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Nitric Oxide Insufficiency, Platelet Activation, and Arterial Thrombosis

Regulation of Vascular Bed-Specific Prothrombotic Potential

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Insights Into Mechanisms of Thrombosis From Genetic Models

Molecular Pathogenesis of Antiphospholipid Antibody Syndrome

Joseph Loscalzo, Guest Editor

Nitric Oxide Insufficiency, Platelet Activation, and Arterial Thrombosis

Joseph Loscalzo

Abstract—Nitric oxide (NO) was originally discovered as a vasodilator product of the endothelium. Over the last 15 years, this vascular mediator has been shown to have important antiplatelet actions as well. By activating guanylyl cyclase, inhibiting phosphoinositide 3-kinase, impairing capacitative calcium influx, and inhibiting cyclooxygenase-1, endothelial NO limits platelet activation, adhesion, and aggregation. Platelets are also an important source of NO, and this platelet-derived NO pool limits recruitment of platelets to the platelet-rich thrombus. A deficiency of bioactive NO is associated with arterial thrombosis in animal models, individuals with endothelial dysfunction, and patients with a deficiency of the extracellular antioxidant enzyme glutathione peroxidase-3. This enzyme catalyzes the reduction of hydrogen and lipid peroxides, which limits the availability of these reactive oxygen species to react with and inactivate NO. The complex biochemical reactions that underlie the function and inactivation of NO in the vasculature represent an important set of targets for therapeutic intervention for the prevention and treatment of arterial thrombotic disorders. (*Circ Res.* 2001;88:756-762.)

Key Words: endothelium ■ nitroglycerin ■ *S*-nitrosothiols ■ nitric oxide synthase ■ oxidative stress

Nitrovasodilators have been used to alleviate myocardial ischemia for more than a century, and the recognition that they simulate endogenous endothelial nitric oxide (NO) represents one of the most important biological discoveries in cardiovascular biomedicine in the last 20 years.¹ Their antianginal effects have been mostly attributed to relaxation of vascular smooth muscle and resulting arterial vasodilation, which leads to improved perfusion and oxygen delivery. This class of drugs also manifests antithrombotic activity, principally by inhibiting platelet function. First shown by Hampton et al² in 1967, the antiplatelet effect of nitroglycerin remained controversial for many years because of the suprapharmacological doses required to inhibit platelet aggregation *in vitro* and the lack of evidence for a direct antiplatelet effect *in vivo*. Akin to its effects in vascular smooth muscle cells,^{3,4} nitro-

glycerin also inhibits platelet aggregation by activating guanylyl cyclase,^{5,6} and this inhibitory action can be potentiated by maintaining intracellular thiol redox state.⁶ These observations, coupled with the recognition of the role of platelet-dependent thrombus formation in acute coronary syndromes, led to renewed interest in the antiplatelet effects of nitrovasodilators and, eventually, of NO itself.

In 1985, we reported the case of a 29-year-old woman with a hypertensive crisis treated with sodium nitroprusside for blood pressure control who sustained an intracerebral hemorrhage after being normotensive on therapy for 24 hours.⁷ We showed that her bleeding time was prolonged, *in vitro* platelet aggregation was attenuated in the presence of *N*-acetyl-L-cysteine, and these abnormalities returned to normal with discontinuation of the sodium nitroprusside. These

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data were confirmed in a prospective assessment of platelet function in patients treated with intravenous nitroglycerin; only on restoring platelet thiol stores by addition of *N*-acetyl-L-cysteine *ex vivo* was the inhibitory action of nitroglycerin apparent.⁸ Furthermore, in an animal model of acute platelet-mediated coronary thrombus formation, pharmacologically relevant concentrations of intravenous nitroglycerin can inhibit platelet-dependent cyclic flow reductions, and this effect can be potentiated by *N*-acetyl-L-cysteine.⁹ Since these initial observations, several studies have confirmed the antiplatelet effects of nitrovasodilators *in vivo* and the importance of the vascular redox state and oxidative stress on nitrovasodilator metabolism and action.^{10,11}

