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Endothelium-derived nitric oxide and vascular physiology and pathology

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Abstract. In 1980, Furchgott and Zawadzki demonstrated that the relaxation of vascular smooth muscle cells in response to acetylcholine is dependent on the anatomical integrity of the endothelium. Endothelium-derived relaxing factor was identified 7 years later as the free radical gas nitric oxide (NO). In endothelium, the amino acid L-arginine is converted to L-citrulline and NO by one of the three NO synthases, the endothelial isoform (eNOS). Shear stress and cell proliferation appear to be, quantitatively, the two major regulatory factors of eNOS gene expression. However, eNOS seems to be mainly regulated by modulation of its activity. Stimulation of specific receptors to various agonists (e.g., bradykinin, serotonin, adenosine, ADP/ATP, histamine, thrombin) increases eNOS enzymatic activity at least in part through an increase in intracellular free Ca^{2+}. However, the mechanical stimulus shear stress appears again to be the major stimulus of eNOS activity, although the precise mechanisms activating the enzyme remain to be elucidated. Phosphorylation and subcellular translocation (from plasmalemmal caveolae to the cytoskeleton or cytosol) are probably involved in these regulations. Although eNOS plays a major vasodilatory role in the control of vasomotion, it has not so far been demonstrated that a defect in endothelial NO production could be responsible for high blood pressure in humans. In contrast, a defect in endothelium-dependent vasodilation is known to be promoted by several risk factors (e.g., smoking, diabetes, hypercholesterolemia) and is also the consequence of atheroma (fatty streak infiltration of the neointima). Several mechanisms probably contribute to this decrease in NO bioavailability. Finally, a defect in NO generation contributes to the pathophysiology of pulmonary hypertension. Elucidation of the mechanisms of eNOS enzyme activity and NO bioavailability will contribute to our understanding the physiology of vasomotion and the pathophysiology of endothelial dysfunction, and could provide insights for new therapies, particularly in hypertension and atherosclerosis.

Key words. Nitric oxide; nitric oxide synthase; endothelium; hypertension; atherosclerosis.

Introduction

Endothelium is a cell monolayer which constitutes the internal structure of the entire circulatory system. It has long been considered as a 'cling film' which at most prevents coagulation. In 1980, Furchgott and Zawadzki [1] demontrated that the relaxation of vascular smooth muscle cells in response to acetylcholine is dependent on the anatomical integrity of the endothelium. They named the factor responsible for this intercellular relationship endothelium-derived relaxing factor (EDRF). In the late 1980s, EDRF was recognized as the free radical gas nitric oxide (NO) [2, 3].

In addition to NO, endothelium generates several other mediators involved in the regulation of vasomotion. Certain arachidonic acid derivatives, in particular prostacyclin, elicit, like NO, relaxation of the underlying smooth muscle cells and the inhibition of platelet aggregation. Endothelium also generates an as yet uncharacterized vasodilative factor, the endothelium-derived hyperpolarizing factor (EDHF). The respective

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importance of NO, prostacyclin and EDHF varies among species, vascular beds, and the physiological or pharmacological stimuli used [4]. Endothelium can also, particularly in pathological circumstances, generate vasoconstrictive factors such as endothelin.

Endothelium is an anatomical barrier between blood and interstitium at the capillary level, but also throughout the vascular tree. Endothelium can express cell adhesive molecules which drive the adhesion and subsequent trans-endothelial migration of leukocytes into the intima. This physiological process is involved, for instance, in clearance of low-density lipoprotein (LDL) excess in the arterial wall [5]. LDLs penetrate the intima to provide cholesterol to smooth muscle cells. An abnormality in the regulation of the monocyte/macrophage traffic leads to fatty streak lesion, the first step in atherosclerosis. More generally, the endothelium controls the inter- and transcellular traffic of numerous nutrients, hormones, and cells, although these functions have been poorly studied so far, because of the absence of adequate tools.

Finally, endothelium prevents exposure of the thrombogenic sub-endothelium to the circulating factors of coagulation. The inhibition of platelet aggregation by NO and prostacyclin represents another aspect of the antithrombotic properties of endothelium.

