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Nitric Oxide and Its Role in the Cardiovascular System

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Nitric oxide (NO) is a ubiquitous, naturally occurring molecule found in a variety of cell types and organ systems. In the cardiovascular system, NO is an important determinant of basal vascular tone, prevents platelet activation, limits leukocyte adhesion to the endothelium, and regulates myocardial contractility. NO may also play a role in the pathogenesis of common cardiovascular disorders, including hypotension accompanying shock states, essential hypertension, and atherosclerosis. In this review, we discuss the biochemistry of NO and focus on its biology and pathophysiology in the cardiovascular system.
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NITRIC OXIDE SYNTHESIS

NITRIC OXIDE (NO) synthesis occurs in a wide variety of cell types and tissues including the vascular endothelium, platelets, macrophages, and neuronal cells. The substrate for NO synthesis is the terminal guanidino nitrogen of the amino acid L-arginine,[1] which undergoes a 5-electron oxidation to form L-citrulline and the free radical NO$^{\cdot}$.[2] Molecular oxygen[3] and NADPH[2] are cosubstrates in this reaction that is catalyzed by the enzyme (family) NO synthase (NOS). NOS contains both flavin adenine dinucleotide and flavin mononucleotide[4] and requires the presence of several oxidative cofactors, including tetrahydrobiopterin,[5] reduced glutathione,[6] and a heme complex.[7] There is considerable homology between NOS and the enzyme cytochrome P450 reductase.[8] Moreover, it has been suggested that NOS facilitates electron flow between reducing equivalents analogous to cytochrome P450 but does so in a unique manner that involves bound calcium-calmodulin complexes.[9]

There are two main isoform classes of NOS (Fig 1). One is a constitutive enzyme (cNOS) class that is present in the vascular endothelium (eNOS or *Nos3*), neuronal cells (nNOS or *Nos1*) and several other cell types. cNOS is regulated by Ca^{2+} and calmodulin. In the vascular endothelium, agonists such as acetylcholine and bradykinin stimulate inositol 1,4,5-trisphosphate (IP_3) production by activating the phosphoinositide second messenger system.[10] IP_3 binds to receptors on the endoplasmic reticulum and causes Ca^{2+} release from intracellular stores[10]; this transient elevation in intracellular Ca^{2+} promotes Ca^{2+} binding to calmodulin, and, once formed, the Ca^{2+}/calmodulin complex activates cNOS.[11-13] cNOS produces modest amounts of NO until Ca^{2+} levels decrease. The rapid and transient production of NO by cNOS allows NO to function in neuronal tissue as a neurotransmitter. Similarly, in the vascular endothelium, cNOS is well suited for its role in maintaining basal vascular tone,[14] owing to its capacity to generate low-level, intermittent release of NO.

The other main isoform class of NOS is an inducible enzyme (iNOS or *Nos2*). iNOS has been found in macrophages[15] and neutrophils,[16] and is immunologically activated by exposure to bacterial endotoxin or cytokines such as interleukin-1 (IL-1) or interferon-γ.[17-19] iNOS activity is regulated at the transcriptional level[20] and is not affected by intracellular Ca^{2+} levels. Macrophage iNOS induction after cytokine exposure

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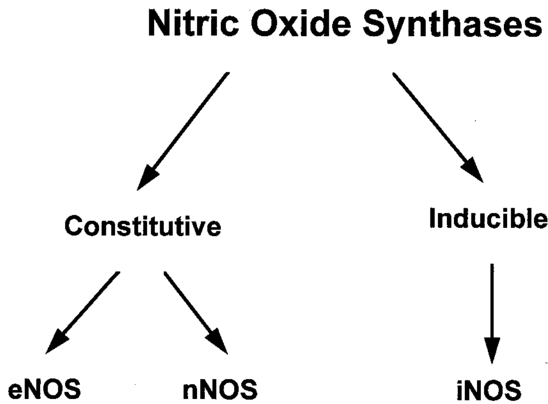

Fig 1. The family of NO synthases is composed of two broad classes, the constitutive class, which is made up of the endothelial isoform (eNOS) and the neuronal isoform (nNOS), and the inducible class, which contains the inducible isoforms (iNOS) found in macrophages, neutrophils, fibroblasts, and hepatocytes.

requires several hours, and, once induced, iNOS is capable of generating far greater quantities of NO per mole of enzyme per minute than is cNOS.[21] At high concentrations, NO is cytotoxic, and it appears that NO plays a key role in the immune response of macrophages to bacteria and other pathogens.[22]

It has recently been shown that macrophage iNOS also contains a calmodulin-binding consensus sequence.[23] Cho and colleagues[23] have reported that the calmodulin subunit is very tightly bound to iNOS, requiring very low levels of Ca^{2+} for activation; therefore, iNOS activity is not affected by calcium transients. cNOS binds calmodulin via a reversible, Ca^{2+}-dependent mechanism and is, therefore, activated by elevation of intracellular calcium.[2]

There are several other putative mechanisms of NOS regulation that are believed to have physiological relevance. nNOS is phosphorylated by cyclic adenosine 5′-monophosphate–dependent protein kinase, protein kinase C (PKC), and also a Ca^{2+}/calmodulin-dependent protein kinase, and it appears that phosphorylation by PKC substantially reduces NOS catalytic activity.[4] Breakdown of phosphoinositide (PIP_2) stimulates concurrent production of both IP_3 and activation of PKC by diacylglycerol, and Bredt and colleagues[4] have suggested that PKC-induced NOS phosphorylation is an example of negative feedback or "cross-talk" between the PIP_2 and NO signalling systems. Rengasamy and Johns[24] have recently shown in vitro that authentic NO inhibits NOS activity, and these investigators have suggested that this inhibition occurs as a result of the formation of an NO-iron complex with the heme iron of NOS. This example of negative feedback between NO and NOS may represent an important physiological regulatory mechanism for NO synthesis.[24]

Recently, Resnick and colleagues[25] have shown that a shear-stress–response element (GAGACC) exists in the promoter region of the genes of several proteins synthesized by and released from endothelial cells in response to increases in shear stress, including the B-chain of platelet-derived growth factor (PDGF-B), intercellular adhesion molecule-1, transforming growth factor-β, and tissue-type plasminogen activator. The nucleotide sequence of the genomic DNA of eNOS[26] shows the presence of nine of these shear-stress–response elements, suggesting that mechanochemical regulation of the transcription of eNOS may modulate NO production, as well.