Endothelium-Derived NO and Inhibition of Platelet Function

In Vitro Studies

The identification of endothelium-derived relaxing factor as NO or a closely related derivative thereof^{12,13} and the demonstration that endothelial NO has antiplatelet effects^{14,15} raised the question of mechanism. Analogous to assessing the importance of thiols in the action of nitrovasodilators, we first examined the effects of thiols on endothelial NO and demonstrated that in addition to undergoing oxidation to nitrite and nitrate, reacting with superoxide anion to form peroxy-nitrite, and reacting with heme iron to form the charge-transfer complex required to activate guanylyl cyclase, NO and oxygen or peroxy-nitrite can react with thiols to form *S*-nitrosothiols.^{16,17} These latter compounds serve as stable reservoirs of NO, which can be transferred to and from protein-bound pools, such as *S*-nitroso-serum albumin,¹⁸ by *trans*-*S*-nitrosation reactions.^{19,20} Recent studies also suggest that *S*-nitrosothiols can be stored by platelets and released during heterotypic cellular interactions.²¹

N-Acetyl-L-cysteine potentiates the antiplatelet effect of endothelial NO,²² and this action can be mimicked by the *S*-nitrosothiol *S*-nitroso-*N*-acetyl-L-cysteine.²³ *S*-nitroso-*N*-acetyl-L-cysteine inhibits both thrombin-induced and U-46619 (a stable thromboxane A₂ analogue)-induced expression of platelet surface P selectin (a granule protein), CD63 (a lysosomal protein), and the calcium-dependent active conformation of the heterodimeric fibrinogen-binding integrin glycoprotein IIb/IIIa (α_{2b}/β_3).²⁴ This suppression of the conformational change in glycoprotein IIb/IIIa required for fibrinogen binding is associated with suppression of intracellular calcium flux and demonstrable reduction in both the affinity (2.7-fold increase in K_d) and number (50% decrease) of fibrinogen-binding sites on the platelet surface.²³ Inhibition of cytosolic calcium flux with exposure to strong platelet agonists like thrombin or U46619 seems to be a consequence of inhibition of capacitative calcium influx resulting from enhanced sarcoplasmic reticulum/endoplasmic reticulum calcium-ATPase-dependent refilling of calcium stores.²⁵ *S*-Nitroso-*N*-acetyl-L-cysteine-dependent reduction in fibrinogen binding is dose-dependent and correlates strongly with NO-dependent activation of platelet guanylyl cyclase and cGMP accumulation.²³

Activation of platelets is associated with activation of another important signaling pathway, the phosphoinositide 3-kinase

(PI3-kinase) pathway. PI3-kinase represents a family of ubiquitous enzymes involved in a variety of cell functions, including cytoskeletal rearrangements. These enzymes have the capacity to phosphorylate lipids and proteins. They catalyze the phosphorylation of the inositol ring at the D3 position in a variety of phosphoinositide substrates as well as the phosphorylation of protein serine moieties. The p85/p110 isoform of PI3-kinase was the first to be identified in platelets and is activated by several G protein-linked receptors, including the thrombin receptor protease-activated receptor-1 (PAR-1).^{26,27} Activation may involve either a nonreceptor tyrosine-phosphorylated intermediate²⁸ or may occur directly by some isoforms of G protein subunits.^{29,30} Activation of platelets by thrombin results in translocation of PI3-kinase to the cytoskeleton at sites of integrin-dependent focal adhesions, where the enzyme is believed to play an important role in the cytoskeletal reorganization and conformational change in glycoprotein IIb/IIIa required for irreversible fibrinogen binding.³¹ Inhibition of this enzyme in platelets by the fungal metabolite wortmannin leads to impaired platelet aggregation and enhanced disaggregation.

Having previously shown that nitrovasodilators can induce platelet disaggregation³² and platelet PI3-kinase renders platelet aggregation irreversible, we recently examined the effect of the *S*-nitrosothiol *S*-nitroso-glutathione on platelet PI3-kinase.³³ These studies showed that the NO donor inhibits the thrombin receptor-activating peptide stimulation of PI3-kinase activity associated with tyrosine-phosphorylated proteins in immunoprecipitates and of p85/PI3-kinase associated with the src family kinase member lyn. The activation of PI3-kinase complexed with lyn requires the activation of lyn itself and other tyrosine kinases, and inhibition of this process by the NO donor is cGMP-dependent and likely involves inhibition of the dephosphorylation of lyn required for its activation. The effect of PI3-kinase activity on platelet Akt^{34,35} and its relationship to platelet NO synthase activity have not yet been reported.

