJ, Graf GA, McNiven MA, Sessa WC, Engelman JA, Scherer PE, Okamoto T, Lisanti MP 1999. Caveolins, liquid-ordered domains, and signal transduction. *Mol Cell Biol 19*: 7289-7304.

Yokomori H, Oda M, Ogi M, Sakai K, Ishii H 2002. Enhanced expression of endothelial nitric oxide synthase and caveolin-1 in human cirrhosis. *Liver 22*: 150-158.

Yokomori H, Oda M, Yoshimura K, Nomura M, Wakabayashi G, Kitajima M, Ishii H 2003. Elevated expression of caveolin-1 at protein and mRNA level in human cirrhotic liver: relation with nitric oxide. *J Gastroenterol 38*: 854-860.