Yet another level of regulation of iNOS and eNOS activity has recently been identified that involves the cofactor tetrahydrobiopterin. Cytokines have been shown to increase NO production in monocytes both by inducing iNOS transcription and by augmenting guanosine-5′-triphosphate cyclohydrolase I,[27] the first committed step in the pteridine synthetic pathway. More recently, two reports confirm that cytokine-stimulated tetrahydrobiopterin production is an important determinant of eNOS activity, as well.[28,29] Thus, intracellular cofactor concentrations can both be modulated by and, in turn, can modulate NOS activity and NO production.

NO BIOCHEMISTRY

NO is a free radical gas that is moderately stable in aqueous media and functions as a biological messenger in physiological solutions. The free radical, $NO^{\cdot}$, is well suited for its role as a biological messenger because it is a small, reactive diatomic molecule with an available unpaired electron. $NO^{\cdot}$ is also able to diffuse readily across biological membranes.[30]

The redox biochemistry of nitrogen monoxide involves an array of interrelated, biologically active redox forms, NO^+ (nitrosonium), NO^-

(nitroxyl anion), and the free radical $NO^{\cdot}$.[31] These NO redox species interact in a manner that is analogous to the redox biochemistry of diatomic oxygen. O_2, $O^{\cdot}{}_2^{-}$ (superoxide), and O_2^{2-} (which forms H_2O_2 in solution) are the three redox species of dioxygen. Unlike O_2, O_2^{2-} and H_2O_2 (hydrogen peroxide) are very cytotoxic and possess very distinct and different chemical properties than does O_2. In an analogous manner, each redox species of NO has its own distinctive set of chemical properties and reactivities.

The free radical $NO^{\cdot}$ has a single electron in its $2p - \pi$ antibonding orbital.[32] The primary biologic reactions of $NO^{\cdot}$ involve oxygen, superoxide anion, and redox metals, including transition metals (especially iron) and heme-containing proteins such as hemoglobin.[31] $NO^{\cdot}$ also reacts rapidly with superoxide anion ($O_2^{\cdot -}$) in biological solutions to form peroxynitrite ($OONO^-$).[33] Peroxynitrite is a powerful oxidant that has been implicated in a number of protein oxidation reactions that occur physiologically, including nitration of tyrosine and oxidation of sulfhydryl groups.

NO^+ (nitrosonium) is formed by loss of an electron from $NO^{\cdot}$, and, conversely, NO^- (nitroxyl anion) is formed after addition of an electron to the $2p - \pi$ antibonding orbital.[31] In biological systems, the redox biochemistry of NO^+ is characterized by nitrosation reactions involving nucleophiles such as amides, sulfite, and thiols.[31] NO^- reacts with hemoglobin[30] and may also react with thiols in a reaction that produces S-nitrosothiols as a minor product.[34]

It has also been shown that, under physiological conditions, endogenous NO (or an oxidized derivative, such as NO^+, N_2O_3, or nitrosylated metals) reacts in the presence of molecular oxygen with low molecular weight thiol groups to form S-nitrosothiols.[35] In human plasma, S-nitrosothiols are the primary redox form of NO and have a concentration in the low micromolar range.[36] In contrast, the plasma concentration of free NO is approximately 3 nmol/L, and we have, therefore, suggested that S-nitrosothiols, which are significantly more stable in plasma than free NO, serve as the major plasma reservoir of NO.[36] S-nitrosothiol formation may also attenuate NO toxicity because S-nitrosothiols are much less reactive than NO and, therefore, are less likely to react with oxygen to form toxic oxidizing species;[31]; this chemistry is probably important in the alveolus.[37]

After production, NO readily diffuses across cell membranes to interact with specific molecular targets (Fig 2). NO regulates protein activity by reversibly binding to available acceptor functionalities, including heme iron and thiols.[31] The interaction between NO and the enzyme guanylyl cyclase, which mediates target cell responses such as vascular smooth muscle relaxation and platelet inhibition, has been well characterized.[38] After entering the target cell, NO binds to the heme moiety of guanylyl cyclase and activates the enzyme by inducing a conformational change that displaces iron out of the plane of the porphoryrin ring.[39] Guanylyl cyclase then catalyzes the production of cyclic guanosine 3′,5′ monophosphate (cGMP) from guanosine-5′-triphosphate.[40,41]

cGMP is believed to elicit vascular smooth muscle relaxation through several mechanisms. One important mechanism involves phosphorylation of the enzyme myosin light chain kinase (MLCK). MLCK phosphorylates the regulatory (molecular mass, 20,000 d) set of myosin light chains (also known as LC_{20}).[42] Phosphorylation of LC_{20} activates cross-bridge cycling and initiates contraction.[42] LC_{20} phosphorylation also markedly increases actinomyosin adenosine 5′-triphosphatase activity.[43] cGMP modulates

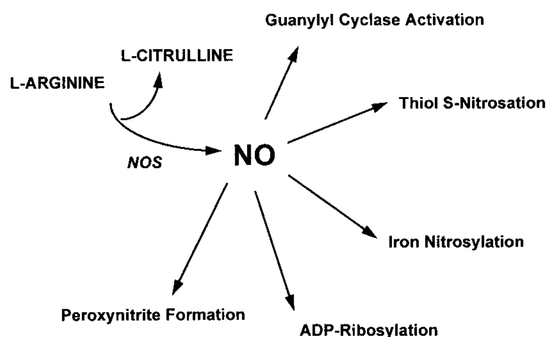

Fig 2. The formation of NO from L-arginine by the NOSs is depicted, and the subsequent reactions that underlie the biological effects of NO are listed. Relevant reactions in the cellular and extracellular milieux include activation of guanylyl cyclase; S-nitrosation reactions of low molecular weight and protein thiols in the intracellular and extracellular environments; complex formation with transition metals, especially iron (iron nitrosylation); activation of a cytosolic ADP ribosyltransferase, leading to transfer of ADP ribose to GADPH (ADP-Ribosylation); and peroxynitrite formation through the reaction of NO with superoxide.