The inhibitory effect of endothelial NO, *S*-nitrosothiols, and nitrovasodilators on platelet activation is analogous to that of prostacyclin and its analogues. However, there are two notable differences between the antiplatelet effects of these two classes of inhibitor: unlike NO, prostacyclin-mediated platelet inhibition is cAMP-dependent; and, also unlike NO, prostacyclin has no effect on platelet adhesion.³⁶ In contrast to the clear inhibitory effect of NO donors on the expression of the active conformation of platelet glycoprotein IIb/IIIa, NO donors have no effect on the expression of the integrin glycoprotein Ib/IX,²⁴ suggesting that inhibition of platelet adhesion may be a consequence of inhibiting interactions between von Willebrand factor and glycoprotein IIb/IIIa. We recently synthesized an *S*-nitrosated derivative of the recombinant von Willebrand factor fragment AR545C and found that this molecule effectively and potently inhibited both platelet aggregation and adhesion *in vitro* and *in vivo*.^{37,38} Recent data also demonstrate that inhibition of platelet adhesion to collagen, in particular, seems to depend on the generation of cGMP³⁹ and that this effect can be mimicked by the application of an NO-releasing protein (poly-*S*-nitrosated BSA) to the endothelium.⁴⁰ Owing to their different mechanisms of action, platelet inhibition by these two endothelial products is likely synergistic.³²

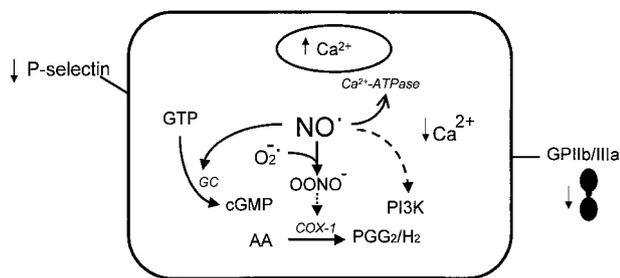


Figure 1. NO effects on platelet signaling and function. NO, derived from endothelial cells or from platelets, suppresses platelet activation by activating guanylyl cyclase (GC), leading to an increase in the conversion of GTP to cGMP, enhancing calcium ATPase-dependent refilling of intracellular calcium stores, and inhibiting the activation of PI3K. As a result of second-order effects mediated by the first two of these signaling systems, intracellular calcium flux (Ca^{2+}) is suppressed, leading to suppression of P-selectin expression and of the active conformation of glycoprotein IIb/IIIa (GPIIb/IIIa) required for binding fibrinogen (trinodular structure). NO also reacts with superoxide to form peroxynitrite (OONO^-), which can react with protein tyrosine residues on cyclooxygenase-1 to inhibit enzyme conversion of arachidonic acid (AA) to prostaglandins G_2 and H_2 (PGG_2/H_2), with a resulting reduction in thromboxane A_2 synthesis. Solid arrows indicate activation; dashed arrows, inhibition.

The importance of the antiplatelet effects of endothelial NO has been additionally confirmed by studies showing that over-expression of endothelial NO synthase in cultured endothelial cells inhibits platelet aggregation.⁴¹ In addition, NO can inhibit both platelet 12-lipoxygenase and cyclooxygenase-1,⁴² in the latter case by reacting with superoxide to form peroxynitrite, which reacts with the enzyme to form a 3-nitro-tyrosine residue.⁴³

In Vivo Studies

The studies reviewed thus far show clearly that NO impairs platelet function in vitro by a variety of mechanisms (summarized in Figure 1). These studies were all performed in erythrocyte-free systems. Some investigators have suggested that the affinity of hemoglobin for NO should render these in vitro observations irrelevant in vivo⁴⁴; however, the hydrodynamic effects of the flowing intravascular red-cell mass lead to a partitioning of platelets close to the low-shear boundary of the endothelial surface, which facilitates direct diffusional access of circulating platelets to endothelial NO, limiting kinetic competition of erythrocytic hemoglobin for the free radical. Consistent with this argument, the in vivo relevance of the in vitro observations was first confirmed in a series of experiments in which the effects of another *S*-nitrosothiol, *S*-nitroso-serum albumin, administered intravenously to dogs with an acutely deendothelialized, stenosed coronary artery (Folts model) was studied.^{45,46} We had previously demonstrated that *S*-nitroso-serum albumin represents an important in vivo reservoir of NO from which low-molecular-weight *S*-nitrosothiols, such as *S*-nitroso-L-cysteine or *S*-nitroso-glutathione, can be derived by thiol-*S*-nitrosothiol exchange reactions.^{19,20} Furthermore, we showed recently that these *trans*-nitrosation reactions can be catalyzed by cell-surface protein-disulfide isomerase to facilitate transfer of NO from an extracellular *S*-nitrosothiol to an