Endothelial NO synthase

Biochemical aspects

In mammalian tissues, NO is derived from the amino acid L-arginine [2, 3]. The conversion of L-arginine to L-citrulline and NO is catalyzed by a family of enzymes termed NO synthases (NOSs), which are complicated proteins [6]. They require several cofactors for the unusual five-electron oxidation of one nitrogen of the L-arginine guanidino group. These mechanisms are detailed by Boucher et al. in this issue. This family of enzymes comprises three members: neuronal NOS, cytokine-inducible NOS and endothelial NOS (eNOS). The major characteristics of these three NOSs are summarized in table 1 and described in more detail in other contributions to this issue. This review will be devoted mainly to eNOS.

Regulation of eNOS gene expression

The initial nomenclature opposed 'inducible' NOS, expressed upon immuno-inflammatory stimuli, to 'constitutive' NOSs, which are constitutively expressed in certain characteristic cell types (neuronal, endothelial). However, it is now known that the level of gene expression of both eNOS and neuronal NOS may also be induced under different physiological conditions (in particular hemodynamic shear stress for eNOS). Thus, the now widely accepted nomenclature identifies the three mammalian enzyme isoforms as neuronal NOS, inducible NOS, and eNOS, reflecting the tissues of origin for the original protein and cDNA isolates [7, 8]. eNOS was originally purified and cloned from vascular endothelium, but its expression has been reported in cardiomyocytes, blood platelets, and hippocampus neurons.

The promotor of human eNOS has a TATA box at nucleotide -1964 with reference to the transcription

Table 1. The three isoforms of NOS.

Specificity	Endothelial (III)	Neuronal (I)	Inducible (II)
Molecular mass	135 kD	155 kD	130 kD
Function of NO	endothelium-derived relaxing factor; antiaggregant	neurotransmitter; neuromodulator; relaxation of smooth muscle	non-specific; host defense
Cofactor Ca^{2+}/calmodulin	dependent	dependent	independent
Other cofactors: NADPH, FAD, FMN, tetrahydrobiopterin	dependent	dependent	dependent
Stimuli	acetylcholine; bradykinin; serotonin; ATP; shear stress	neuro-excitatory amino acids	?
Mechanisms of regulation (+activation, − inhibition)	+Ca^{2+}/calmodulin protein interaction (Cav-1, +HSP 90) + dimerization ? phosphorylation	+Ca^{2+}/calmodulin protein interaction + dimerization ? phosphorylation	+ dimerization phosphorylation
Regulation of gene expression (+activation − inhibition)	+shear stress +proliferation −tumor necrosis factor-α (TNF-α) ? estrogens	+ estrogens	+interleukin-1, +interferon-γ, +TNF α −transforming growth factor β +AMPc, GMPc +NFκB −NO −glucocorticoids
Mechanisms	transcription; mRNA stability	transcription	transcription; mRNA stability

site, probably too far upstream to anchor the assembly of the preinitiation complex. The promotor instead utilizes its GC-rich Sp1 motif as do certain other constitutively expressed genes.

Concensus sites for regulation by activator protein-1, activator protein-2, nuclear factor-1, acute-phase reactants, and shear stress are consistent with evidence that levels of eNOS transcripts can be elevated by shear stress [9], exercise [10], and hypoxia. Regulation by estrogens is still a matter of debate [11, 12]. Cell growth increases eNOS mRNA and protein abundance [13]. Tumor necrosis factor-α decreases eNOS gene expression through a decrease in mRNA stability [14]. Shear stress and endothelial cell proliferation appear to represent, quantitatively, two of the major regulatory factors of eNOS gene expression, through transcriptional and post-transcriptional (increase in mRNA stability) control, respectively.

eNOS: subcellular location and regulation of activity

It is generally accepted that, in response to the stimulation of specific receptors to various agonists (e.g., acetycholine, bradykinin, serotonin, adenosine, ADP/ATP, histamine, thrombin), the increase in intracellular free Ca^{2+} is one of the major mechanisms activating eNOS. Calcium ionophores are thus potent pharmacological activators of eNOS. Relaxation of the underlying vascular smooth muscle is mediated through activation of the soluble guanylate cyclase by NO diffusing from the endothelium.

NO is a labile molecule and may have important biological roles both within the cell in which it is synthesized, and also in interactions with surrounding cells. Since NO may be either stabilized or degraded through its interactions with diverse intracellular or extracellular chemical moieties, the localization of eNOS within the cell might be expected to influence the biological role of the NO produced [8].

