NITRIC OXIDE SYNTHASE: Role in the Genesis of Vascular Disease

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ABSTRACT
The product of nitric oxide (NO) synthase is the most potent endogenous vasodilator known. NO not only is a potent vasodilator, it also inhibits platelet adherence and aggregation, reduces adherence of leukocytes to the endothelium, and suppresses proliferation of vascular smooth muscle cells. A number of disorders are associated with reduced synthesis and/or increased degradation of vascular NO. These include hypercholesterolemia, diabetes mellitus, hypertension, and tobacco use. The endothelial dysfunction caused by these disorders contributes to the alterations in vascular function and structure observed in these conditions. A reduction in the activity of vascular NO likely plays a significant role in the development of atherosclerosis. Insights into the mechanisms by which NO production or activity is altered in these states will lead to new therapeutic strategies in the treatment of a number of vascular disorders, including hypertension, atherosclerosis, restenosis, and thrombosis.

THE NATURE OF NITRIC OXIDE SYNTHASE
Nitric oxide synthase (NOS) is a remarkable molecule that plays a broad role in human biology ranging from homeostasis to pathology. The versatility of this enzyme is even more notable given the simplicity of its action: synthesis of the bimolecular gas nitric oxide (NO).

NOS exists in three isoforms named for the tissues in which they were first cloned and characterized. Endothelial NOS (eNOS), neuronal NOS (nNOS), and macrophage inducible NOS (iNOS) are heme-containing enzymes that catalyze the NADPH- and O₂-dependent five-electron oxidation of L-arginine
to NO and citrulline. These enzymes have high sequence similarity to cytochrome P-450 reductase and are unique in that they are the only mammalian proteins that catalyze both a hydroxylation reaction and an NADPH reduction. Across species, any one of the three isoforms is highly conserved with 85–95% sequence identity. By contrast, the three isoforms share only 50–55% sequence identity with each other. However, in certain regions of the proteins there is high sequence identity, particularly in domains that contain binding sites for various cofactors, including flavin adenine dinucleotide, flavin mononucleotide, tetrahydrobiopterin, heme, and calmodulin.

There are some salient structural differences between the isoforms. The eNOS NH₂ terminus contains a consensus site for N-myristoylation that plays a significant role in the membrane localization of eNOS. This isoform also undergoes palmitoylation. These attributes explain the observation that eNOS is membrane associated, whereas iNOS and nNOS are cytosolic. Although each of the isoforms contains a calmodulin binding site, iNOS binds calmodulin with higher affinity so that calmodulin forms a constitutive subunit to this isoform. This explains the observation that eNOS and nNOS are dependent on exogenous calcium and calmodulin for activation, whereas iNOS is less so.

Generally, nNOS and eNOS are considered constitutive enzymes whereas iNOS is highly regulated by cytokines. However, under certain circumstances, the expression of nNOS or eNOS is inducible. For example, fluid flow (causing endothelial shear stress) up-regulates the expression of eNOS; indeed, six shear stress–responsive elements have been identified in the promoter region of eNOS. Both eNOS and nNOS are up-regulated during pregnancy and with estradiol treatment, and response elements for sex steroids have been recognized in their promoter regions. Transection of the sciatic nerve up-regulates nNOS expression in the ipsilateral dorsal root ganglion, and treatment with neurotoxic agents increases nNOS expression in the hippocampus.

Generally, iNOS is considered to be an enzyme that is induced during immune response. However, under certain circumstances, it may be constitutively expressed. This isoform is detected in fetal tissues where, presumably, its expression is related to endogenous stimuli during development. The enzyme has also been identified in human bronchial epithelium, alveolar macrophages, and rat kidney under conditions where immune activation is not apparent.

All three isoforms have now been found in other tissues. The endothelial isoform is also expressed by kidney tubular epithelial cells and certain neuronal populations (particularly the hippocampal pyramidal cells). The neuronal isoform has been identified in peripheral (nonadrenergic, noncholinergic) neurons, skeletal muscle, pancreatic islet cells, kidney macula densa cells, and
respiratory epithelial cells. Almost any nucleated cell can produce iNOS given the appropriate inflammatory stimulus.