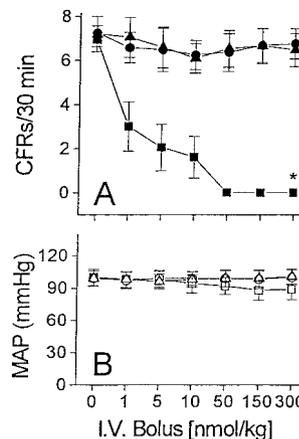


Figure 2. Effect of *S*-nitroso-BSA (SNO-BSA) on cyclic flow reductions (CFRs) in a canine coronary model of periodic platelet deposition. Animals were prepared as described previously.⁴³ After 40 minutes of continuous CFRs, SNO-BSA (1 to 300 nmol/kg) (squares) was administered as an intravenous bolus over 1 to 2 minutes. Controls included the starting materials for synthesizing SNO-BSA, ie, NaNO_2 (triangles) and BSA (circles), prepared in parallel with SNO-BSA. A, Effect of these agents on CFRs (closed symbols). B, Effect of these agents on mean arterial pressure (MAP) (open symbols). Results are mean \pm SEM, determined from 6 animals. * $P < 0.001$ vs NaNO_2 or BSA.

intracellular NO acceptor.⁴⁷ Using this model, we found that *S*-nitroso-serum albumin dose-dependently inhibited platelet-mediated cyclic flow reductions with an IC_{50} of ≈ 0.8 nmol/kg (Figure 2A), which translates into an estimated steady-state plasma concentration of ≈ 5 nmol/L. Importantly, there was only a very modest effect of the NO donor on mean arterial pressure over the range of concentrations used in this study (Figure 2B), suggesting that the antiplatelet effect of *S*-nitroso-serum albumin is more potent than the vasodilator activity in this model of acute coronary syndromes. These animal data were confirmed in patients undergoing coronary angioplasty in whom suppression of platelet activation by the infusion of *S*-nitroso-glutathione occurred at concentrations of the *S*-nitrosothiol that had no effect on blood pressure⁴⁸ and in a rat model of thromboembolic pulmonary hypertension in which inhaled NO inhibited platelet aggregation and platelet-mediated pulmonary thrombosis.⁴⁹ Furthermore, inhibition of endothelial NO synthase shortened bleeding time in human volunteers,⁵⁰ and the coating of artificial surfaces with NO-releasing polymers suppressed platelet adhesion in vivo.^{51,52} Thus, ample data in animals and humans support the view that endothelial NO and NO donors have important antiplatelet effects in vivo, especially in the setting of vascular disease.

Platelet-Derived NO and Inhibition of Platelet Recruitment

Under normal conditions of blood flow and shear stress, the vascular source of NO acting on platelets is likely derived from biochemical agonist- and shear-dependent release of endothelial NO.⁵³ However, under conditions of endothelial dysfunction or denudation, especially in the setting of an acute coronary syndrome, other sources of NO may become important in regulating platelet responses. A constitutive NO

synthase has been found in both human platelets and megakaryocytic cells,^{54,55} and this isoform is active.⁵⁶ Using an NO-selective microelectrode adapted to a platelet aggregometer, Freedman et al⁵⁷ recently showed that this platelet-derived NO not only modestly modulates platelet activation to strong and weak agonists but, more importantly, markedly inhibits platelet recruitment to the growing platelet thrombus. These in vitro findings were confirmed in an animal model of deficient platelet-derived NO, the *Nos3*-null mouse. In this model, Freedman et al⁵⁸ found that there is no detectable *Nos3* gene in marrow cells, that their platelets generate no detectable NO on activation, that the bleeding times of *Nos3*-null mice are correspondingly shorter than those of wild-type mice, and that the bleeding times in wild-type mice rendered thrombocytopenic with carboplatin and transfused with platelets from *Nos3*-null mice are shorter than those of mice transfused with platelets from normocytopenic wild-type mice.⁵⁸ Ex vivo platelet recruitment experiments using flow cytometry and platelets from *Nos3*-null and wild-type mice confirmed the importance of platelet-derived NO in attenuating platelet recruitment to the growing platelet thrombus.⁵⁸ Thus, endothelial- and platelet-derived NO pools both contribute to normal hemostatic function, and a deficiency of either pool enhances hemostatic response to acute vascular injury.

