Endothelial Nitric Oxide Synthase
Host Defense Enzyme of the Endothelium?

Ton J. Rabelink, Thomas F. Luscher

Abstract—This article explores the physiology of superoxide generation by endothelial nitric oxide synthase (eNOS), the so-called “uncoupled” state of the enzyme. The fact that this alternative chemistry of the eNOS enzyme is evolutionary strongly conserved, suggests that it may play a physiological role. It is proposed that this uncoupled state may contribute to defense against infections. As the switch from NO production to reactive oxygen species by eNOS is also the final common pathway in atherogenesis, the uncoupling of eNOS further builds on the hypothesis that atherogenesis is driven by cellular mechanisms that originally serve host defense. The central role of uncoupled eNOS in redox signaling in the endothelium may open up new avenues for therapy to prevent atherosclerosis. (Arterioscler Thromb Vasc Biol. 2006; 26:267-271.)

Key Words: endothelium ■ nitric oxide ■ atherosclerosis

The concept that endothelium-derived nitric oxide (NO) is an important molecule in prevention of (progression of) atherosclerosis has been well established. Consequently, the endothelial enzyme that produces NO, endothelial NO synthase (eNOS) (NOSIII), is considered to be a protective enzyme and loss of NO production to be dysfunctional. In the eNOS enzyme loss of NO release is usually associated with production of reactive oxygen by the enzyme. In the current article the hypothesis is explored that this change in eNOS function represents normal physiology aimed at host defense. This concept would imply that eNOS “dysfunction” in the setting of aging and atherosclerosis is basically about how cardiovascular risk factors use cellular pathways for defense against bacteria.

Endothelial Protection and Permissive Signaling

NO is a reactive molecule that can rapidly diffuse throughout the cell. It can react with sulfur groups on cysteine residues in proteins, particularly when these cysteines are present in an acid–cysteine–base motif. This results in reversible S-nitrosylation of these proteins and in the presence of reducing enzymes, such as GSNO-reductase, may also lead to disulphide bridges between cysteine residues in proteins.1,2 In addition, because of its unpaired electron it can associate with transition metals such as heme leading to nitrosyl modification.3 The rapid access to different parts of the endothelial cell and the reactivity with a wide range of biological molecules makes NO very suitable for a role in coordination and synchronization of cellular processes via posttranslational modification of vital proteins. As was proposed in a seminal article by Carl Nathan redox reactive molecules may play a role in integrating signaling events and determine the duration and strength of signaling.4 Interestingly, eNOS is not a prerequisite for life and genetic deletion of eNOS appears to have little effect on basal vasodilation or basal leukocyte adhesion to the endothelium, supporting a more permissive role for NO under basal conditions.5,6 If one reviews the most important cellular effects of NO they seem to be involved in conservation of metabolism. Probably one important function of NO in the (endothelial) cell is its competition with oxygen as an electron acceptor at complex IV in oxidative phosphorylation.7 This means that with diminishing oxygen availability NO will predominate as an electron acceptor and reduce oxidative phosphorylation thus limiting energy expenditure. NO also participates in defense against hypoxia by increasing hypoxia-inducible factor-1α (HIF-1α) binding activity and HIF-1α protein levels.8 In contrast S-nitrosylation of inflammatory transcription factors including NF-κB, AP1, and DNA methyltransferases generally leads to reduced transcriptional activity.9 In fact also the vasodilator effects of NO serve a state of metabolic preservation by ensuring energy substrate delivery. By S-nitrosylation of HDM2, the protein that regulates p53 degradation, cell cycle arrest will be induced, again inducing a state of quiescence (Figure).

Like for NO, reactive oxygen species may also serve permissive signaling. In the intracellular milieu, where superoxide dismutase is present, the predominant reactive oxygen species will be H2O2.9 H2O2 can also react with cysteine residues leading to formation of sulfenic acid (SOH) and...
disulfide modifications in proteins. This reaction typically would occur in proteins with low pKa cysteine residues. Like NO, H$_2$O$_2$ can also rapidly diffuse throughout the cell. However, the pattern of cellular events that emerges is different from that induced by NO; H$_2$O$_2$ results in phosphorylation of transcription factors such as NF-κB, AP1, and CREB1, it induces chromatin remodeling in the nucleus, thus allowing transactivation of genes by these transcription factors, and it will activate proteases. Besides initiating a coordinated proinflammatory transcriptional response in the endothelium, NF-κB activation also protects the endothelium against cytokine- or infection-induced apoptosis by controlling the intracellular levels and localization of members of the antiapoptotic Bcl-2 protein family. In other words, signaling by reactive oxygen species seems to serve activation of host defense.

A very interesting feature of the NOS enzymes is that they not only generate NO but may also produce reactive oxygen species themselves.