Because of their ubiquitous expression, the NOS enzymes have a great diversity of physiological effects. This review examines the role of eNOS in health and disease. Because eNOS is largely restricted to the vascular endothelium in its expression, we focus on the physiology of endothelium-derived NO and its disruption in pathophysiological states.

THE ENDOTHELIUM

The endothelium is a diaphanous film of tissue one cell layer thick that forms the lumenal surface of all blood vessels. Its apparent fragility belies its substantial role in regulating blood fluidity, lipoprotein metabolism, capillary transport, angiogenesis, interactions of the vessel wall with circulating blood elements, vasomotion, and vascular structure. The normal endothelium elaborates a panoply of proteins, prostanoids, and other paracrine substances to maintain a delicate balance between vasoconstriction and vasodilation, coagulation and blood fluidity, and inhibition and promotion of vascular growth. As an example of the balance between countervailing endothelial mechanisms, the endothelium synthesizes and releases the potent antiplatelet agent and vasodilator, prostacyclin, but it is also capable of elaborating other prostanoids, such as thromboxane A2, which are vasoconstrictors as well as agonists of platelet aggregation. The endothelium also exerts a profound influence on vasomotion by its ability to metabolize circulating vasoactive substances such as serotonin, epinephrine, bradykinin, and angiotensin I.

The endothelium elaborates tissue plasminogen activator, thrombomodulin, and heparan sulfate, which lend anti-thrombogenic properties to the lumenal lining (1–3); however, the endothelium also synthesizes von Willebrand factor, plasminogen activator inhibitor, and, under certain conditions, adhesive glycoproteins, which enhance vessel wall interactions with circulating cells (4–6). This diversity of endothelial synthetic function permits this tissue to maintain blood fluidity but provides for hemostatic mechanisms in the event of intimal injury. A similar complexity exists between the endothelium and the underlying vascular smooth muscle. Vascular smooth muscle proliferation is abetted by growth factors released from the endothelium, such as platelet-derived growth factor, basic fibroblast growth factor, insulin-like growth factor, and other paracrine factors (7–9). These influences are modulated by anti-mitogenic endothelial products such as transforming growth factor beta (10, 11). In addition, the endothelium releases a variety of vasoactive factors that, in addition to regulating vascular tone, are also capable of modulating cellular proliferation. In general, the vasoconstrictors released from the endothelium
(such as endothelin and angiotensin II) are growth promoters (12, 13), whereas the endogenous vasodilators (prostacyclin and endothelium-derived relaxing factor) suppress vascular growth (14, 15).

In sum, the endothelium exerts major control over vascular functions by its elaboration of a diverse array of paracrine substances. In general, synthetic functions of the endothelium that predominate are those that promote vasodilation and that inhibit thrombosis and cellular proliferation. Under certain pathophysiological conditions, the balance shifts to favor vasoconstriction, thrombosis, and/or initiation of cell cycle, and these alterations in endothelial function participate in the pathogenesis of vascular disease. Endothelium-derived NO is a potent regulator of vascular homeostasis. Disturbances in its elaboration or activity play an important role in the initiation and progression of vascular disorders.

ENDOTHELIUM, NITRIC OXIDE SYNTHASE, AND VASCULAR TONE

The discovery of an endothelium-derived relaxing factor (EDRF) was reported by Furchgott & Zawadski in 1980 (16). They found that the intimal lining of the rabbit thoracic aorta released a potent, but short-lived, diffusible vasodilator. This vasodilator was unlike the other known endogenous vasodilator substance, prostacyclin, in that its activity was not inhibited by indomethacin. Because of its short half-life, EDRF defied biochemical characterization for nearly a decade. In 1986, several investigators independently proposed that EDRF was NO, or a NO-containing substance, based on their observations of its similarity to other nitrovasodilators in its biological and physical properties (17, 18). Subsequently, Palmer et al used chemiluminescence and bioassay techniques to provide definitive evidence that NO was synthesized by endothelial cells (19, 20) and was responsible for the relaxing activity of EDRF.

NO induces vasodilation by stimulating soluble guanylate cyclase to produce cyclic GMP (cGMP). NO has a very short half-life and interacts avidly with sulfhydryl-containing proteins, heme proteins, and oxygen-derived free radicals. By virtue of its ability to nitrosylate proteins, it may change their activity or behavior. Recently, NO has been found to interact with two highly conserved sulfhydryl groups on hemoglobin. Formation of the nitrosothiol is favored when hemoglobin is in the oxy-Hb state, whereas dissociation of NO is more likely in the deoxy-Hb state. This interaction with hemoglobin may permit NO to become associated with hemoglobin in the pulmonary vasculature and to be released from hemoglobin in the systemic circulation.