Factors that enhance platelet-derived NO synthesis include α -tocopherol, by inhibiting protein kinase C^{59,60}; statins, by increasing expression of NO synthase in platelets (as in endothelial cells)^{61,62}; L-arginine, by increasing NO synthesis; and intracellular thiol pools, by enhancing synthesis of S-nitrosothiols and limiting oxidative inactivation of NO.⁶³ By reducing intracellular calcium flux required for the activation of platelet NO synthase, cyclooxygenase inhibitors can reduce platelet-derived NO generation,⁶⁴ as can risk factors for atherosclerotic vascular disease^{65,66} (vide infra).

The clinical relevance of the importance of platelet-derived NO in patients with acute coronary syndromes was recently examined. Studying 87 consecutive patients undergoing coronary angiography, 37 with stable angina and 50 with unstable angina or an acute myocardial infarction, we found that platelets from patients with acute coronary syndromes produced significantly less NO than did those from patients with stable angina pectoris (0.26 ± 0.05 versus 1.78 ± 0.36 pmol/ 10^8 platelets, $P=0.0001$).⁶⁷ Because platelet activation has been implicated in the formation of thrombus in patients with acute coronary syndromes, we concluded from this study that an impairment of platelet-derived NO production may contribute to the pathophysiology of this class of atherothrombotic syndromes.

NO Insufficiency and Arterial Thrombosis

The potential in vivo relevance of the antiplatelet effects of NO reviewed thus far have been amply demonstrated in the dog model of acute coronary syndromes (Folts model)^{45,46} and in patients with acute coronary syndromes.⁶⁷ Yet the animal model suffers from the artificiality of its highly platelet-dependent thrombotic response, and the patient study begs the question of causality, because acute coronary syndromes are accompanied by oxidative stress that may itself inactivate NO, rendering it undetectable with the NO-selective electrode used in the study. Thus, these data support

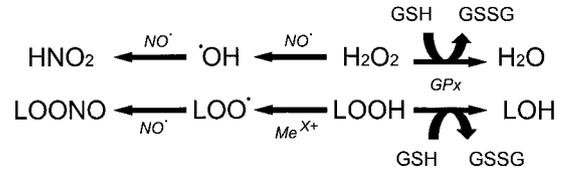


Figure 3. GPx, peroxides, and NO. GPx reduces hydrogen peroxide and lipid peroxides (LOOH) to water and lipid alcohols (LOH), respectively, oxidizing glutathione (GSH) to glutathione disulfide (GSSG) in the process. In the absence of adequate GPx activity, these peroxides can be converted to hydroxyl radical by NO ($\text{NO} \cdot$)⁶⁸ or lipid peroxy radicals by transition metals (Me^{X+}), which then react with NO to form nitrous acid (HNO_2) or LOONO.

but do not prove the hypothesis that a deficiency of vascular NO promotes arterial thrombosis.

