Regulation of NO Synthesis in Endothelial Cells

Endothelial Nitric Oxide Synthase

Localization
The endothelial nitric oxide (NO) synthase (eNOS) is a constitutively expressed 135-kD protein predominantly associated with the particulate subcellular fraction, suggesting that the native enzyme is a membrane-bound protein [1]. Membrane association is achieved by attachment of myristic acid to the amino terminal end of the enzyme and as such is consistent with reports that eNOS cDNA contains a consensus sequence for co-translational modification of the enzyme by N-terminal myristoylation. Preventin of myristic acid incorporation in site-directed mutagenesis experiments conversed the membrane-associated eNOS to a cytosolic form but has little effect on enzyme activity. Myristoylation alone, however, provides barely enough energy to anchor a protein to a lipid bilayer, therefore other factors are important in determining the association of the enzyme with the plasma membrane, e.g., additional hydrophobic interactions owing to reversible palmitoylation of a nearby cysteine residue. A recent detailed analysis of the membrane association of eNOS has demonstrated that this enzyme is localized to specific structures in the plasmalemmal membrane identified as caveolae and that this membrane compartmentalization requires both myristoylation and palmitoylation. In cases of such a dual protein acylation the combined hydrophobic interactions of the two covalently attached lipid moieties anchor the protein firmly to the plasma membrane and attachment could be regulated by palmitate turnover. Although eNOS is reported to be reversibly palmitoylated in response to certain stimuli, the role of actively regulated eNOS palmitoylation in determining its cellular localization remains controversial.

Functional Properties
eNOS is classified as a Ca^{2+}/calmodulin-dependent enzyme and exhibits low NO output at resting levels of [Ca^{2+}]. Ca^{2+} concentration-dependently increases activity of the purified eNOS and an increase in [Ca^{2+}], such as that observed following agonist stimulation, enhances endothelial NO production while both the agonist-induced NO formation and the subsequent vasodilatation are abolished in the absence of extracellular Ca^{2+} [3]. A basal enzyme activity, which is sensitive to the NO synthase inhibitor, NO-nitro-L-arginine, is however evident event at Ca^{2+} concentrations as low as 10 nM indicating that approximately 75% of basally produced NO may be formed via a Ca^{2+}-independent pathway. Little physiological relevance was attributed to this phenomenon and the identification of a calmodulin-binding domain in the primary structure of the eNOS together with the finding that calmodulin-binding proteins inhibited enzyme activity strengthened the hypothesis that the binding of a Ca^{2+}/calmodulin complex is essential to acti-
vate the constitutive enzyme. However, recent evidence indicates that mechanical stimulation of native and cultured endothelial cells by fluid shear stress results in the formation of NO via a pathway which comprises $\text{Ca}^{2+}$-dependent as well as $\text{Ca}^{2+}$-independent components [4, 5]. In native endothelial cells, the shear stress-induced NO production consists of an initial peak followed by a sustained plateau phase which is maintained as long as shear stress is applied. The initial component of this response can be abolished by removal of $\text{Ca}^{2+}$ from the extracellular medium and coincides with an increase in $[\text{Ca}^{2+}]_i$, observed in cultured endothelial cells exposed to shear stress in a parallel plate flow chamber. Moreover, the sustained phase of NO production is insensitive to the chelation of extracellular $\text{Ca}^{2+}$.

Moreover, the sustained phase of NO production [5]. Enhancing effects of these inhibitors on $\text{Ca}^{2+}$ signalling. There are, for instance, fundamental differences in the role played by phosphotyrosine in mediating endothelial responsiveness to agonist-induced stimulation and mechanical stimulation as the tyrosine kinase inhibitor, erbstatin A, only slightly attenuates the vasodilator response to acetylcholine in perfused carotid arteries, while it abolishes the $\text{Ca}^{2+}$-independent, shear stress-induced NO production [5]. Enhancing cellular levels of phosphotyrosine by inhibiting protein tyrosine phosphatases also elicited the $\text{Ca}^{2+}$/calmodulin antagonistic-independent, erbstatin A-sensitive formation of NO [7]. Thus the endothelial response to the application of tyrosine phosphatase inhibitors exhibits characteristics identical to those of the shear stress-induced activation of eNOS. The sensitivity of the shear stress-induced activation of eNOS to tyrosine kinase inhibitors led to the suggestion that eNOS activity may be regulated by tyrosine phosphorylation. Although the tyrosine (and serine) phosphorylation of eNOS does increase transiently following the application of fluid shear stress, this appears to be a $\text{Ca}^{2+}$-dependent phenomenon and phosphorylation levels return rapidly to basal levels although the $\text{Ca}^{2+}$-independent output of NO is maintained [8]. Examination of the effects of shear stress and tyrosine phosphatase inhibitors on eNOS revealed that both stimuli result in the time-dependent decrease in the recovery of the enzyme from the Triton X-100-soluble cell fraction and a concomitant increase in its recovery in the insoluble fraction [7]. A similar change in detergent solubility was observed with three tyrosine-phosphorylated proteins, i.e., the focal adhesion kinase, the cytoskeletal-associated protein paxillin, and the caveolae marker protein caveolin. The shear stress-induced tyrosine phosphorylation and redistribution of these proteins to the detergent-insoluble fraction was attributed to activation of an integrin-coupled signal transduction pathway since both effects were abolished by interfering with the association of integrins with the extracellular matrix. Given that a change in detergent solubility is frequently indicative of the formation of a protein:protein or a protein:lipid complex, it is conceivable that shear stress elicits the formation of such a multimeric complex comprised of eNOS, tyrosine kinases and phosphatases as well as cytoskeletal and caveolar proteins. This complex, which could regulate the maintained $\text{Ca}^{2+}$-independent activation of eNOS, may also include Hsp90 as geldanamycin, which prevents the association of Hsp90 and eNOS [6], abrogates the shear stress-induced $\text{Ca}^{2+}$-independent activation of eNOS and attenuates the alteration in eNOS detergent solubility [7].