Endothelium-derived NO is the most potent endogenous vasodilator known. The physiological importance of this endothelial factor has been dem-
demonstrated in animals and humans. Administration of inhibitors of NOS elevates blood pressure because of an increase in systemic vascular resistance (25, 26). These studies indicate that NO plays a major homeostatic role in modulating vascular resistance. It is released from the endothelium in response to a wide variety of neurohormonal agents or physical forces. The vasoconstrictor effects of norepinephrine, serotonin, vasopressin II, and endothelin are opposed by the endothelium. The activation of specific receptors on the endothelium by these agonists induces the release of NO, which mitigates the direct effect of the vasoconstrictors on vascular smooth muscle (27–29). The same phenomenon is observed with the effects of aggregating platelets on vascular tone (30, 31). Aggregating platelets release adenosine diphosphate and serotonin to induce vasoconstriction in the absence of the endothelium. However, when the endothelium is intact, these agents interact with purinergic and serotoninergic receptors on the endothelial surface to induce NO synthesis and release.

There is great heterogeneity from one vascular bed to another in the response of endothelium to these neurohormonal agents. In the human renal artery, acetylcholine, thrombin, serotonin, and adenosine diphosphate all induce endothelium-dependent relaxations (32). By contrast, in human limb arteries, only acetylcholine of the agents mentioned induces vasodilation. An even greater contrast is seen between arteries and veins. In general, endothelium-dependent relaxations are more prominent in arteries than in veins (33). Human internal mammary arteries are capable of prominent endothelium-dependent relaxations, whereas the ability of human veins to respond with endothelium-dependent relaxation is limited (34). Since NO is not only a vasodilator but has antiplatelet and antimitogenic properties (see below), one might speculate that the enhanced patency of internal mammary artery grafts compared with saphenous venous grafts for coronary artery bypass is, in part, due to the greater NO activity in these vessels.

The endothelium also responds to hemodynamic forces. Blood vessels dilate in response to increases in blood flow (Figure 1). The integrity of the endothelium is essential for this flow-mediated vasodilation (35–37). Flow-mediated vasodilation is largely due to the release of NO, although in some vascular beds prostacyclin, endothelium-derived hyperpolarizing factor, or direct activation of potassium channels may contribute (38–41).

Disturbances of flow-mediated vasodilation may have pathophysiological consequences. In patients with coronary artery disease and impairment of endothelial vasodilatory function, increases in blood flow through the diseased coronary artery cause a paradoxical vasoconstriction, which can contribute to myocardial ischemia during exercise or mental stress (42, 43). Long-term changes in flow induce remodeling of the vessel. With sustained increases in
flow rate through a conduit artery, the vessel restructures itself to achieve a larger diameter, as in the arterial segment proximal to an arteriovenous fistula. The opposite effect is seen with chronic reductions in flow rate. This remodeling is dependent on the presence of the endothelium (44). NO is known to modulate vascular muscle growth; it is possible that changes in the release of NO may contribute to these structural alterations (see below).

Under normal conditions, NO is a major regulator of vascular resistance, and the major source of this vasodilator is the endothelium. However, the marked reduction in vascular resistance that is associated with septicemia is now known to be a result of NO derived from activated macrophages and vascular smooth muscle (45, 46). Monocytes and vascular smooth muscle cells do not produce NO constitutively. However, the expression of NOS can be induced in these cells during sepsis. The subsequent production of NO by these activated cells greatly exceeds that of the endothelium, accounting in part for the hypotension observed in septic shock. In animal models of septic shock, administration of NOS antagonists reverses the blood pressure drop and increases survival (46). However, the therapeutic dose range is narrow; administration of higher doses of the antagonists increases the mortality in these conditions.