Although eNOS does not contain any hydrophobic transmembrane domain, it is targeted to the particulate subcellular fraction, and more precisely to plasmalemmal caveolae. Caveolae are small invaginations in the plasma membrane characterized by the presence of the transmembrane protein caveolin [15]. In many tissues, caveolae may serve as sites for sequestering signaling molecules such as receptors (including muscarinic M2 and bradykinin B2 receptors), G proteins, as well as eNOS. The presence of these receptors within the caveolae may facilitate the activation of eNOS by establishing local caveolar domains in which NOS-coupled signaling molecules are in propinquity. The targeting of eNOS to plasmalemmal caveolae might also influence the local concentration of the substrates and cofactors of the enzyme, and thereby confound the interpretation of enzymological studies analyzed in cell-free systems [16].

Phosphorylation represents an important mechanism for the post-translational regulation of various cellular proteins. Phosphorylation of eNOS on serine residues has been reported in endothelial cells in response to bradykinin [17] and to hemodynamic shear stress [18]. Phosphorylation of eNOS on tyrosine residues is more controversial. In any case, the biological consequences of eNOS phosphorylation are unclear. eNOS phosphorylation could be involved in intracellular eNOS translocation that follows agonist-induced activation [8].

eNOS is unique among the NOS isoforms in being dually acylated by the saturated fatty acids myristate and palmitate. These co- and post-translational modifications play a key role in the anchorage of eNOS in the plasma membrane (caveolae). Whereas myristoylation is irreversible, palmitoylation is reversible. Depalmitoylation, which occurs after agonist-induced activation of eNOS, probably contributes to the dissociation of the enzymes from the caveolae [8].

eNOS interacts directly with caveolin-1 in endothelial cells, leading to an inhibitory effect under basal conditions. The inhibitory effect of caveolin-1 on eNOS activity can be completely reversed by Ca^{2+}/calmodulin, a complex elicited after the stimulation of endothelial cells by several agonists [19]. The activated eNOS-calmodulin complex synthesizes NO until the intracellular free Ca^{2+} concentration decreases to the point where the calmodulin dissociates and the inhibitory eNOS-caveolin-1 complex reforms. These mechanisms probably account for the short-term effect of shear stress, but not for the sustained release of NO in response to sustained shear stress. In this latter case, the targeting or association of eNOS with the cytoskeleton could play a prominent role in the sustained activation of eNOS [20].

NO and vascular physiology

Endothelial control of vasomotion

As mentioned in the Introduction, Furchgott et Zawadzki [1] first demontrated that endothelium reversed the intrinsic constrictive effects of acetylcholine on the vascular smooth muscle though the release of EDRF, subsequently identified as NO (and thus often named EDRF/NO). Numerous latter experimental and clinical studies confirmed that all the arterial endothelium, and to a lesser extent vein endothelium, releases EDRF/NO. Several pathophysiological states (see below) induce endothelial dysfunction, i.e., a decrease in endothelium-dependent vasodilation [21]. When this endothelial dysfunction is severe, leading to the abolition of EDRF release, the agonists which normally induce an endothelium-dependent vasodilation induce a vasoconstriction

through the direct activation of smooth muscle receptors. Indeed, the increase in intracellular free Ca^{2+} in smooth muscle cells elicits their contraction. When the endothelium is normal, this latter effect is counteracted by the potent vasorelaxing effect of EDRF/NO. This is true for numerous agonists, such as acetycholine, bradykinin, serotonin, adenosine, ADP/ATP, histamine, and thrombin. Even angiotensin II stimulates NO release from macrovascular endothelium, which may modulate the vasoconstrictor effect of angiotensin II on smooth muscle cells [22]. However, this beneficial effect may be counteracted by the simultaneous production of peroxynitrite ($ONOO^-$), which could contribute to several pathological processes in the vascular wall [22].

In fact, EDRF/NO is not the sole endothelium-derived vasodilator. Endothelium generates prostacyclin (PGI2), which relaxes the underlying vascular smooth muscle though activation of adenylate cyclase and subsequent generation of cyclic AMP. Endothelium also generates a hyperpolarizing factor (EDHF), which could be a product of cytochrome P450. The importance of EDRF/NO in vasodilation varies between vascular beds and among animal species. Finally, under some pathophysiological circumstances (e.g., hypoxia-anoxia, high blood pressure), endothelium-derived vasoconstrictive factors can be released and contribute to a paradoxical vasoconstrictive effect. Certain prostaglandins (in particular PGH2), superoxide anion (O_2^-) and endothelin are the best characterized endothelium-derived contracting factors (EDCFs) [23].