**The Catalytic Conundrum**

This capacity to release reactive oxygen species is related to the basic chemistry that occurs at the enzyme. Basically, eNOS, as well as the other NOS isoforms, are NADPH-consuming enzymes. As a consequence there will be electrons flowing in the enzyme from the reductase domain toward the heme containing oxygenase domain. In the eNOS enzyme, the cofactor calmodulin is an important checkpoint in this chemistry as it is required for shuttling of electrons toward the heme group. In the presence of oxygen a very reactive superoxy ferrous–peroxy ferric complex is formed. The eNOS cofactor tetrahydrobiopterin (BH4), another important checkpoint in the chemistry, facilitates the reaction of electrons and oxygen with L-arginine leading to the formation of L-citrulline and NO. However, if either BH4 or the substrate L-arginine are lacking, the superoxy ferrous–peroxy ferric complex may dissociate to yield superoxide (if BH4 is lacking) or H$_2$O$_2$ (if L-arginine is lacking). This latter state is referred to as the so-called “uncoupled” state of eNOS. This uncoupled state has been associated with risk factors for atherosclerosis and consequently has been regarded as an abnormality of eNOS function. For example, models of diabetes, hypertension, and atherosclerosis have been associated with reduced tissue BH4 levels, increased superoxide generation, and impaired endothelial function.

As explained before, uncoupling of eNOS implies that the endothelial cell switches from a quiescent state (NO) into a state that is adapted for host defense (H$_2$O$_2$). In this respect generation of reactive oxygen species by uncoupled eNOS could be regarded as physiological signaling during injury and infection, and in fact could even be an essential effector mechanism in the host defense response. If one considers that the NO synthases are highly conserved in evolution, and the same BH4-dependent catalytic chemistry is present in plants, fish, insects, and mammals, eNOS uncoupling may represent a widely spread physiological mechanism. The knock-out animal experiments have taught us that eNOS-derived NO does not seem to be critical for normal cell physiology; one can raise, however, the question whether uncoupling of eNOS is an essential physiological mechanism in the setting of host defense.

**Uncoupling and Host Defense**

A model to look at host defense testing both innate immunity as well as adaptive immune responses is organ transplantation. Instead of bacterial antigens, innate immune cells such as monocytes and dendritic cells in the adventitia present allo-antigens that with proper costimulation give a T cell and B cell response leading to rejection of the transplanted organ. In such a MHC class mismatched allograft model we found that increasing BH4 availability by administering the precursor compound sepiapterin leads to reduction of reactive oxygen species and nitrotyrosine while NO production by the allograft increases at the same time. This strongly suggests that increased coupling of NO synthases had occurred. This coupling was associated with a profound immune suppressive effect. Macrophage influx in the transplanted organ went down 60% despite the fact that no immune suppressive drugs were given. These observations could not be explained by a nonspecific antioxidant effect of BH4, as sepiapterin had no effect on superoxide production and inflammation in transplantation of isografts, whereas ischemia-reperfusion injury was present but no antigen presentation takes place. It thus appeared that uncoupling of NOS is an important proinflammatory mechanism in the immune response to allografts.

As vascular inflammation involves endothelial cell activation as well as concomitant influx of leukocytes, both uncoupling of eNOS as well as iNOS induction in leukocytes could modulate this immune response. The role of iNOS induction in host defense is well recognized. Recently, however, it has been suggested that iNOS induction may in fact be secondary to eNOS uncoupling. For example, during endotoxemia a marked reduction in inducible NO synthase (iNOS) protein was observed in the heart of eNOS knock-out mice compared with wild type, indicating that for iNOS induction initial eNOS activation is required. Although the article did not directly address whether such activation of eNOS implied uncoupling and subsequent redox signaling, this is a plausible scenario as iNOS induction is dependent on redox signaling.

If uncoupling of eNOS is involved in host defense one would also expect increased susceptibility to infection in the absence of the eNOS enzyme. This issue had not extensively been addressed, but a recent study on colitis in mice indeed demonstrated increased bacterial invasion of the colon in eNOS knock-out mice.

Another consequence of this hypothesis is that the eNOS enzyme should have a Janus face, which allows the enzyme to switch from the coupled to the uncoupled mode when it encounters infectious agents—from cardiovascular homeostasis to host defense. Indeed, activation and endothelial deposition of the complement cascade, a first-line bacterial defense component of the innate immune system, causes redox signaling in the endothelial cell. Also, phagocytic cells take up bacteria through toll-like receptors or Fcy receptors and activate NADPH oxidase. Such activation of NADPH oxidases in phagocytes has been shown to induce...
downstream redox signaling in endothelial cells when these phagocytes adhere to endothelium. Many cell types including endothelial cells, furthermore, produce low levels of $\text{H}_2\text{O}_2$ in response to cytokines (transforming growth factor [TGF]-$\beta$, tumor necrosis factor [TNF]-$\alpha$, and interleukin [IL]), peptide growth factors (PDGF; EGF, VEGF, bFGF), and agonists of G protein–coupled receptors (eg, angiotensin II, thrombin, lysophosphatidic acid, sphingosine 1-phosphate, histamine, and bradykinin) involved in inflammation. An important feature of BH4 is that it is very susceptible to oxidation and in fact can autooxidize leading to accelerated degradation of BH4 under conditions of oxidative stress and redox signaling. In this way activation of the innate immune system will also induce uncoupling of eNOS and subsequent proinflammatory transcription in the endothelial cell (Figure).