Figure 1  Photographs of original records from one experiment, representative of five, demonstrating that flow-mediated vasodilation is endothelium dependent. Intact endothelium: (left) Rabbit iliac artery segment contracted by norepinephrine (10-6 M). Intraluminal perfusate flow is 0 ml/min. Intraluminal pressure is 60 mm Hg and was held at this level throughout the experiment. (second from left) Immediately after recording the previous photograph, the flow of perfusate [physiological saline solution (VEH)] was increased to 2 ml/min for 2 min and this photograph was recorded. The vessel has visibly dilated. After endothelial denudation: (second from right) The endothelium was removed using saponin. The vessel was again contracted by norepinephrine (10-6 M), and this photograph was taken when the contraction had stabilized at an intraluminal flow of 0 ml/min and a pressure of 60 mm Hg. (right) Immediately after recording the previous photograph, the flow of perfusate was increased to 2 ml/min for 2 min and this photograph was taken. Flow-mediated vasodilation has been abolished. From Reference 38.
animals, presumably as a result of the blockade of endothelial NOS attended by local vasoconstriction, tissue ischemia, and necrosis. Preliminary studies in humans indicate that NOS antagonists may reverse the hypotension associated with septic shock (47). NOS antagonists may become useful therapeutic adjuncts in septicemia; antagonists that are selective for the induced enzyme may avoid adverse effects on tissue perfusion and platelet aggregability.

NITRIC OXIDE SYNTHASE AND PLATELET REACTIVITY

The adherence of platelets to the vessel wall, and their aggregation, is suppressed by nitrovasodilators. These effects are associated with, and mediated by, an increase in intracellular cGMP. Therefore, it is not surprising that endothelium-derived NO has also been shown to have these antiplatelet properties (48–50). Like other nitrovasodilators, NO acts synergistically with prostacyclin to inhibit platelet aggregation and adherence, contributing in a major way to the anti-thrombogenic properties of the endothelium.

ADP and serotonin released from aggregating platelets, as well as thrombin, act on specific endothelial receptors to induce the release of NO (30, 31). Accordingly, NO is an important component of the negative feedback loop that prevents the propagation of thrombus. As previously mentioned, increases in flow rate also release NO. This phenomenon may be important in suppressing platelet adherence or aggregation favored by the rheologic changes induced by increases in blood flow. Indeed, NO inhibits the aggregation of circulating platelets. Platelets traversing the vascular bed of the perfused heart exhibit an elevation of intracellular cGMP, which leads to the phosphorylation of cGMP-dependent phosphoproteins (such as vasodilator-stimulated phosphoprotein), which suppress platelet reactivity. These effects on circulating platelets are blocked by antagonists of NO activity or synthesis (51, 52). Autocrine NO produced by platelets may also act as a brake on platelet activation. Human platelets contain small amounts of NOS, which resembles the neuronal isoform. Indeed, administration of arginine in vitro or in vivo enhances platelet synthesis of NO with increase in intraplatelet cGMP associated with reduced platelet reactivity. These effects of arginine are reversed by L-N-monomethyl arginine, an antagonist of NOS.

NITRIC OXIDE SYNTHASE AND VASCULAR GROWTH

The endothelium plays an important role in maintaining the underlying vascular smooth muscle in a quiescent state. Damage to the vessel wall with endothelial denudation occurs during balloon angioplasty. Loss of the endothelium, and injury to the vascular smooth muscle, initiates a series of events that result
in myointimal hyperplasia. This lesion is due to the migration of vascular smooth muscle cells into the intima, with proliferation of these cells and alteration in their phenotype from a contractile to a secretory form (53). These altered smooth muscle cells elaborate extracellular matrix, which contributes to the thickness of the lesion. Platelets adhering to the vessel wall and injured vascular smooth muscle cells release growth factors that play a major role in inciting and sustaining this response to injury (54). A number of endothelial products inhibit cell proliferation (55). One of these antiproliferative agents is NO. The growth of vascular smooth muscle cells in culture is inhibited by agents that release NO (15). In addition, the stable analogue of cGMP, 8-bromo-cGMP, has the same effect (15). Like NO, atrial natriuretic peptide increases levels of intracellular cGMP; this effect is associated with an inhibition of growth in cultured vascular smooth muscle cells stimulated by angiotensin II (56).

In animal models of restenosis after balloon angioplasty, endogenous NO appears to modulate myointimal hyperplasia. Although the endothelial source of NO synthesis is removed by angioplasty, the inducible isoform is expressed by vascular smooth muscle in the vicinity of the injury. This may explain the observation that systemic administration of L-arginine (the NO precursor) can reduce the degree of myointimal hyperplasia after balloon angioplasty in animal models. The effect of arginine is blocked by coadministration of NOS antagonists (57, 58).

