Cytoprotective Effects of Nitric Oxide

John P. Cooke, MD, PhD; Philip S. Tsao, PhD

In the myocardium it subserves, thrombosis of a coronary artery precipitates metabolic alterations that are characterized by progressive reductions in high-energy phosphate stores and a build-up in toxic metabolites that will ultimately lead to cell death unless perfusion is restored. Although reperfusion is mandatory to achieve myocardial salvage, it is often associated with a paradoxical acceleration of myocardial dysfunction and perhaps even myocyte death.* In an era of myocardial revascularization and thrombolysis, it has become critical to understand the cellular mechanisms of reperfusion injury so as to derive new therapeutic strategies.

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A key concept derived from experimental work is that reperfusion injury is triggered by dysfunction of the reperfused endothelium. During the ischemic period, endothelial ATP levels decrease, intracellular hypoxanthine increases, calcium metabolism and permeability are altered, and xantine dehydrogenase is converted into the oxidant-generating xanthine oxidase. With reperfusion comes an influx of calcium and molecular oxygen that precipitates a sudden burst of oxygen-derived free radicals, released as endothelial xantine oxidase converts molecular oxygen into superoxide anion. Superoxide anion, and free radicals derived from it (hydrogen peroxide and hydroxyl anion), attack polyunsaturated fatty acids in the endothelial cell membrane to generate lipid peroxides, thereby altering cell membrane fluidity, increasing calcium permeability, and activating phospholipase A. The subsequent increase in intracellular calcium and activation of phospholipase A cause the endothelium to elaborate platelet-activating factor (PAF), leukotriene B, (LTB), thromboxane A (TXA), and complement component C. These fluid-phase mediators are potent amplifiers of inflammation: PAF induces the expression of the endothelial adhesion molecule P-selectin and upregulates the activity of the neutrophil adhesion molecule CD (a member of the integrin family of adhesion molecules); in addition, PAF, LTB, TXA, and C, act as potent chemoattractants that mediate neutrophil activation, adherence, and diapedesis. Neutrophils entering this disordered milieu become activated and adhere to the altered endothelium. Incited by the paracrine factors released by the endothelium, the neutrophils also begin to generate superoxide anion (via NADPH oxidase) as well as proteases (such as elastase) and cytokines (TNF, interleukin-). The oxygen-derived free radicals released by the neutrophils compound the endothelial injury and induce myocyte necrosis as the neutrophils migrate into the subjacent tissue. Their emigration is dependent on neutrophil-derived elastase; other neutrophil-derived proteases contribute to myocyte necrosis. In addition, the cytokines released from the activated neutrophils induce the expression of other endothelium-derived adhesion molecules (e.g., ICAM-I and E-selectin) that sustain the inflammatory process.

Inhibitors of Reperfusion Injury

Reperfusion injury may be viewed as an imbalance between endogenous forces that promote and those that inhibit cell adherence and activation. Two potent endogenous inhibitors of cell adherence and activation are prostacyclin and nitric oxide (NO). and prostacyclin not only are potent vasodilators but also inhibit leukocyte adherence and act synergistically to inhibit platelet interaction with the vessel wall. Elaboration by the endothelium and/or activity of both substances is dramatically reduced in reperfusion injury. Indeed, one of the earliest observed abnormalities in reperfusion injury is an endothelial dysfunction manifested by a loss of NO-dependent vasodilatation. This phenomenon occurs within 2.5 minutes of reperfusion in animal models.

The importance of NO as an inhibitor of neutrophil-endothelial cell interaction has been demonstrated by Kubes and colleagues. These investigators observed and quantitated neutrophil adherence to the vessel wall in the perfused rat mesenteric venule using videomicroscopy. Infusion of the NO synthase antagonist -N-methylarginine induced a 10-fold increase in neutrophil adherence to the vessel wall; this effect was mediated by the neutrophil adhesion molecule CD11/18 since a monoclonal antibody directed against the chain of this glycoprotein adhesive complex blocked the effect of the NO synthase antagonist. Restoration of NO synthesis by infusion of the precursor -arginine also restored the antiadhesive property of the endothelium. Treatment with the NO precursor also improves NO-dependent vasodilatation of the reperfused feline coronary artery; this effect is associated with a reduction in neutrophil accumulation and myocyte necrosis of the reperfused region. Similarly, beneficial effects of the NO precursor have been observed in the reperfused feline intestine. Other investigators have shown that NO prevents neutrophil aggregation, an effect that is potentiated by

*From the Division of Cardiovascular Medicine, Stanford University School of Medicine, Stanford, Calif.

†Correspondence to John P. Cooke, MD, PhD, Division of Cardiovascular Medicine, Stanford University School of Medicine, 300 Pasteur Dr, Stanford, CA 94305.