MLCK activity by activating a cGMP-dependent protein kinase that phosphorylates MLCK.[44,45] Phosphorylation of MLCK diminishes its affinity for calmodulin and, as a consequence, decreases the phosphorylation of myosin light chain, which in turn stabilizes the inactive form of myosin.[44,46] In this manner, cGMP may induce vasorelaxation by indirectly decreasing myosin light chain-dependent myosin activation. It has also been suggested that cGMP may elicit vascular smooth muscle relaxation by affecting Na^+/Ca^{2+} exchange or perhaps by a cGMP-mediated effect on phosphodiesterase.[10]

NO also interacts with target cells by cGMP-independent mechanisms. NO activates a cytosolic adenosine 5′-diphosphate (ADP)-ribosyltransferase in human platelets that catalyzes the transfer of ADP ribose to glyceraldehyde 3-phosphate dehydrogenase (GADPH), which is an enzyme integrally involved in glycolysis.[47] The addition of an ADP ribose group to GADPH (a chemical process known as ADP ribosylation) inactivates the enzyme and thereby slows glycolysis and decreases adenosine 5′-triphosphate formation.[10] NO-mediated injury in processes such as myocardial stunning or neurotoxicity may be secondary to NO stimulation of GADPH ADP-ribosylation, with the resultant inhibition of glycolysis.[10]

Detecting biologically derived NO has proven challenging, owing principally to its instability and high degree of reactivity with other biological compounds, such as thiols and hemoglobin. The most commonly used techniques for measuring NO· are chemiluminscence, electron paramagnetic resonance (EPR), and spectrophotometry of methemoglobin.

Chemiluminescence relies on the reaction of NO with ozone.[36] This oxidative reaction produces nitrogen dioxide in an excited state; relaxation from this high energy state generates light with a distinctive wavelength that is detected by reaction with ozone in the chemiluminescence spectrometer.[9] Luminescence is directly proportional to NO· concentration,[48] with a typical detection threshold in the range of 20 to 50 pmol/L.[48] A major limitation of the technique is that extraction of NO· from biological solutions requires extensive chemical pretreatment, rendering it difficult to distinguish NO· from NO-related adducts that form during pretreatment.[36]

EPR detection of NO· relies on the fact that NO· is a paramagnetic gas with an unpaired electron.[49] Application of a magnetic field and a discrete amount of microwave energy excites the unpaired electron to a higher energy state; relaxation to the ground state releases energy with a characteristic EPR spectrum.[48] NO· cannot be directly detected by EPR because the relaxation time of the excited electron is too rapid.[50] However, it is possible to use other compounds, such as hemoglobin, to form stable NO· adducts that are detectable by EPR. This technique is called "spin-trapping" and has a typical detection threshold in the nanomolar range.[48]

The methemoglobin spectrometry assay is based on the rapid oxidation of oxyhemoglobin to methemoglobin by NO·.[9] NO· is detected by observing a characteristic shift in the visible spectrum peak absorbance from 433 nm to 406 nm.[51] The oxidation of reduced hemoglobin (Fe^{2+}) to methemoglobin (Fe^{3+}) occurs in less than 100 milliseconds and is, therefore, rapid enough to measure continuous NO· production during NO· synthesis.[52] The detection threshold of methemoglobin spectrophotometry is also in the nanomolar range.[52]

ENDOTHELIUM-DERIVED RELAXING FACTOR AND NO

In 1980, Furchgott and Zawadzki[53] discovered that the vasodilator action of acetylcholine required the presence of an intact endothelium. They showed that the binding of acetylcholine to muscarinic receptors on endothelial cells triggers the release of a potent vasodilator that has subsequently been termed endothelium-derived relaxing factor (EDRF).[53] EDRF can, then, be viewed as a paracrine vascular hormone that relaxes underlying vascular smooth muscle[53] and inhibits local platelet adhesion and aggregation.[54]

It has subsequently been observed that EDRF and NO· possess very similar biological and chemical properties; therefore, several investigators have proposed that EDRF is NO·. The experimental evidence for this identity is compelling. Two separate groups of investigators have shown using a bioassay system that EDRF and

NO· cause equipotent vasorelaxation of arterial and venous smooth muscle.[55,56] The in vitro half-life ($t_{1/2}$) of NO· and EDRF, as measured by bioassay, also appears to be identical ($t_{1/2}$ = 30 seconds).[55] Both substances activate guanylyl cyclase via heme-dependent mechanisms[41] in vascular smooth muscle and platelets and, thereby, evince vasorelaxation[51] and inhibition of platelet aggregation, respectively.[57] Lastly, EDRF and NO· appear to have the same susceptibility to a variety of inhibitors and potentiators. EDRF and NO· are potentiated by superoxide dismutase (SOD)[55] and inactivated by methylene blue,[51] oxygen[41] superoxide anion,[14] hemoglobin,[55] and myoglobin.[56]

However, there are several important differences between EDRF and NO· that have continued to fuel the controversey over the exact chemical identity of EDRF. Proximal and distal canine coronary arteries differ in their response to EDRF but not to NO·.[58] NO· appears to relax nonvascular smooth muscle consistently, whereas EDRF does not elicit this effect in all vascular smooth muscle beds.[59] Finally, Kelm and Schrader[60] have shown that the in vivo half-life of authentic NO· is 0.1 second, which is in sharp contrast to the estimated 6- to 30-second half-life of EDRF.

The short half-life of NO· in plasma is reflective of the existence of several reactants in the plasma milieu that inactivate NO·. In comparison, EDRF is comparatively more stable in plasma, with a half-life of 6 to 30 seconds, and this observation has led some investigators to suggest that NO· is stabilized in vivo by formation of NO adducts that have the similar biological activity as NO· but a much longer half-life.[35]

Biological thiols have been proposed as candidates for this stabilizing role, and we have shown that low molecular weight thiols readily combine with oxides of nitrogen to form S-nitrosothiols.[35] S-nitrosothiols have significantly longer half-lives than NO·, and they also possess EDRF-like platelet inhibitory and vasorelaxant properties in vivo.[35] Myers et al[61] have found that the biological potency and half-life of EDRF in a bioassay system far more closely resemble that of S-nitroso-L-cysteine ($t_{1/2}$ = 15 to 30 seconds) than that of NO· and have, therefore, proposed that EDRF is S-nitroso-L-cysteine.[61]

There is substantial evidence that S-nitrosothiols are active intermediates in the metabolism of organic nitrates[62,63] and in the cellular metabolism of NO.[35,36,64-66] We have shown that endogenous NO (probably as NO^+) reacts with serum albumin to form S-nitroso-albumin.[64] This comparatively stable, large pool of S-nitrosothiol has EDRF-like vasorelaxant properties and also inhibits platelet aggregation.[64] The S-nitrosation of other extracellular and intracellular proteins also likely occurs, thus representing a form of posttranslational modification that modulates protein function and cell phenotype.