Cytokines released by phagocytes will not only reduce BH4 availability. They can also induce arginase in the endothelium and shuttle L-arginine metabolism toward urea production. This may reduce L-arginine availability for the eNOS enzyme and further contribute to eNOS uncoupling. Whereas binding of ADMA itself to the enzyme probably decreases the accessibility of O$_2$ to bind to the heme, thus basically shutting down the enzyme, other methylated arginines such as L-NMMA can induce uncoupling of NOS.

**Atherosclerosis: Hijacking the Host Defense System**

The concept that uncoupling of eNOS is part of the normal physiology of endothelial cell activation also implies that aging and atherogenesis may be the price we pay for an efficient host defense system. Indeed, molecular pathways activated in these conditions may have originally evolved for defense against bacteria, at times when this was the primary threat and long-living organisms were unforeseen. It is quite interesting that one can indeed explain many of the effects of cardiovascular risk factors through this mechanism. For example, cytokines released from adipocytes in the metabolic syndrome constitute an important cardiovascular risk. This is reflected in the poor cardiovascular outcome that is associated with elevated levels of the acute phase reactant C-reactive protein (CRP). Such inflammatory cytokines (TNF-$\alpha$, IL-6) and possibly also CRP itself can directly activate NADPH oxidases in endothelial cells. The ensuing oxidative signaling would then lead to uncoupling of eNOS. Also, metabolic risk factors may primarily lead to redox signaling in the endothelial cell. Increased glucose and free fatty acid uptake by endothelial cells in insulin resistance and diabetes will cause overload of the mitochondria with substrate for oxidative phosphorylation resulting in incomplete reduction of O$_2$ and production of $\text{H}_2\text{O}_2$. Again the net result would be uncoupling of eNOS, and in agreement uncoupling of eNOS has been demonstrated in diabetes models. Also hypercholesterolemia, and in particular oxidized lipoproteins, can directly through the LOX-1 receptor activate NADPH oxidases in the endothelial cell and lead to uncoupled eNOS.

**What Are the Implications?**

Rather than blocking one risk factor or one pathophysiological mechanism and be confronted with therapy failure attributable to redundancy in risk factors and atherogenic mechanisms, this concept suggests that we should rather aim at the final common pathways of aging and atherogenesis. One of the main paradigm shifts in treatment of cardiovascular disease will be to prevent patients with cardiovascular risk factors from becoming high-risk patients. The endothe-
lrium is a central transducer by which risk factors can mediate progression to clinically overt cardiovascular disease, and uncoupled eNOS could be the critical checkpoint here that leads to endothelial cell activation.

With this concept in mind administration of L-arginine has been explored in conditions such as hypercholesterolemia, diabetes, and coronary artery disease. Whereas in acute studies generally an increase in NO bioavailability was shown,39,40 chronic L-arginine administration could not demonstrate a benefit.41 It was postulated by Loscalzo that possible beneficial effects of chronic L-arginine administration may be offset by simultaneous increments in S-adenosyl-L-homocysteine as a side product of the L-arginine associated creatinine production.42 Vascular increments in S-adenosyl-L-homocysteine may lead to accumulation of homocysteine and ADMA and reduce NO bioavailability at the same time. An alternative strategy to maintain an NO-dominated state in the endothelial cell in the presence of risk factors could be the administration of BH4 or its precursor protein sepiapterin. This has proven to be a very effective strategy in animal models of cardiovascular disease.43 Also in humans acute administration of tetrahydrobiopterin could restore NO activity in hypercholesterolemia,16 diabetes,44 and coronary artery disease.45 Unfortunately no studies on chronic administration of biopterins on endothelial function or atherosclerosis progression have been published to date. This may be a particularly interesting option as chronic oral treatment with BH4 has been used successfully for treatment of patients with hyperphenylalaninemia for more than 10 years with no side-effects reported.46

There is irony in the idea that we may have to pay the price of atherosclerosis for an enzyme system that so beautifully serves adaptation to metabolic as well as immunologic demands. The appreciation of this irony may, however, help discover new therapeutic avenues in cardiovascular disease.

References


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