*Myocyte necrosis due to reperfusion injury has been demonstrated in animal models, but it is not yet clear whether this degree of reperfusion injury occurs in humans.
superoxide dismutase.\textsuperscript{18} In addition to its activity against neutrophil aggregation and adherence, NO inhibits NADPH oxidase, the key enzyme responsible for neutrophil-derived superoxide anion.\textsuperscript{19}

To summarize, reperfusion injury is initiated by an endothelial dysfunction that can be characterized as an imbalance between endogenous factors that promote and those that inhibit the activation and interaction of neutrophils with the endothelium. On the basis of this paradigm, three therapeutic strategies have been explored and have received experimental support:

- Inhibit the production of or chemically inactivate superoxide anion or other reactive oxygen intermediates derived from endothelial cells or neutrophils. Agents of this class that have shown experimental promise include superoxide dismutase, allopurinol, catalase, and iron chelators.\textsuperscript{5}
- Inhibit the production or antagonize the action of lipid or protein mediators of the inflammatory response triggered by reactive oxygen intermediates. Compounds of this sort include inhibitors of phospholipase A\textsubscript{2}, lipooxygenase, and elastase, as well as antagonists of receptors for PAF or LT\textsubscript{B}, or monoclonal antibodies to neutrophil or endothelial cell adhesion molecules.\textsuperscript{5}
- Replace or augment the synthesis of endogenous inhibitors of cell adhesion and activation. Agents of this class include prostacyclin or its derivatives, defibrotide (an enhancer of prostacyclin action), L-arginine (the NO precursor), and NO donors.\textsuperscript{16,20,21}

**Cytoprotective Effects of an NO Donor**

In this issue of *Circulation*, Lefer and coworkers\textsuperscript{22} have provided further evidence for a cytoprotective role of NO. In this elegantly executed study, an open-chest anesthetized canine model of ischemia-reperfusion was used to investigate the efficacy of the NO donor SPM-5185 (a cysteine-containing mononitrate that releases NO). In this model, ligation of the left anterior descending coronary artery (LAD) for 1 hour followed by reperfusion for 4.5 hours induced marked elevation of creatinine kinase (CK) activity, reflecting necrosis of about 40\% of the ischemic myocardium. By contrast, when the LAD was reperfused with blood containing SPM-5185 rather than vehicle, there was a significant reduction in plasma CK associated with necrosis of only 10\% of the ischemic myocardium. Inhibition of myocardial necrosis was also associated with a reduction in segmental stiffness and an improvement in postischemic segmental work.

The investigators hypothesized that the effect of the NO donor to limit reperfusion injury was due to its ability to abrogate neutrophil adherence to and diapedesis through the reperfused endothelium, thereby protecting the subjacent myocytes from neutrophil-generated, oxygen-derived free radicals. One measure of neutrophil infiltration is tissue myeloperoxidase. This oxidative enzyme is enriched in neutrophils but is normally found in low levels in the myocardium. After reperfusion injury, Lefer and coworkers observed that levels of myeloperoxidase rose dramatically in the ischemic myocardium of vehicle-treated animals but to a much lesser degree in animals receiving the NO donor. The mechanism by which SPM-5185 suppressed neutrophil accumulation may be related to its action on neutrophil and endothelial adhesiveness. To examine this hypothesis, Lefer’s group studied the effect of this agent on neutrophil-endothelial interaction using a functional binding assay in vitro. They discovered that neutrophils stimulated by leukotriene B\textsubscript{2} adhered to the endothelium of coronary arteries isolated from normal dogs. This endothelium-neutrophil interaction was associated with pronounced contraction of the coronary artery rings as well as with endothelial injury manifested by attenuation of NO-dependent vasodilation. Neutrophil adhesion was blocked by a monoclonal antibody directed against the common \(\beta\)-chain of the CD11-CD18 neutrophil adhesion molecule; inhibition of neutrophil binding was associated with inhibition of constriction and prevention of endothelial injury. SPM-5185 had identical effects on neutrophil adherence and vascular reactivity.

To summarize, the salutary effects of the NO donor appear to be due to its ability to suppress neutrophil adherence to the endothelium. However, the NO donor also reduced myocardial oxygen demand during ischemia-reperfusion (largely due to reduction in heart rate) and increased myocardial blood flow. Therefore, inhibition of myocardial necrosis by this agent may have been due in part to reduction in myocardial work load and increase in myocardial oxygen supply. The effect of SPM 5185 on myocardial blood flow may have been due in part to its modest vasodilatory action at the dose used in this study. However, an action of the NO donor on neutrophil adherence in the microvasculature may have played a greater role since the no-reflow phenomenon after reperfusion is mediated by leukocyte adherence to and obstruction of the resistance vessels.\textsuperscript{3,5}