Further evidence that NO can act as an inhibitor of proliferation of vascular smooth muscle cells in vivo has been provided by our group using an in vivo gene transfer approach. In the rat carotid artery, we have successfully transferred and overexpressed the gene encoding NOS to the injured and de-endothelialized vessel wall. This has the effect of restoring local release of NO and suppressing vascular lesion formation after balloon angioplasty (59).

Proliferation of vascular smooth muscle is seen in states of endothelial dysfunction that are characterized by reduced release of NO, providing further support for its role in regulating vascular structure. It is often observed after balloon injury that the myointimal proliferation continues even after the vessel wall has been resurfaced by endothelium. Examination of this endothelium reveals that it is morphologically abnormal (60). Cells are irregularly sized, polygonal, and not aligned with blood flow. These cells appear to release less NO, as manifested by an attenuation of endothelium-dependent relaxations in these vessels. It is possible that this and other alterations in endothelial function are permissive in the sustained proliferation of vascular smooth muscle. This endothelial dysregulation is presumably responsible for the cellular proliferation in atherosclerosis and other disease states.
ALTERATIONS IN NITRIC OXIDE SYNTHASE ACTIVITY IN DISEASE

*Hypercholesterolemia and Atherosclerosis*

In hypercholesterolemic animals and man, endothelium-dependent relaxation is reduced and vasoconstriction is enhanced. This is largely due to a reduction in NO activity (61–64) and not to the physical absence of the endothelium. The same effect of hypercholesterolemia is also observed in resistance vessels of atherosclerotic animals and man (64, 65). Indeed, this abnormal vasoreactivity is seen well before any morphological changes of atherosclerosis. Coronary arteries isolated from pigs fed a high cholesterol diet for eight weeks exhibit an attenuation of endothelium-dependent relaxation without any significant pathology demonstrable by light microscopy (66).

Endothelium-dependent vasodilation is reduced in hypercholesterolemic human epicardial coronary and resistance vessels (63, 67). These abnormalities likely contribute to angina during exertion or emotional stress. Indeed, human coronary arteries exhibiting an abnormal response to acetylcholine also vasoconstrict with mental stress (42) and exertion (43) or in response to the cold pressor test (68). An improvement in endothelial function would likely be of clinical benefit in such patients by restoring normal vascular reactivity, preventing vasospasm, and inhibiting platelet adherence and aggregation. Cholesterol reduction has been shown to improve endothelial vasodilatory function (69). It is possible that this improvement in vascular function may explain, in part, some of the reduction in vascular events seen in patients receiving aggressive anti-lipid treatment. In addition, the administration of antioxidants (70), estrogen (71), and L-arginine (67, 72, 73) have all been shown to enhance endothelial vasodilatory function in humans; their role in the treatment of coronary artery disease has not been defined, but they may present alternative therapeutic strategies.

Vessels isolated from normal animals exhibit an endothelial dysfunction within minutes of exposure to cholesterol in vitro (74, 75). This abnormality appears to be due to the low-density lipoprotein (LDL) component of cholesterol, especially the oxidized form. The mechanism by which oxidized LDL inhibits endothelium-dependent relaxations is under investigation. Oxidized LDL or other oxygen-derived free radicals may chemically combine with and inactivate NO (75, 76). Controversy exists as to whether or not oxidized LDL can inhibit the activity of NOS. Hypercholesterolemia also induces the endothelium itself to generate superoxide anion (77). This oxidative stress may play an important role in the pathogenesis of atherosclerosis by oxidizing lipoproteins and lipid membranes, as well as by altering endothelial function.
Specifically, alterations in the redox state of the endothelium appear to enhance endothelial adhesiveness for monocytes (78, 79).

Increasing evidence indicates that endothelium-derived NO may act to oppose the hypercholesterolemia-induced alterations in endothelial redox state. In normocholesterolemic rabbit thoracic aorta, there is a basal production of superoxide anion by non-endothelial cells (80). Exogenous NO donors significantly reduce the generation of superoxide anion. Administration of a NO precursor, benzoyl-L-arginine ethyl ester, decreases alloxan-stimulated superoxide production by rabbit aorta, an effect that is reversed by the NOS antagonist, L-N-monomethyl arginine (81).