**Transcriptional Regulation of eNOS**

Although generally referred to as a constitutive enzyme eNOS can be induced or upregulated in response to a number of stimuli. Indeed, the promoter of the eNOS gene contains a number of recognition sites for transcription factors, including SP1, AP-1, AP-2 CF-1 and NF-1 as well as a putative acute phase response/shear stress, and sterol-regulatory cis elements.
Fluid shear stress plays a role in the chronic regulation of eNOS, an effect which may account for the fact that the basal release of NO is significantly greater from native endothelial cells, continuously exposed to shear, than from confluent cultured endothelial cells maintained under static conditions. A similar effect in endothelial cells in situ is thought to underlie the beneficial effects of exercise of eNOS levels. Apart from shear stress, eNOS expression can be enhanced by basic fibroblast growth factor and appears to be negatively regulated by protein kinase C. Insulin also enhances the expression of eNOS mRNA and protein in native endothelial cells via a signalling cascade involving the activation of Erk1/2, Jak2, and STAT1 as well as increased DNA-binding activity of the transcription factors AP-1 and SP1 [9]. Vascular endothelial growth factor (VEGF_{165}) also increases eNOS mRNA and protein levels. However, the intracellular pathway resulting in this increase in eNOS expression and increased basal NO production differs from that of insulin as eNOS mRNA levels remained elevated in VEGF-treated cells in the presence of actinomycin D. Thus it is likely that the VEGF-induced upregulation of eNOS expression is the consequence of a post-transcriptional effect on eNOS mRNA stability [10]. Cytokines also effect the expression of eNOS. TNF-α, for example, decreases NOS mRNA levels by increasing the rate of mRNA degradation via a process that involves de novo protein synthesis [11]. The combination of IFN-γ plus TNF-α or IL-1β has, on the other hand, been shown to paradoxically enhance eNOS activity, despite a concurrent decrease in NOS mRNA, an effect which may be accounted for by increased endogenous BH4 levels [12].

References

1 Fleming I, Busse R: Vascular effects of NO: in Goligorsky M, Gross SS (eds): Nitric Oxide and the Kidney: Physiology and Pathophysiology. New York, Chapman & Hall, 1997, pp 161–175.
2 Michel T, Feron O: Nitric oxide synthases: Which, where, how and why? J Clin Invest 1997;100:2146–2152.
3 Fleming I, Bauersachs J, Busse R: Calcium-dependent and -independent activation of the endothelial NO synthase. J Vasc Res 1997;34:165–174.
4 Kuchan MJ, Frangos JA: Role of calcium and calmodulin in flow-induced nitric oxide production in endothelial cells. Am J Physiol 1994;266:C628-C636.
5 Ayajiki K, Kindermann M, Hecker M, Fleming I, Busse R: Intracellular pH and tyrosine phosphorylation but not calcium determine shear stress-induced nitric oxide production in native endothelial cells. Circ Res 1996;78:750–758.
6 Garcia-Cadena G, Fan R, Shah V, Sorrentino R, Cirino G, Papapetropoulos A, Sessa WC: Dynamic activation of endothelial nitric oxide synthase by Hsp90. Nature 1998;292:821–824.
7 Fleming Im Bauersachs J, Fisslthaler B, Busse R: Calcium-independent activation of the endothelial nitric oxide synthase in response to tyrosine phosphatase inhibitors and fluid shear stress. Circ Res 1998;81:686–695.
8 Fleming I, Fisslthaler B, Busse R: Shear stress-induced phosphorylation of the endothelial NO synthase (abstract). Nitric Oxide 1998;2:76.
9 Benzing T, Fleming I, Bouloumié A, Mülsch A, Busse R: Insulin enhances the expression of the constitutive nitric oxide synthase in native porcine aortic endothelial cells via activation of Erk1/2 and AP-1 (abstract). Pflügers Arch 1998;435:R83.
10 Bouloumié A, Schini-Kerth V, Busse R: Vascular endothelial growth factor upregulates nitric oxide synthase expression in cultured and native endothelial cells. Cardiovasc Res 1998; (in press).
11 Yoshizumi M, Perrella MA, Burnett JC Jr, Lee M-E: Tumor necrosis factor downregulates an endothelial nitric oxide synthase mRNA by shortening its half-life. Circ Res 1993;73:205–209.
12 Rosenkranz-Weiss P, Sessa WC, Milstien S, Kaufman S, Watson CA, Pober JS: Regulation of nitric oxide synthesis by proinflammatory cytokines in human umbilical vein endothelial cells. J Clin Invest 1994;93:2236–2243.