The mechanism whereby NO is transferred from S-nitroso-albumin to the intracellular level remains unclear. S-nitroso-albumin is too large to cross endothelial cell membranes readily, and the 12-hour half-life of S-nitroso-albumin argues against spontaneous NO· release as the mechanism of NO· delivery to target cells.[64] We have recently obtained evidence that S-nitroso-L-cysteine may be an active intermediate in the transfer of NO from S-nitroso-albumin to the intracellular milieu by a mechanism of thiol-S-nitrosothiol exchange or transnitrosation.[66]

NO AND THE CARDIOVASCULAR SYSTEM

Physiological Effects

NO has been implicated in a wide range of physiological roles in the cardiovascular system (see Table 1). NO is involved in regulating vascular tone and myocardial contractility; maintaining endothelial integrity; and, also, inhibiting platelet aggregation. Dysregulation of NO production may play a role in the pathogenesis of several cardiovascular disorders including essential hypertension, reperfusion injury, atherosclerosis, and the myocardial depression associated with (septic) shock.

Regulation of vascular tone. NO is continuously produced by vascular endothelial cells, and this basal release of NO appears to regulate vascular tone.[67] In a rabbit model, systemic

Table 1. Cardiovascular Effects of NO

Regulation of vascular tone
Regulation of myocardial contractility
Antithrombotic effects in the vasculature
Regulation of endothelial-leukocyte interactions
Regulation of endothelial integrity and permeability
Regulation of vascular cell proliferation

doses of N^G-monomethyl-L-arginine (L-NMMA), a specific inhibitor of NOS, cause a marked increase in systolic blood pressure, and this observation suggests that continuous endothelial production of NO is involved in blood pressure regulation.[68] Vallance and colleagues[69] performed a similar experiment with normal human volunteers. Using L-NMMA locally infused into the brachial artery, they showed that endothelial-derived NO is integrally involved in regulating peripheral arteriolar tone.[69] By contrast, blocking angiotensin II production with a local infusion of angiotensin-converting enzyme inhibitors or by locally inhibiting prostaglandin synthesis has only minimal effects on peripheral vascular tone.[70] Thus, unlike these other vasoactive paracrine hormones, NO is a major determinant of basal vascular tone.[71] NO also plays a major role in determining the resting vascular tone of coronary resistance vessels,[52] regulates basal pulmonary vascular resistance,[72] and is responsible for autoregulating blood flow in several organ systems including the brain, heart, and kidney.[67]

NO production appears to be highest in arteries of smaller diameter (resistance).[14] NO may coordinate blood flow distribution between small arterioles and microvascular networks by regulating the diameter of these resistance vessels.[71] There is also evidence from rat experiments that NO controls the resting tone of different vascular beds, including the mesenteric, renal, and internal carotid systems.[73]

Vascular endothelial cells produce NO at a basal rate and also in response to physiological stimulation. Physiological stimuli for NO release include platelet products (such as ADP and serotonin), thrombin, shear stress, and changes in oxygen tension.[74] Shear stress, in particular, seems to be a major stimulus for NO release,[75] and this effect is regulated by a calcium-sensitive potassium channel that serves as a mechanochemical transducer in the endothelium.[76] NO activity is highest in larger diameter arteries that are subject to greater variations in pulsatile flow and shear stress.[71] NO appears to be involved in autoregulation of blood flow, both in large arteries and at the microcirculatory level, and may, thereby, be a critical determinant of the distribution of flow among the various vascular networks.[69,74]

NO release from the endothelium also appears to be regulated in part by the autonomic nervous system. NOS has been found in parasympathetic nerves located in the adventitia of cerebral and retinal arteries, where it may be involved in the regulation of vascular tone.[77] There is also recent evidence that NO attenuates arterial response to noradrenergic stimulation.[78] It remains unclear as to whether endothelial NO release is secondary to the noradrenergic stimulation itself or whether basal NO production is unmasked by a vasoconstrictive stimulus.[78]

Lastly, in addition to its role as a vasorelaxant, NO appears to modulate vascular tone by regulating the expression of endothelial vasoconstrictors and growth factors.[79] Hypoxia has been shown to induce the expression and secretion of endothelin-1 (ET-1),[80] a potent endothelial vasoconstrictor, and PDGF-B, a potent mitogen with some vasoconstrictive properties.[81] Hypoxia is also known to induce pulmonary artery vasoconstriction, and it has been suggested that ET-1 and PDGF-B may mediate hypoxic pulmonary vasoconstriction.[79] Kourenbanas and colleagues[79] have recently shown that NO suppresses ET-1 and PDGF-B production in an hypoxic environment by regulating the expression of these gene products at the level of transcription.[79] Interestingly, it has also been shown that PDGF-B suppresses endogenous NO production during atherogenesis[82]; therefore, it would appear that both NO and PDGF-B are involved in a negative feedback loop.

Regulation of myocardial contractility. NO also appears to be directly involved in the regulation of myocardial contractility. Brady and colleagues[83] have shown in vitro that endothelial-derived NO, and also sodium nitroprusside (a direct NO donor), cause a substantial reduction in cardiac myocyte contraction via a cGMP-dependent mechanism. These results suggest that the microvascular endothelium, which is in close proximity to cardiac myocytes in vivo, may influence myocardial contractility through NO release.[83]

NO production has also been shown within cardiac myocytes. Shultz and colleagues[84] have proven that cNOS and iNOS are present in adult rat ventricular myocytes. Ventricular cNOS does not appear to generate enough basal

release of NO to affect myocardial contractility because the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) has no effect on the contractility of normal myocytes in vitro.[85] By contrast, myocyte iNOS is readily inducible under conditions of experimental endotoxemia and, once induced, produces a level of NO release that reduces myocyte contractility significantly.[85]

NO also appears to play an important role in the autonomic modulation of myocardial function. Balligand and colleagues[86] have recently shown that an endogenous NO signaling pathway modulates rat myocyte responsiveness to adrenergic and cholinergic stimuli. They also showed that NOS inhibition significantly enhanced the inotropic effect of the β-agonist isoproterenol on rat ventricular myocytes, but had no effect on basal ventricular contractility.[86] These observations suggest that β-adrenergic stimulation of myocytes activates a countervailing NO signaling pathway that attenuates the effects of adrenergic stimulation.[87]