**Cytoprotective Effects of Endogenous NO**

Endothelium-derived NO is a potent endogenous nitrovasodilator, and it plays a major role in modulating vascular tone. This paracrine factor exerts its effect by stimulating soluble guanylate cyclase in the underlying vascular smooth muscle cells, thereby elevating intracellular levels of cGMP and inducing relaxation of the vascular smooth muscle.\textsuperscript{23} Exogenous nitrovasodilators such as nitrogllycerin exert their effects via the same mechanism. Endogenous NO is now known to be derived from the guanidino nitrogen atoms of L-arginine, which is metabolized by NO synthase to L-citrulline and NO.\textsuperscript{24-26}

NO not only is a potent vasodilator but also inhibits interaction of the vessel wall with circulating blood elements. Indeed, platelet adhesion and aggregation induced by intimal injury of the canine coronary artery are attenuated by nitroglycerin (exogenous NO donor) as well as by intravenous infusion of L-arginine (the NO precursor).\textsuperscript{27} When human platelet-rich plasma is incubated ex vivo with aspirin-treated endothelial cells, an inhibition of platelet aggregation is observed that can be reversed by hemoglobin (which scavenges NO) or methylene blue (which inhibits the action of NO on soluble guanylate cyclase).\textsuperscript{6} The effect of NO in inhibiting platelet adhesion and aggregation is associated with increases in the level of platelet cGMP.\textsuperscript{7,8} Because NO inhibits platelet adhesion to the vessel wall and aggregation within the lumen, it is likely that this endothelial factor plays an important role in preventing coronary
thrombosis. Therefore, novel therapeutic agents that enhance endogenous NO synthesis or supplement its activity will be useful in platelet-mediated ischemic syndromes such as unstable angina and coronary thrombosis.

NO is also a potent modulator of monocyte interaction with the vessel wall. Monocyte adherence to endothelial cells in culture is inhibited by introduction of NO into the culture medium.\textsuperscript{9} Migration of monocytes induced by the chemotractant peptide fmlp in vitro is also inhibited by exogenous NO.\textsuperscript{9} Furthermore, NO abrogates the ability of macrophages to oxidize low-density lipoprotein.\textsuperscript{28}

**Importance of NO in Other Cardiovascular Disorders**

These properties of NO may explain the antiatherogenic effects of its precursor, l-arginine. Early in the course of hypercholesterolemia, an endothelial dysfunction occurs that is manifested by reduction in NO-dependent vasodilation.\textsuperscript{29-31} This abnormality can be acutely reversed by intravenous administration of l-arginine, in hypercholesterolemic animal models as well as in humans.\textsuperscript{32-35} Moreover, chronic dietary administration to hypercholesterolemic rabbits is also associated with improvement in NO-dependent vasodilation, which is sustained throughout the course of treatment.\textsuperscript{36} This improvement in endothelial function is associated with a striking reduction in the intimal lesions in the rabbit thoracic aorta.\textsuperscript{37} Evidence from our laboratory leads us to propose that this salutary effect of dietary l-arginine is mediated in large part by inhibition of endothelial-monocyte interactions. We have studied these interactions by observing the binding of fluorescent-labeled mononuclear cells to vascular segments in vitro. Vascular segments from hypercholesterolemic rabbits exhibited a greater affinity for mononuclear cells than did segments from normocholesterolemic animals. Dietary arginine treatment did not affect lipoprotein levels in hypercholesterolemic animals but was associated with a marked reduction in the affinity of the endothelium for mononuclear cells (unpublished studies).

Restenosis is another cardiovascular disorder that may be modulated by endogenous NO. Balloon injury to the vessel wall removes the endothelial source of NO, derived from the constitutive form of NO synthase. However, the cytokines elaborated by the injured vessel wall and circulating blood elements activate synthesis in the vessel wall of the inducible isof orm of NO synthase.\textsuperscript{37,38} That this new enzyme activity can modulate the progression of the intimal lesion has recently been shown by McNamara and colleagues.\textsuperscript{39} These investigators subjected the iliac artery of the New Zealand White rabbit to balloon angioplasty. The NO precursor was administered to one group of animals; in comparison to the vehicle-treated group, the l-arginine-treated animals exhibited 40% less intimal thickening. The salutary effect of l-arginine was blocked by the antagonist of NO synthase, L-nitroarginine methyl ester.

In summary, endogenous NO plays a dominant role in modulating conduit vessel diameter and vascular resistance by virtue of its ability to potently relax vascular smooth muscle. For this reason, alterations in its synthesis or activity may play an important role in some vasospastic disorders and hypertensive states. Moreover, because of its ability to inhibit interactions of circulating blood elements with the vessel wall, a deficit of endogenous NO may promote vascular thrombosis, restenosis, atherosclerosis, and reperfusion injury. Novel therapeutic strategies for these cardiovascular disorders will certainly be derived from fresh basic insights regarding measures to enhance the synthesis or effect of endogenous NO or to supplement its activity with exogenous NO donors that are designed to circumvent the deficiencies of available nitrovasodilator therapy.

**References**

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J P Cooke and P S Tsao

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