At all times, vascular tone is influenced by the constrictive and relaxing factors released by endothelium. Chemical modifications of the guanidino group of L-arginine result in compounds which inhibit NO synthase. N^G-monomethyl-L-arginine (L-NMMA), N^G-nitro-L-arginine (L-NA), and N^G-nitro-L-arginine methyl ester (L-NAME) inhibit the release of NO from endothelial cells and aortic rings, indicating that there is continuous release of NO, which maintains vasodilator tone in this tissue. In vivo, short-term administration of L-NMMA or L-NA induces an increase in blood pressure of about 30 mm Hg in rats and rabbits [24]. Thus, under normal conditions, relaxing factors (and in particular EDRF/NO) predominate over constrictive factors.

Why is hemodynamic shear stress, i.e., friction of blood flow on the endothelium, the main stimulus of NOS activity? As the metabolic activity of a tissue increases, the local metabolic changes (e.g., decrease in PO_2, increase in PCO_2, decrease in pH) promote the relaxation of precapillary sphincters, the first mechanism causing an increase in capillary blood flow. Blood flow also increases in the upstream arterioles and arteries. This increased blood flow is accompanied by an increase in endothelial shear stress, which in turn enhances endothelial NO synthesis and release, and also prostacyclin synthesis and release. These factors diffuse locally and relax the underlying smooth muscle cells. This flow-dependent vasodilation occurs in arteries and veins and allows the adaptation of the diameter of the vessel to the flow. Flow-dependent vasodilation (proximal), combined with metabolic vasodilation (distal), allows a perfect adaptation between tissue O_2 consumption and tissue O_2 supply.

In addition to regulating eNOS activity, blood flow has been recognized as an important stimulus of eNOS gene expression both in vitro and in vivo. Repeated exercise increases eNOS gene expression [10]. This increase in NO production could represent one of the mechanisms by which training allows an increase in aerobic capacity though an improvement of blood flow supply to working skeletal muscles.

Effect of NO on platelet and leukocyte functions

Endothelium plays an essential role in the control of platelet aggregation through the release of NO and PGI2 [25]. Platelets also synthesize NO when activated by collagen, arachidonic acid, ADP, or thrombin because they express eNOS. cGMP production and the decrease in intracellular calcium induced by the autocrine generation of NO represent an inhibitory feedback mechanism of aggregation, in addition to the antiaggregant effect of paracrine endothelial NO.

Endothelium-derived NO inhibits leukocyte adhesion through inhibiting the expression of adhesive molecules. Alteration of the physiological release of NO by endothelium would favor the adhesion of monocytes to endothelium, and the recruitment and infiltration of the neointima leading to fatty streak deposits. Finally, NO also inhibits smooth muscle cell proliferation.

Thus, endothelium-derived NO is a pluripotent molecule controlling not only vasomotion, but also protecting the intima from platelet aggregation and infiltration of the intima by foam cells and smooth muscle cells.

NO and vascular pathology

NO has in fact been used in cardiovascular therapy for more than 100 years. Indeed, nitroglycerin acts through the release of NO. The last decade revealed that NO plays a key role in homeostasis of the circulation, and that a decrease in NO production and/or bioactivity contributes to the pathophysiology of several major diseases of the cardiovascular system.

NO and systemic high blood pressure

Vascular control and the control of blood pressure are under a myriad of influences. It was therefore reasonable to hypothesize that inhibition of NO release might have only a minor effect on systemic blood pressure. Over the past few years, the effects of chronic inhibition of NOS on blood pressure regulation has been extensively studied. Chronic blockade of NOS with L-NAME in Wistar rats induces a time- and dose-dependent increase in blood pressure [26]. Aortic cGMP decreases in reciprocal fashion with increasing L-NAME doses from 0 to 10 mg/kg per day, culminating in a ten-fold drop in arterial wall cGMP [26]. It is noteworthy that the plasma levels of cGMP do not change significantly despite the dramatic decrease in arterial wall cGMP, and correlate with plasma atrial natriuretic factor levels [26]. Thus, chronic blockade of NOS with L-NAME induces a dose-dependent increase in blood pressure and a recriprocal decrease in aortic cGMP, which is mainly dependent on NOS soluble guanylate cyclase activity. The inhibition of endothelial-derived NO promotes early monocyte infiltration of the arterial wall [27]. Thus, any condition leading to a decrease in endothelium-derived NO generation could, through the inflammatory-elicited expression of inducible NOS, lead to a compensatory production of NO.