Regulation of antithrombotic properties of the endothelium. It is also apparent that NO is intimately involved in maintaining the integrity of the vascular endothelium by modulating platelet-vessel wall interactions. Endothelial NO production is stimulated by shear stress and by substances released during platelet activation (such as ADP or serotonin) or by determinants of the coagulation cascade, such as thrombin.[88] Once produced, NO diffuses from the endothelial cell into the vessel lumen, where it interacts with platelets. There is substantial in vitro evidence that NO is a potent inhibitor of platelet function. Mellion et al[57] have shown that NO inhibits ADP-induced platelet aggregation and also effectively disaggregates platelets; both of these effects are mediated by a cGMP-dependent mechanism.[57] We have shown EDRF is a potent inhibitor of platelet function and that this effect is potentiated by thiols.[89] Radomski et al[90] recently showed that human platelets contain an NOS that is activated when platelets are stimulated to aggregate. Platelets themselves also have the enzymatic capacity to synthesize NO,[90] with both a constitutive and inducible form of NOS identified in human megakaryoblasts.[91] NOS activity increases with platelet activation, and this response appears to modulate platelet aggregation, thereby potentially limiting the self-amplification of platelet thrombus formation in vivo. It also appears that human neutrophils inhibit platelet aggregation by releasing an NO-like factor.[92]

The antithrombotic properties of the endothelial surface may, in large part, be a consequence of the synergistic actions of NO and prostacyclin. Prostacyclin is a potent local vasodilator and as potent an inhibitor of platelet aggregation as NO.[54] By contrast with NO, the physiological antiplatelet effects of prostacyclin are mediated by an increase in cyclic adenosine 5′-monophosphate levels.[93] NO also causes vasodilation and inhibits platelet aggregation, but, unlike prostacyclin, NO also inhibits platelet adhesion to the vascular endothelium.[94] Radomski et al[95] and we[96] have shown the synergistic antiaggregatory effects of NO and prostacyclin on platelets. NO and prostacyclin may act in concert to oppose local vasospasm or thrombus formation at sites where platelets aggregate and the coagulation cascade is activated.[88] It has also been proposed that the antiplatelet effects of endothelial-derived NO may prevent thromboembolic events during administration of potent prostacyclin inhibitors such as aspirin.[14]

Regulation of endothelial-leukocyte interactions and vascular permeability. In addition to its antiaggregatory effects on platelets, NO has been reported to inhibit neutrophil aggregation in vitro,[92] and there is growing evidence that NO acts in vivo to inhibit leukocyte adhesion to the endothelium.[98] It has been reported that NO inhibition promotes leukocyte adhesion and emigration[97] and also causes a rapid increase in microvascular permeability and vascular protein leakage,[99] features characteristic of an acute inflammatory response.

Gaboury and colleagues[100] have shown that the antiadhesive effects of NO on neutrophils are related to its interaction with superoxide anion. Superoxide promotes leukocyte adhesion to the vascular endothelium,[101] and it has been postulated that the antiadhesive effects of NO are a chemical consequence of its ability to inactive this reactive oxygen derivative anion.[100] Kubes and coworkers[102] have proposed that, in the absence of NO, superoxide anion activates mast cells, causing degranulation that promotes leukocyte adhesion to the endothelium. Thus, a

relative imbalance in the levels of superoxide anion and/or NO may promote mast cell degranulation, leukocyte adherence, and leukocyte emigration, thereby inducing acute inflammation.[102]

Regulation of cell proliferation. NO can inhibit cell proliferation by a variety of mechanisms (see Table 2). By interacting with the tyrosyl radical at the active site of ribonucleotide reductase, NO can inactivate this enzyme, which is rate-limiting for nucleic acid synthesis. By interacting with cytochrome heme prosthetic groups, NO can impair electron transport and uncouple oxidative phosphorylation. Similarly, by promoting ADP-ribosylation of GADPH, NO can impair glycolysis.[10]

Endogenous NO can clearly function to modulate vascular smooth muscle proliferation, probably through these mechanisms and possibly others, as well. Garg and Hassid[103,104] have shown that exogenous NO inhibits vascular smooth muscle mitogenesis and proliferation in vitro, and that this mechanism is cGMP-dependent. Similar effects have been observed with fibroblasts.[105] We have shown that S-nitroso-albumin delivered at a site of mechanical endothelial denudation in rabbit femoral arteries can markedly attenuate platelet adhesion to the site and subsequent neointimal proliferative response.[106] Similarly, L-arginine, the substrate for NOS, has also been shown to impair neointimal proliferation after vascular balloon injury in rats,[107] and the overexpression of cNOS at a site of vascular injury has been shown to impair neointimal proliferation.[108] Recent evidence also suggests that PDGF impairs NO production in vitro[81]; therefore, it is possible that counteracting mechanisms prevail after injury, an imbalance among which may promote pathophysiological vascular responses.

Pathophysiological Effects

Under normal physiological conditions, NO plays a major role in regulating myocardial contractility, vascular tone, platelet-endothelial interactions, and leukocyte adhesion. Therefore, it is not surprising that dysregulation of NO production has widespread pathophysiological implications (see Table 3). Excess NO production has been implicated in the pathogenesis of sepsis-induced hypotension and myocardial depression.[10] In contrast, it has been suggested that a defect in endothelial NO production may contribute to the pathogenesis of essential hypertension.[109] There is also evidence that diminished NO production accelerates atherogenesis by allowing unopposed platelet-endothelial interactions and through loss of NO-mediated inhibition of vascular smooth muscle proliferation.[10]

Table 2. Antiproliferative Effects of NO in the Vasculature

Inhibition of ribonucleotide reductase
Inhibition of electron transport
Impairment of glycolysis
Impairment of endothelial-leukocyte interactions
Inhibition of platelet adhesion, secretion, and aggregation
Increases in intracellular cGMP

Table 3. Pathophysiological Cardiovascular Conditions in Which NO Plays a Role

Septic shock
Myocardial contractile dysfunction states
Ischemia-reperfusion injury
Atherosclerosis
Hypertension (essential and pulmonary)