The mechanism by which NO reduces oxidative stress remains undefined. It is possible that NO reacts directly with lipid peroxy radicals, thereby disrupting the chain of autocatalytic reactions involved in the oxidation of intracellular lipid (82). Indeed, NO donors inhibit copper-catalyzed oxidation of LDL cholesterol in vitro (83). Another mechanism by which NO may exert its effects is by inhibiting the generation of oxygen-derived free radicals by oxidative enzymes. Clancy and colleagues (84) found that when neutrophils are exposed to NO, their ability to generate superoxide anion is limited. This appears to be due to a direct effect of NO on the multimeric oxidative enzyme NADPH oxygenase. NO may interact with sulfhydryl or heme moieties within the membrane-bound component of this enzyme to prevent its activity. These observations are concordant with the observation of Yates and colleagues (85), who found that autocrine NO regulates macrophage oxidation of LDL cholesterol. This effect was reversed by an antagonist of NOS. In preliminary studies, we have shown that the stimulation of endothelial cells by fluid flow reduces their generation of superoxide anion; this effect was NO dependent. Taken together, these studies indicate that NO reduces oxidant stress. The critical role of NO in modulating endothelial redox state has also been demonstrated by Niu and coworkers (86). These investigators used intracellular fluorophores responsive to changes in redox state to demonstrate that antagonists of NOS precipitate oxidative stress.

The effect of NO to reduce the generation of reactive oxygen species may explain its repression of NFkB-mediated gene expression. Antioxidants such as α-n-acetylcysteine and pyrrolidine dithiocarbamate are known to inhibit the dissociation of NFkB from its inhibitor, IkBa (78, 87). In cultured human umbilical venous endothelial cells, interleukin-1 induced expression of the vascular cell adhesion molecule (VCAM-1) is selectively antagonized by exposure of the cells to the antioxidant pyrrolidine dithiocarbamate (78). NO donors mimic the effect of antioxidants in suppressing NFkB-mediated gene expression (88). The activation of NFkB by tumor necrosis factor alpha in cultured human saphenous vein endothelial cells is blocked by exogenous NO,
whereas NO donors have little effect on other nuclear binding proteins (AP-1 and GATA). Immunoprecipitation studies indicated that exogenous NO stabilizes the NFkB/IkBα complex. Moreover, exogenous NO enhances the transcription of IkBα but not NFkB. These observations likely explain previous findings that exogenous NO donors inhibit interleukin-1–stimulated VCAM-1 expression and monocyte adhesion (89).

Since fluid flow is a potent stimulus for the release of endothelium-derived NO, we speculated that the effect of flow to inhibit atherogenesis may be mediated, in part, by NO. In preliminary studies, we found that previous exposure to flow inhibited lipoprotein- or cytokine-mediated superoxide production, NFkB activation, VCAM-1 expression, and endothelial adhesiveness for monocytes. The effect of fluid flow appears to be due to shear-induced release of NO since coincubation with l-nitro-arginine completely abolished, whereas an NO donor mimicked, these effects of flow.

The tractive force exerted by fluid flow (shear stress) may directly influence gene expression by acting upon shear stress-responsive elements (SSRE) in the promoter region of specific flow-responsive genes. One of these (GA-GACC) was first defined by Resnick et al (90) in the 5′ promoter region of the gene encoding the platelet-derived growth factor B chain. This SSRE is positively regulated by shear stress. This same SSRE is found in the promoter region of a number of genes regulated by flow. Six repeats of the SSRE are found in the 5′ promoter region of the gene encoding endothelial NOS (ECNOS) (24). Exposure of endothelial cells in vitro to fluid flow enhances the expression of ECNOS (24, 91).

Flow-stimulated NO has acute effects on monocyte-endothelial cell interaction as well as the more chronic effects on redox regulation of gene expression. Monocyte adherence to endothelial cells in culture is inhibited by administration of NO with a time course that implies an effect on signal transduction of adhesion pathways (92). We have also shown that the adherence of monocyteoid cells to bovine aortic endothelial cells is inhibited by a brief (i.e. 15 min) exposure to flow in a NO-dependent manner in the absence of any changes in the expression of endothelial molecules, indicating that, acutely, flow-released NO inhibits the signal mechanism of adhesion (93). Longer-term exposure to flow (e.g. 72 h) reduces monocyte adhesion in association with the down-regulation of VCAM-1.