Using a similar model of L-NAME-induced hypertension, Bayliss et al. [28] have shown that when L-NAME treatment was extended beyond 8 weeks, all animals developed lesions of nephroangiosclerosis and a concomitant increase in plasma renin activity. These renal alterations probably participate to further increase the level of hypertension. Sixteen weeks of L-NAME treatment produces nearly 100% mortality, demonstrating the severity of this novel model of hypertension. Another surprising feature of this hypertension is the development of specific neurological lesions. About two-thirds of the rats with L-NAME-induced hypertension developed asymmetric paralysis predominating on the anterior limbs. Histological examination in all cases revealed infarction of the spinal cord, and infrequent cerebral infarctions [29].

In summary, it is clear that blockade of the arginine NO system can produce severe hypertension in the rat (fig. 1). However, L-NAME administration to mice also induces hypertension, although this is delayed and less severe [30]. In contrast, chronic NO blockade in most other species such as rabbit, dog, or guinea pig does not significantly increase blood pressure, whereas acute L-NAME increases blood pressure in all species. The recruitment of compensatory mechanisms such as the induction of cyclooxygenase-2 in these animal species probably helps to normalize the blood pressure level.

Hypertension in the spontaneously hypertensive rat (SHR) occurs independently of sodium intake. Acute

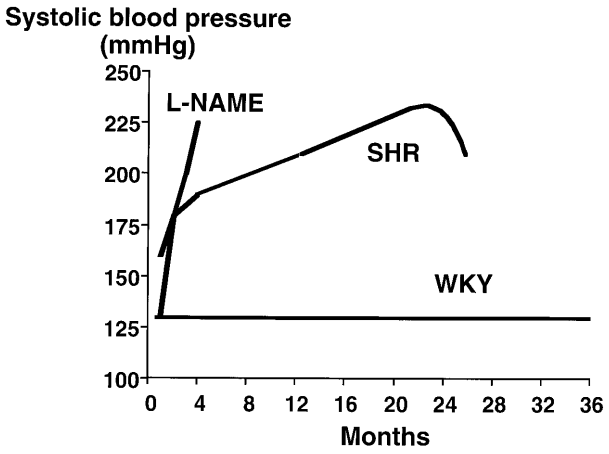

Figure 1. Evolution of systolic blood pressure in normotensive Wistar-Kyoto rats (WKY), spontaneously hypertensive rats (SHR) and L-NAME-treated rats (chronic blockade of NO production), with survival rates averaging 36, 24 and 3 months, respectively.

studies in which inhibitors of NOS were administered intravenously have suggested that NO production is preserved in SHRs [31]. We evaluated the importance of NO release in SHRs by studying the effect of chronic blockade of NOS for 2 weeks [32]. L-NAME induced a time-dependent increase in blood pressure in the SHR. This aggravated hypertension was accompanied by a very high mortality rate (55% at the end of the 2 weeks). In addition, the increase in blood pressure in response to L-NAME in SHRs was at least as great as that observed in Wistar-Kyoto rats. These studies suggest that basal NO release is not impaired in SHRs, but represents a major counter-regulatory mechanism in this genetic model of arterial hypertension.

Two other well-characterized models of hypertension are the desoxycorticosterone acetate (DOCA)/salt model, which involves unilateral nephrectomy, administration of DOCA, and high sodium intake (0.9% NaCl in the drinking water), and the two-kidney/one clip model (renovascular hypertension). However, no deficit in NO production has been documented in either model.