Septic shock. Excess NO production may mediate the hypotension and myocardial depression associated with septic shock. Overwhelming infection with Gram-negative bacteria results in the release of endotoxin, a lipopolysaccharide component of bacterial cell walls that stimulates production of proinflammatory cytokines.[110] These cytokines are capable of activating iNOS in macrophages and vascular smooth muscle cells.[10] Interferon γ, tumor necrosis factor, IL-1, and IL-2 have all been shown to induce transcription of the iNOS gene.[111] Endotoxin has also been shown to induce transcription of iNOS in both vascular smooth muscle cells[112] and macrophages.[113] Once synthesized, iNOS produces abundant amounts of NO, resulting in the vasodilation and hypotension that characterize septic shock. Gonzales and colleagues[78] have reported that the vascular response to sympathetic nerve stimulation is modulated by NO, and they have suggested that overproduction of NO during sepsis attenuates the vascular response to noradrenergic nerve stimulation leading to vasodilation. Similarly, the potent vasodilatory effects of NO may underlie the attenuated vasoconstrictor response to agents, such as dopamine, that occur in sepsis.[114]

Because it is apparent that NO is integrally involved in the pathophysiology of septic shock, several new therapeutic strategies are directed at antagonizing NO overproduction in this syndrome. L-arginine analogues have been used as specific inhibitors of iNOS.[115] Glucocorticoids also appear to inhibit iNOS activity[116]; there may also be a role for methylene blue, which inhibits soluble guanylyl cyclase, modulates the redox state of NO, and directly inhibits NOS.[117,118]

Glucocorticoids have been shown to inhibit induction of iNOS after exposure to endotoxin.[116] In addition, in an experimental model of sepsis, dexamethasone markedly reduced endotoxin-induced NO synthesis and prevented the development of hypotension.[118] Methylene blue has recently been shown to reverse hypotension secondary to sepsis in association with normalization of plasma NO levels,[117] results consistent with inhibition by methylene blue of guanylyl cyclase or, perhaps, as the investigators suggest, with methylene blue-mediated inhibition of NO synthase in vivo.[116,117]

NOS inhibition can not only reverse endotoxin-induced hypotension,[117-119] but also improves the blood pressure of patients with sepsis and hypotension refractory to medical therapy. In these patients, NOS inhibition led to a rapid increase in mean arterial pressure from 50 to 90 mm Hg and also caused a dramatic increase in systemic vascular resistance.[120] However, despite hemodynamic improvement, the mortality in these patients was 100%.[120]

Although it appears that NOS inhibition may be an effective therapy for the treatment of hypotension secondary to sepsis, there is no evidence that NOS inhibitors reduce mortality, and there is concern that complete, nonselective inhibition of NO synthesis may have deleterious side effects. Nava and colleagues[118] have shown that, in an animal model of sepsis, higher doses of the NOS inhibitor L-NMMA are associated with a substantially higher mortality, suggesting that there may be a narrow therapeutic window for NOS inhibitors. Shultz and Raij[121] have shown that NOS inhibition during experimental endotoxemia may result in glomerular thrombosis. Thus, NO release during sepsis may be necessary to ensure adequate local perfusion to vital organs and to prevent vascular thrombosis in small arterioles, both by minimizing vascular resistance and by impairing platelet activation.[107]

Myocardial contractile dysfunction states. Septic shock is often accompanied by myocardial contractile dysfunction, and there is evidence that cytokines mediate this effect through activation of a constitutive NO synthase.[122] Finkel and coworkers[122] have recently shown that tumor necrosis factor-α, IL-6, and IL-2 have negative inotropic effects on cardiac muscle in vitro. The onset of the negative inotropic effect is rapid and reversible, suggesting that these effects of cytokines are mediated through activation of a constitutive myocardial NO synthase.[122] In contrast, Balligand and colleagues have shown that lipopolysaccharide-induced NO production by cardiac myocytes causes contractile dysfunction, and they have suggested that a myocardial iNOS mediates these effects.[123] These findings are most consistent with the presence of both cNOS and iNOS isoforms in ventricular myocytes.[123]

Finkel and colleagues[122] have also proposed that cytokines may be involved in the pathogenesis of postischemic myocardial depression ("myocardial stunning"). During reperfusion, activated leukocytes and macrophages infiltrate into ischemic myocardium. Release of proinflammatory cytokines from these immune cells may activate myocardial iNOS leading to excessive NO production and secondary myocardial stunning.[122] Elevated concentrations of IL-6 have been found in samples of pulmonary venous blood after cardiopulmonary bypass, and it has been suggested that myocardial stunning, a frequent occurrence after bypass surgery, may also be mediated by cytokine release.[124]

Reperfusion injury. Reperfusion-induced myocardial injury is mediated by release of oxygen-derived free radicals from activated leukocytes. Superoxide anion ($O_2^{\cdot -}$) is thought to be intimately involved in reperfusion-induced injury because SOD has been shown to reduce postischemic injury.[125] Beckman and colleagues have proposed that, during reperfusion, production of both $O_2^{\cdot -}$ and $NO^{\cdot}$ is stimulated, and these two substances rapidly react to form peroxynitrite ($ONOO^-$).[126] Peroxynitrite is a very powerful oxidant that has been implicated in membrane lipid peroxidation and protein

oxidation reactions[33] in biological systems. It may be that reperfusion injury is mediated by peroxynitrite formation, and that the protective effects of SOD relate to its ability to prevent formation of the reactant superoxide required for peroxynitrite formation during reperfusion.[126]

There is evidence that inhibition of NO synthesis at the onset of reperfusion reduces subsequent injury. Chen and colleagues[127] administered an NOS inhibitor at the time of reperfusion in a mouse middle cerebral artery occlusion model and showed a substantial reduction in infarct size. This protective effect may reflect the fact that NOS inhibition probably reduces peroxynitrite formation.

However, there is equally compelling evidence that NO functions as a protective agent during ischemia-reperfusion. In a cat model of myocardial ischemia-reperfusion, infusion of NO during reperfusion significantly reduced the extent of myocardial necrosis as compared with that of control.[128] There is also evidence that NOS inhibition can increase tissue damage during cerebral ischemia-reperfusion.[129]

There are a number of possible mechanisms whereby NO may provide protection against reperfusion injury. First, neutrophils have a well-defined role in the inflammatory response to tissue injury. NO limits inflammatory injury by preventing leukocyte emigration and adherence[98] and the leukocyte-dependent increase in microvascular permeability.[99] NO also inactivates superoxide anion, which is a strong promoter of leukocyte adhesion.[100] Lastly, NO inhibits neutrophil superoxide anion production through a direct effect on NADPH oxidase.[130]

Atherosclerosis. Hypercholesterolemia is a well-known risk factor for atherosclerosis, and it has been suggested that endothelial dysfunction secondary to hypercholesterolemia may be involved in the early pathogenesis of the disease process.[131] Recent evidence suggests that hyperlipoproteinemia also impairs endothelial function in the absence of atherosclerosis. Takahashi and colleagues[132] have shown in vitro that lipoproteins (high-density lipoprotein, low-density lipoprotein, and very low-density lipoprotein) inhibit endothelium-dependent relaxation. Cohen and coworkers[133] have shown that impaired endothelium-dependent relaxation occurs in the coronary arteries of swine fed a high cholesterol diet in the absence of any histologic evidence of atherosclerosis.