The acute and chronic effects of NO upon endothelial adhesiveness may play an important role in atherogenesis. The effect of chronic exercise in inhibiting atherosclerotic lesion formation (94, 95) may be, in part, due to the effect of exercise to intermittently increase flow and, thereby, enhance the vascular expression of ECNOS and the elaboration of NO (96). Similarly, the predisposition for lesion formation at sites of branching (where shear stress is
low) may be, in part, due to the reduced elaboration of NO at these sites (97). We speculate that NO is an endogenous anti-atherogenic molecule, which exerts its effects, in part, via its modulation of endothelial redox state.

We have tested this hypothesis in animal models. In hypercholesterolemic New Zealand white rabbits, NO-dependent relaxations can also be normalized by administration of the NO precursor, L-arginine (98, 99). Intravenous administration of L-arginine normalizes endothelium-dependent vasodilation in the hind limb resistance vessels, as well as in the thoracic aorta (Figure 2). This effect is likely due to the metabolism of L-arginine, since its inactive enantiomer D-arginine does not mimic the beneficial effect. In man, infusions of L-arginine normalize endothelium-dependent vasodilation to acetylcholine in resistance vessels of the forearm and the coronary circulations (67, 73).

Chronic administration of L-arginine to hypercholesterolemic animals also improves endothelium-dependent relaxations. This improvement in vascular NO activity is associated with a striking inhibition of intimal lesion formation (Figure 3) (100). This effect appears to be due to a direct action of vascular NO on endothelial adhesiveness for monocytes. In a separate study, we used a functional binding assay to examine the adhesiveness of the endothelium for normal rabbit mononuclear cells, or a human monocytoid cell line (101). After two weeks of dietary interventions, the thoracic aorta was harvested, segments of the vessel were bisected longitudinally, and the endothelium was exposed ex vivo to a solution containing mononuclear or monocytoid cells. After ex vivo incubation for 30 min, nonadherent cells were removed with fresh media, and the preparations were fixed and the bound cells were counted. Thoracic aortae from hypercholesterolemic animals exhibited a threefold increase in adhesiveness for monocytes. By contrast, hypercholesterolemic animals receiving dietary arginine manifested a significant reduction in monocyte binding. Conversely, animals receiving normal diet with the addition of nitro-arginine (an antagonist of NOS) exhibited a tenfold increase in cell binding (even more binding was observed than in hypercholesterolemic animals).

In some animals, RNA was isolated from the thoracic aorta and probed for monocyte chemotactic protein-1 (MCP-1) mRNA. MCP-1 is a 76 amino acid chemokine thought to be a major chemotactic factor for monocytes in both inflammation and atherogenesis (102). The expression of MCP-1 is induced by lipopolysaccharides, cytokines (tumor necrosis factor alpha and interleukin-1 and -4), and phorbol esters. Human aortic endothelial and smooth muscle cells exposed to minimally modified LDL cholesterol express MCP-1; this protein accounts for virtually all the chemotactic activity produced under these conditions (103). The expression of MCP-1 could not be detected in aorta from normal animals but was present in tissues from hypercholesterolemic rabbits. Administration of L-arginine attenuated the expression of MCP-1. Animals fed
Figure 2  Endothelium-dependent relaxation is inhibited by hypercholesterolemia and normalized by L-arginine. Tracings of original records showing endothelium-dependent relaxation in isolated thoracic aortae from normal and hypercholesterolemic rabbits. (A) Vascular ring from a normal rabbit is contracted by norepinephrine (NE) and then relaxed with increasing concentrations of acetylcholine (ACh). w/o, Washout (replacement of bathing solution with fresh physiological saline solution). (B) Vascular ring from a hypercholesterolemic rabbit that had received an intravenous infusion of D-arginine. Note impaired relaxation to ACh. (C) Vascular ring from a hypercholesterolemic rabbit that had received an intravenous infusion of L-arginine is exposed to protocol described in A. Note normalized response to ACh. From Reference 99.
New Zealand white rabbits were fed a 1% cholesterol diet plus vehicle or 2.25% L-arginine in the drinking water. After 10 weeks, the thoracic aortae were harvested for histomorphometry. Arginine treatment significantly reduced intimal lesion formation. (Upper) Microphotograph of representative cross section of thoracic aorta from a hypercholesterolemic animal. Arrows designate intimal lesion. (Lower) Microphotograph of a representative cross section of thoracic aorta from a hypercholesterolemic animal receiving supplemental arginine. Intimal lesion formation is significantly reduced.
a normal diet plus the endothelial antagonist nitroarginine exhibited a dramatic increase in MCP-1 mRNA level. Recently, Zeiher and colleagues have also provided evidence that NO inhibits MCP-1 expression in cytokine-stimulated human venous endothelial cells, in a cGMP-independent fashion (104).