Thus, in general, there is no evidence for an alteration in the endothelial NO pathway as a primary cause of hypertension in experimental animals. The only exception is the salt-sensitive hypertension of the Dahl-Rapp rat. In this model, Chen and Sanders [33] provided evidence that hypertension was completely prevented by the administration of L-arginine but not D-arginine. L-Arginine also prevents the renal failure and death from hypertensive nephrosclerosis which commonly occur in these animals [34]. Increases in dietary sodium

chloride increase NO production (estimated from urinary nitrates) in the salt-resistant Dahl-Rapp rat and the Sprague-Dawley rat, but not in the salt-sensitive rat, unless it is provided with arginine. Dexamethasone completely prevented the increase in excretion of cyclic GMP and nitrate, and hypertension resulted despite concomitant treatment with L-arginine. Recent work suggests that a defect in eNOS (which was recently shown to be suppressible by glucocorticoids) in the kidney could be responsible for this hypertension (J. Pollock, personal communication).

As discussed above, most animal models have failed to demonstrate an abnormality in NO production as the underlying cause of hypertension. The possibility remains, however, that such a defect exists in at least some subsets of human hypertension. It has not yet been possible to examine these issues at a molecular level, although the role of EDRF-NO has been studied in essential hypertension in humans by comparing either brachial or coronary artery vasodilator responses to acetylcholine and nitroprusside [35–37]. In most studies, the responses to acetylcholine were decreased while the response to endothelium-independent vasodilators was unaltered. However, the endothelium-dependent vasodilation in response to increased blood flow, a more physiological stimulus, is usually normal. Thus, a defect in NO production in essential hypertension in humans remains to be clarified.

NO and atherosclerosis

Several animal models have already provided insights into the pathophysiology of atheroma. Rabbits fed a cholesterol-rich diet constitute a useful model of atheroma, developing fatty streak lesions within a few weeks as well as progressive endothelial dysfunction [38]. In this model, the blockade of NO production increases fatty streak deposits [39]. However, in apolipoprotein-E-deficient mice, another model of atherosclerosis, the inhibition of NOS does not alter the extent of atheroma [40]. Thus, the role of NO in the early stages of atheroma remains to be clarified.

Several clinical studies have demonstrated an abnormality of endothelium-dependent vasodilation in atherosclerosis [41]. Thus, the vasodilation of atheromatous coronary arteries in response to acetylcholine is impaired compared to that of control normal coronary arteries, whereas nitroglycerin vasodilates both groups in a similar fashion [21]. Interestingly, an impairment of acetylcholine-induced vasodilation is observed even in the presence of minimal coronary artery lesions, as well as in response to physiological stimuli such as increased blood flow. The mechanisms of endothelial dysfunction can be summarized as follows.

Alteration of NOS substrate availability. During the past few years, there has been some interest in manipulating the in vivo activity of NOS by administering L-arginine. From a biochemical point of view, it seems unlikely that arginine administration could increase the activity of the endothelial cell enzyme. The Michaelis constant (K_m), the substrate concentration at which the reaction velocity is half maximal, is 2.9 μM [42] whereas the intracellular level of L-arginine is several hundred times this value both in vivo (0.8–2 mM) and in cultured cells (0.1–0.8 mM). Under these conditions, it is very unlikely that the cellular concentration of the substrate could be a rate-limiting factor. Furthermore, endothelial cells recycle L-citrulline to L-arginine, putatively helping to maintain sufficient levels of L-arginine during periods of prolonged EDRF release [43].

Studies of endothelial cells in culture and of both animals and humans in vivo suggest, however, that L-arginine can influence NO production. L-Arginine partially reverses the impairment of endothelium-dependent vasodilation in response to acetylcholine in hypercholesterolemic patients and animal models [44, 45].

This unexpected response to arginine in what seems to be a large intracellular excess has been termed the arginine paradox [46]. However, we do not know (i) the true K_m of the eNOS in the cell, or (ii) the arginine concentration in the vicinity of the enzyme. Indeed, arginine is actively transported into the endothelium by the cationic amino acid transporter $Y+$, which is located in microdomains of the plasma membrane, the caveolae, i.e., in the same place as eNOS itself [16]. One can hypothesize that the arginine uptake velocity in this sub-cellular compartment could be crucial to substrate availability. The existence of endogenous inhibitor of NOS during hypercholesterolemia could account for the beneficial effect of arginine supplementation in this pathophysiological state [41]. Finally, another paradox is that endothelium is able to recycle arginine from citrulline, thereby leading to the independence of NO production from extracellular arginine upon certain stimuli, such as a calcium ionophore, but not others, such as acetylcholine or bradykinin [47].