In normal endothelium, NO production causes vasodilation, inhibits platelet aggregation, and attenuates vascular smooth muscle proliferation; hypercholesterolemia appears to interfere with these normal endothelial functions. Shimokawa and Vanhoutte[134] have shown that hypercholesterolemia impairs endothelium-dependent relaxation in response to aggregating platelets. Lefer and Ma[135] have shown that hypercholesterolemia leads to a reduction in basal NO production by rabbit coronary artery endothelium that, in turn, promotes increased leukocyte adherence. Through these mechanisms, it is possible in part that, by impairing endothelial function, hypercholesterolemia may induce or accelerate atherogenesis. Endothelial dysfunction may also promote atherogenesis by allowing unopposed platelet aggregation and vascular smooth muscle proliferation.[136]

Creager and colleagues[137] have shown in humans that hypercholesterolemia impairs vasodilation in resistance vessels. The mechanism by which this effect occurs remains to be elucidated. It has been proposed that either a reduction in NO synthesis or release or possibly an increase in NO inactivation accounts for the impairment of endothelium-dependent relaxation in these individuals.[136] In support of the former hypothesis, Creager's group has recently shown that infusion of L-arginine acutely improves endothelial-dependent vasodilation in hypercholesterolemic patients, possibly by increasing NO synthesis.[137]

It has also been shown that endothelium-mediated vasodilation is attenuated in atherosclerotic human coronary arteries.[138] This impairment in endothelium-dependent relaxation may be a consequence of decreased production or impaired diffusion of NO owing to the presence of a thickened intima.[138] Diminished NO production at atherosclerotic sites may further promote atherogenesis by allowing unopposed platelet aggregation and adhesion of platelets and leukocytes.[74] Recently, Johnstone and colleagues[139] have shown that patients with insulin-dependent diabetes mellitus have impaired endothelium-dependent vasodilation, and it may

be that the high prevalence of vascular disease in this patient population is related to the loss of NO's protective effects.[139]

Hypertension. Panza and coworkers[109] have recently shown that patients with essential hypertension have a defect in EDRF (NO) release both under basal conditions and during activation of the endothelium. This defect does not appear to be caused by reduced availability of substrate for NO production but, rather, may be a consequence of impaired NO synthesis or release by endothelial cells.[140] Interestingly, animal studies have shown that antihypertensive therapy corrects the impairment in endothelium-dependent vasodilation, suggesting that hypertension may induce endothelial dysfunction rather than result from it.[141]

CARDIOVASCULAR THERAPEUTICS

Organic nitrates and other nitrosovasodilators serve as an exogenous source of NO and, therefore, exert many of the same physiological effects, including vasorelaxation, inhibition of platelet aggregation, and attenuation of vascular smooth muscle proliferation. In addition to their conventional use, there are several relatively novel clinical cardiovascular settings in which NO donors might be useful. NO donors may be useful for the inhibition of the vascular smooth muscle proliferation during atherogenesis or after balloon angioplasty. Nitrosovasodilators have proven efficacy in acute coronary syndromes, and this effect may be a consequence of their antiplatelet effect as well as vasodilator properties.[142] They may also be useful in treating disease processes as diverse as reperfusion injury, pulmonary hypertension, and adult respiratory distress syndrome (ARDS). In addition, dysregulation of NO production has been implicated in the pathogenesis of sepsis and myocardial stunning, and NOS inhibitors have been used experimentally in these conditions as discussed above.

Antiplatelet Therapy

During its metabolism, nitroglycerin is denitrified and reduced to form NO.[143] In contrast, sodium nitroprusside spontaneously releases NO, probably as NO^+.[31] Both nitroglycerin and nitroprusside have antiaggregatory effects on platelets and promote platelet disaggregation in vitro.[57,144] Nitroglycerin also reduces local vasoconstriction and platelet adherence after in vivo arterial wall injury.[145]

There is substantial in vivo evidence that organic nitrates are potent inhibitors of platelet aggregation. Nitroglycerin infusion has been shown to prolong bleeding times significantly.[146,147] Decaterina and colleagues[148] infused isosorbide dinitrate into 11 volunteers and observed marked inhibition of platelet aggregation ex vivo.[148] Kuritsky[149] showed that sublingual nitroglycerin successfully disperses platelet-rich thrombi in the retinal circulation. In the model by Folts et al[150] of canine coronary stenosis mimicking unstable angina pathophysiology, continuous nitroglycerin infusion inhibits platelet aggregation within 40 minutes and at doses that have only a modest effect on blood pressure.

Platelets metabolize organic nitrates in a manner analogous to vascular smooth muscle. NO generation is catalyzed by sulfhydryl species and involves both denitrification and reduction.[151] Loscalzo[63] and Ignarro et al[62] have proposed that, during denitrification, organic nitrates react with thiols to form active S-nitrosothiol intermediates. These S-nitrosothiol species have been shown to inhibit platelet aggregation in a cGMP-dependent mechanism.[142]

Mendelsohn and colleagues[152] have proposed a biochemical mechanism by which S-nitrosothiols inhibit platelet aggregation. S-nitrosothiols activate guanylyl cyclase, leading to an increase in intraplatelet cGMP that is inversely correlated with fibrinogen binding to the platelet surface integrin, glycoprotein IIb/IIIa.[152] Elevation of cGMP is also associated with a reduction in the intracellular calcium flux that usually accompanies platelet activation, an ion transient required for the induction of a conformational change in the fibrinogen-binding integrin.[152]

Organic nitrates and other nitrosovasodilators are of proven benefit in acute coronary syndromes, and this efficacy is widely believed to be a principal consequence of vascular smooth muscle relaxation. However, in vivo studies have shown the significant antiplatelet effects of

organic nitrates and other nitrosovasodilators, suggesting that the antithrombotic actions of these drugs may contribute substantially to their therapeutic efficacy. A meta-analysis by Yusuf et al[153] has shown that intravenous nitroglycerin (and also nitroprusside) used in the setting of acute myocardial infarction reduces mortality by 35%, provided systemic blood pressure reduction is not excessive (systolic blood pressure, ≥ 90 mm Hg). This substantial reduction in mortality is comparable with the results of trials using antiplatelet therapy alone and, therefore, suggests that the antithrombic properties of these drugs contribute substantially to their clinical efficacy.[142] Unfortunately, two recent, large randomized trials failed to confirm these benefits of nitrates used in the setting of acute myocardial infarction.[154,155] Therefore, the notion that their antiplatelet action improves clinical outcome remains only a theoretical possibility at this time.