These studies indicate that vascular NO is an important regulator of endothelial adhesiveness and monocytes, in part, by regulating the expression of MCP-1. Our findings are consistent with recent investigations revealing that chronic administration of NOS antagonists accelerates intimal lesion formation in hypercholesterolemic rabbits (105, 106).

Although these investigations indicate that NO can inhibit or slow the process of monocyte adhesion and infiltration, they leave unanswered the question of how enhancement of NO activity could affect preexisting lesions. In a separate study to answer this question, New Zealand white rabbits were placed on a high cholesterol diet for 10 weeks. Subsequently, half of the animals were given supplemental arginine whereas the others received only vehicle. Some animals in each group were sacrificed at 10, 14, 18, or 22 weeks. In the arginine-treated animals, we observed regression of lesions related to the degree of improvement in NO activity. The mechanism by which enhanced NO activity induces regression is under study. Our preliminary findings indicate that apoptosis of intimal macrophages may contribute to this process. This would be consistent with in vitro observations that NO donors induce apoptosis in isolated foam cells.

**Hypertension**

EDRF plays a major role in the regulation of systemic vascular resistance. It is, therefore, conceivable that endothelial vasodilatory dysfunction could contribute to hypertension. Indeed, endothelial dysfunction has been demonstrated in animal and human hypertension (107–109). Depending on the experimental model, the reduction in endothelium-dependent relaxation is due to an attenuation of NO activity, or to the augmented elaboration of an endothelium-derived contracting factor (possibly a prostanoid). Data suggest that the endothelial dysfunction is secondary and reversible with the treatment of hypertension (110, 111). Conversely, infusions of NOS antagonists produce marked increases in blood pressure in experimental animals (112). These inhibitors have been considered nonspecific, and the effect on blood pressure could conceivably be due to an effect on the neuronal NOS. However, more definitive data for a primary role of NO in the regulation of blood pressure was recently provided by Huang et al (113). They report that inactivation of the mouse endothelial NOS gene by homologous recombination produces mice that are significantly hypertensive.

Two recent studies have provided evidence for endothelial vasodilatory dysfunction in hypertensive humans (114, 115). In both studies, forearm blood
flow was measured by strain-gauge plethysmography in response to intra-arterial infusions of endothelium-dependent and -independent vasodilators. In these young patients with mild essential hypertension, endothelium-independent vasodilation was relatively undisturbed. By contrast, cholinergic vasodilation (presumably endothelium dependent) was attenuated. Whether this is a primary or secondary phenomenon is not known. There is preliminary evidence suggesting that this endothelial deficit may precede the appearance of essential hypertension (116). In young normotensive individuals with hypertensive parents, cholinergic forearm vasodilation is impaired; by contrast, endothelium-independent vasodilation is normal.

There are a number of other disorders associated with endothelial vasodilator dysfunction (Table 1). The mechanisms of the endothelial vasodilator dysfunction in these disorders are under investigation. To what extent NO contributes to the pathophysiology of these disorders is not yet known.

SUMMARY

In summary, endothelium-derived NO is derived from the metabolism of arginine. It is a potent vasodilator and plays a key role in modulating conduit and resistance vessel tone. NO also has important effects on cell growth and on the interactions of circulatory blood cells with the vessel wall. As a consequence, disturbances of EDRF activity may initiate or contribute to diverse disease states such as septic shock, hypertension, vasospasm, toxemia, and atherosclerosis.

Table 1 Disease states characterized by endothelial dysfunction

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