Alteration of eNOS gene expression. Another factor which could be responsible for the alteration of endothelial NO production during atherosclerosis could be a decrease in eNOS gene expression, which can be altered by several factors [48]. eNOS gene expression is increased in response to shear stress, in vitro as well as in vivo. This hemodynamic factor plays a key role in the pathophysiology of atheroma. Indeed, atheroma preferentially occurs in areas where the level of shear stress is low and/or turbulent (e.g., carotid sinus, branches).

Conversely, exposure of cultured endothelial cells to pro-inflammatory cytokines such as tumor necrosis fac-

tor-α [14] (the expression of which is increased in fatty streak lesions) or to high concentrations of oxidized LDL decreases eNOS gene expression. These factors could account for the decrease in eNOS gene expression reported in advanced atherosclerosis [49]. However, in most cases, the expression of eNOS appears to be preserved.

Alteration in the signaling pathway. In the aorta of hypercholesterolemic rabbit, the endothelium-dependent vasodilation in response to a calcium ionophore (which activates eNOS through an increase in intracellular free calcium) is less altered than vasodilation in response to receptor-mediated agonists (such as acetylcholine) [50–52]. Interestingly, similar alterations have been reported in other models of endothelial dysfunction, such as that induced by pro-inflammatory cytokines [53] or that associated with neointimal hyperplasia [54]. The continuous generation of NO induced by pro-inflammatory mediators seems to play a major role in the inhibition of endothelium-dependent relaxation [53]. These studies suggest the existence of an abnormality either at the level of trans-membrane receptors, or in the transduction signaling pathway (such as G-coupling proteins) between receptor activation and eNOS activation [55]; phosphorylation and/or subcellular location of the eNOS could contribute to such alterations in the signaling pathway [8].

Increased breakdown of NO by oxidative stress. All mammalian cells generate superoxide anions ($O_2^{-\bullet}$), reactive oxygen species, which are thought to be mainly inactivated by the superoxide dismutases (SOD) family [56]. Endothelium generates substantial amounts of $O_2^{-\bullet}$ although the mechanisms of production have not been extensively characterized [57, 58]. $O_2^{-\bullet}$ is known to react rapidly with NO in solutions, the rate constant, k, being 6.7×10^9 M^{-1} s^{-1}, which is about three times higher than that of SOD-catalyzed dismutation (2×10^9 M^{-1} s^{-1}) [59].

Evidence from experimental studies suggests that overproduction of $O_2^{-\bullet}$ and subsequent oxidative inactivation of NO may both be important in the pathophysiology of atherosclerosis. For example, treatment of cholesterol-fed rabbits with cell-targeted forms of SOD partially restores endothelium-dependent vascular relaxation [60, 61]. One major question remains the molecular and cellular source of the $O_2^{-\bullet}$ overproduced in atherosclerotic vessels. Macrophage-derived foam cells represent the natural candidate for most of this $O_2^{-\bullet}$ production, because they express an enzymatic complex, NADPH oxidase, which is the major producer so far characterized [62]. However, both the endothelium and the vascular smooth muscle cell contain membrane-bound oxidases that use NADH and NADPH as substrates for $O_2^{-\bullet}$ generation [63]. Recent data support a role for p22phox, one of the components of the vascular smooth muscle NAD(P)H oxidase, in the control of vascular $O_2^{-\bullet}$ production [63, 64]. Furthermore, both p22phox expression and $O_2^{-\bullet}$ production are both increased in smooth muscle stimulated by angiotensin II. The physiological role of this vascular NAD(P)H oxidase remains to be understood, as does its potential role in LDL oxidation. Finally, under certain conditions such as tetrahydrobiopterin deficiency, eNOS can itself generate superoxide anion. In this case, the site of generation of superoxide anion could be critical for NO breakdown [65].

NO and pulmonary hypertension

The pulmonary circulation is characterized by its low vascular tone at rest, and its reactivity to hypoxia. There is evidence to suggest that background release of NO contributes to the normally low pulmonary vascular tone in normoxia [66]. Infusion into the main pulmonary artery of both methylene blue and the arginine analog, L-NAME, significantly increases basal normoxic pulmonary vascular resistance of isolated perfused lungs from humans, whereas indomethacin has no effect [67]. Direct infusion of L-NMMA into the pulmonary circulation of living individuals also significantly increases pulmonary vascular resistance and decreases pulmonary blood flow velocity in healthy adults [68] as well as in infants with congenital heart disease [69]. These results are consistent with those obtained by Cremona et al. [67] and suggest that basal release of NO, but not of prostanoid products, might account at least in part for the low pulmonary vascular tone observed in humans. The architecture of the pulmonary vascular tree is probably also important, however, and further investigations are needed to assess the respective importance of NO and the particular anatomical disposition of the pulmonary circulation.