Antiproliferative Therapy

In addition to their antiaggregatory effects on platelets, nitrosovasodilators have an inhibitory effect on vascular smooth muscle proliferation. S-nitroso-N-acetylpenicillamine and isosorbide dinitrate have been shown to inhibit fibroblast mitogenesis in vitro.[105] Nitroprusside, isosorbide dinitrate, and S-nitroso-N-acetylpenicillamine have been shown to inhibit vascular smooth muscle mitogenesis and proliferation, suggesting that endogenous NO inhibits vascular smooth muscle cell growth under normal circumstances.[103]

When the endothelium is damaged, whether by atherosclerosis or balloon angioplasty, local production of NO is decreased resulting in unopposed platelet aggregation and vasoconstriction.[74] Platelet activation and aggregation lead to growth-factor release and subsequent vascular smooth muscle proliferative responses.[156] Intravenous nitroglycerin has been used successfully to prevent platelet adherence and vasoconstriction after balloon angioplasty.[145] Thus, organic nitrates and other NO donors may have potential therapeutic benefit as inhibitors of vascular smooth muscle proliferation during atherogenesis and after angioplasty.

Therapy of Ischemia-Reperfusion

There is also evidence that NO donors may be useful in attenuating ischemia-reperfusion injury. In addition to limiting platelet-endothelial interactions and inhibiting vascular smooth muscle proliferation, NO inhibits neutrophil chemotaxis and adhesion.[98] Ma and colleagues[157] have shown that decreased basal release of NO after myocardial ischemia-reperfusion promotes neutrophil adhesion to the coronary endothelium, which, in turn, may lead to neutrophil-induced reperfusion injury.[157] The investigators also suggest that loss of basal NO release may exacerbate reperfusion injury by promoting vasoconstriction, platelet aggregation, and release of platelet mediators.[157] Recently, Lefer and colleagues[158] showed that infusion of the NO donor, 5PM-5185, at the time of reperfusion in a canine model of ischemia-reperfusion resulted in a reduction in neutrophil accumulation and myocardial necrosis. The protective effects of this NO donor appeared to be related to inhibition of neutrophil adherence to the coronary endothelium.[158]

The role of NO in the pathogenesis of ischemia-reperfusion remains controversial. In a rat model of middle cerebral artery occlusion, Kuluz and colleagues[159] showed that NOS inhibition before arterial occlusion worsens ischemia-reperfusion injury and increases infarct volume. By contrast, Beckman and Crow[160] showed in a rat model of middle cerebral artery occlusion that NOS inhibition at the time of reperfusion substantially reduces infarct volume. The discrepancy between these results may reflect the timing of NOS inhibition. NOS inhibition increases blood pressure but may also reduce cerebral perfusion substantially; therefore, preischemic treatment with NOS inhibitors may increase ischemic injury.[127] By contrast, NOS inhibition at the time of ischemia may reduce infarct size by preventing NO's deleterious biochemical side effects discussed above.[160]

Other Therapeutic Considerations

Recognition of the therapeutic potential of exogenous NO donors has lead to the development of a new class of nitrovasodilators called sydnonimines. The sydnonimines include mol-

sidomine and its metabolite 3-morpholino-sydnonimine (SIN-1).[161] SIN-1 is the active intermediate of molsidomine, and it reacts with molecular oxygen to form NO through a process that involves a 1-electron abstraction.[162] SIN-1 induces vascular smooth muscle relaxation and inhibits platelet aggregation through a cGMP-dependent mechanism[163]; prolonged exposure to SIN-1 does not cause tolerance.[161] Bath[163] has shown that SIN-1 and nitroprusside, two spontaneous NO donors, inhibit monocyte chemotaxis in vitro. By contrast, isosorbide dinitrate and nitroglycerin have no effect on monocyte function, suggesting that monocytes are unable to metabolize directly isosorbide dinitrate and nitroglycerin.[163] These results suggest that SIN-1 might be useful to inhibit monocyte function during atherogenesis.[163] SIN-1 has also been shown to have a cardioprotective effect in an animal model of myocardial ischemia-reperfusion.[164]

The S-nitrosothiols are another therapeutic class of NO donors that appear to have great therapeutic potential. S-nitrosothiols are potent vasodilators and antiplatelet agents and, therefore, may be useful in treating hypertension or acute coronary syndromes.[165] Loscalzo's group have synthesized an S-nitrosated derivative of captopril, S-nitroso-captopril (SnoCap), that combines the properties of an angiotensin-converting enzyme inhibitor with those of a direct nitrosovasodilator.[166] In vivo hemodynamic studies in anesthetized dogs have shown the efficacy of SnoCap, raising the possibility that SnoCap may be useful in treating vascular disorders such as essential hypertension, coronary artery disease, and congestive heart failure.[167]

Recent investigative work has focused on the therapeutic use of NO in pulmonary hypertension and ARDS. Frostell and colleagues[168] have shown that inhaled NO reverses hypoxic pulmonary vasoconstriction in humans without inducing systemic vasodilation. Pepke-Zaba and coworkers[169] treated eight patients with severe pulmonary hypertension with inhaled NO and showed an average reduction in pulmonary vascular resistance of greater than 30% without significant change in systemic vascular resistance. Finally, Rossaint and colleagues[170] treated nine patients with severe ARDS with inhaled NO and showed a reduction in pulmonary-artery pressure and an increase in PaO_2. Oxygenation presumably improves because NO is distributed by ventilation and, therefore, selectively improves perfusion to well-ventilated regions.[171]

CONCLUSIONS

An understanding of the biology of NO has expanded rapidly and dramatically over the past decade. In particular, the role of NO in the cardiovascular system has proven to be multifaceted and important for both normal function and pathophysiological response. A clearer understanding of the molecular mechanisms by which NO exerts its beneficial and adverse cardiovascular effects will very likely lead to judicious and novel therapeutic strategies in the future.

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