Although there are theoretical grounds to support the hypothesis that hypoxia reduces the synthesis of NO, lack of NO does not seem to account for the acute hypoxic pulmonary vasoconstriction. Indeed, in isolated perfused rat lungs, treatment with various NOS inhibitors also markedly enhances the pulmonary pressor response to acute hypoxic challenges [70–75]. These results rule out the hypothesis that blunted NO production causes acute hypoxic pulmonary vasoconstriction. Instead, they suggest that NO activity is increased during acute alveolar hypoxia. They also indicate that production of NO by pulmonary endothelium possibly acts as a brake to limit the excessive vasoconstriction induced by various chemical and physical stimuli [76, 77]. This protective mechanism of NO, modulating pulmonary vasoreactivity and preventing disproportionate pulmonary vasoconstriction, might be of significance in both health and pulmonary vascular disease.

More consistent, as concerns the role of NO, are data gathered from studies performed in chronic hypoxia, a leading cause of pulmonary hypertension [78]. In experimental conditions, exposure to hypoxia impairs endothelium-dependent relaxation of isolated pulmonary vascular rings [79] and the release of NO from cultured pulmonary endothelial cells [80]. In patients with chronic hypoxic pulmonary hypertension, endothelium-dependent relaxation is impaired compared with that of control patients [79], whereas contractile responses to α-adrenergic agonists are significantly greater in rings from patients with pulmonary hypertension than in control rings [81]. Treatment with L-NMMA eliminates this difference, increasing the tension in control rings but not in rings from patients with pulmonary hypertension [82]. This suggests that lack of NO not only impairs relaxation but also favors excessive pulmonary vasoconstriction in response to contractile stimuli. The reduced endothelium-dependent relaxation is also related to structural changes affecting the intima and the media [82, 83], thereby supporting the view that functional alteration of the endothelium not only alters pulmonary vasoreactivity but is also likely to affect remodeling of the pulmonary circulation. Reduced endothelium-dependent relaxation in chronic hypoxic pulmonary hypertension is observed with a variety of agonists, including a non-receptor-mediated agent, the calcium ionophore A23187 [84]. This suggests that the abnormality responsible for this impairment is located beyond the receptor-agonist coupling mechanisms. The impaired endothelium-dependent relaxation of pulmonary arteries from patients with pulmonary hypertension cannot be restored by an excess of L-arginine although the latter readily reverses the inhibitory effects of L-NMMA [84]. This suggests that reduced endothelium-dependent relaxation is not caused by a defect of L-arginine availability and/or transport. Circumstantial evidence suggests that lack of oxygen probably accounts for reduced NO release in chronic hypoxic pulmonary hypertension, which is consistent with the involvement of molecular oxygen in NO synthesis. As functional alteration of pulmonary endothelium is likely to affect both reactivity and growth of pulmonary vessels, endothelium-derived NO probably has a pivotal role in modulating pulmonary vascular tone and controlling pulmonary vascular remodeling in health and disease.

Conclusion

eNOS appears to play a key role in vascular physiology, as has been largely demonstrated in pharmacological experiments. Abrogation of eNOS expression by targeted gene inactivation confirmed the crucial role of eNOS in the control of arterial blood pressure [85]. However, clear demonstration of an abnormality in eNOS expression and/or activity in human hypertension remains to be established. In contrast, a decrease in endothelium-derived NO bioactivity has been shown in both animal models and in human atherosclerosis. This abnormality could contribute to the terminal events that complicate atheroma (vascular spasm and thrombosis) as a consequence of the loss of the vasorelaxant and antiaggregant properties of NO. The role of NO in the early phase of atheroma, i.e., fatty streak deposit, is more controversial. The hierarchy in the mechanisms responsible for the endothelial dysfunction of atherosclerotic vessels remains to be established. Further studies are required to understand the complex mechanisms that regulate NO production and bioavailability in vascular physiology and pathology.

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