As evidence for the causal relationship between vascular NO insufficiency and arterial thrombosis, we were fortunate to have studied two brothers who presented to our colleague Dr Alan Michelson (University of Massachusetts) with the syndrome of childhood stroke. One boy sustained two separate thrombotic cerebrovascular accidents at ages 13 and 22 months, whereas the other sustained a transient ischemic attack at age 15 months. Routine analysis for known genetic risk factors for arterial thrombosis was negative. In an effort to assess optimal treatment for these children, we evaluated the effect of an NO donor, S-nitroso-N-acetyl-L-cysteine, on their platelets. In contrast to the platelets of their unaffected sister, mother, and father, as well as age-matched control subjects, the NO donor was completely unable to impair platelet P-selectin expression in response to thrombin and was completely unable to prevent ADP-induced aggregation. Mixing experiments showed that the defect lies in the patients' plasmas; resuspending their platelets in age-matched control plasma led to normal levels of inhibition by S-nitroso-N-acetyl-L-cysteine. After an extensive search for possible culprit molecular mediators of this inactivation of an NO donor in plasma, results showed that the patients and their mother had a deficiency of the plasma isoform of glutathione peroxidase (GPx-3).⁶⁸ This enzyme belongs to a family of selenocysteine-containing proteins, four of which have peroxidase activity. Each of these peroxidases reduces hydrogen peroxide and lipid peroxides to its corresponding alcohol. GPx-3 is the only one of the selenocysteine-containing peroxidases found in the extracellular space and is responsible for most of the hydroperoxide-reducing activity of plasma.^{69,70} Previous studies demonstrated that GPx activity potentiates inhibition of platelets by S-nitrosothiols and does so both by reducing lipid peroxides to lipid alcohols,⁷¹ thereby preventing the generation of lipid peroxy radicals that can inactivate NO by forming lipid peroxynitrites⁷² (Figure 3), and by catalyzing the liberation of NO from low-molecular-weight S-nitrosothiols.⁷¹ The importance of GPx-3 in the regulation of platelet-dependent thrombus formation rests in the fact that activated platelets are rich sources of reactive oxygen species, including superoxide and 12-hydroperoxy-5,8,10,14-eicosatetraenoic acid [12(S)-HpETE]^{71,73}; and the importance of the enzyme deficiency in the two brothers originally studied was shown by demonstrating the restoration of platelet-inhibitory activity of the NO donor on addition of exogenous (cellular) GPx to their plasmas.⁶⁸

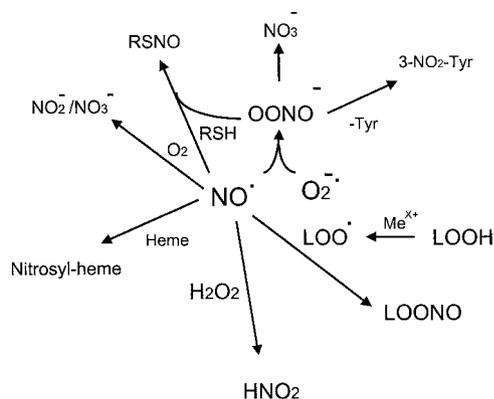


Figure 4. Reactive oxygen species and NO. NO can undergo several fates within platelets, including oxidative inactivation to nitrite (NO_2^-) and nitrate (NO_3^-) and reaction with superoxide anion to form peroxynitrite (OONO^-). Peroxynitrite can react with tyrosyl residues in proteins to form 3-nitrotyrosyl residues and with thiols to form S-nitrosothiols (RSNO); the latter can also form by the reaction of NO with thiols in the presence of oxygen. By reacting with heme iron, nitrosyl-heme charge-transfer complexes can form, accounting for the biological activation of guanylyl cyclase by NO. Lipid peroxides (LOOH) can yield lipid peroxy radicals ($\text{LOO}\cdot$), which can react with NO to form lipid peroxynitrites (LOONO), and hydrogen peroxide can react with NO to form nitrous acid (HNO_2) and hydroxyl radical.⁸³ Each of these reactions and the reactants that engage in them are present in platelets and in the extracellular microenvironment of activated platelets.

Recent studies expanded this initial observation in a single family to seven families with (familial) childhood stroke. In each of these families, a deficiency of GPx-3 was detected in affected family members, and pedigree analysis suggested that the deficiency is inherited as an autosomal dominant trait. Interestingly, the magnitude of the deficiency correlated with the extent of P-selectin expression measured by flow cytometry in response to thrombin.⁷⁴

In an attempt to identify the basis for the molecular defect, we used single-strand conformational polymorphism (SSCP) analysis of the GPx-3 gene. The gene structure comprises 5 exons, the second of which contains the selenocysteine codon TGA⁷⁵; the 3'-untranslated region contains an element of putative secondary structure (a stem loop) that is required for recognition of TGA as a selenocysteine codon rather than as a stop codon.⁷⁶ We performed SSCP using primers for the five exons, the putative promoter, and the 3'-untranslated region of the GPx-3 gene on DNA from 100 individuals with stroke younger than 35 years of age to identify potential mutations or polymorphisms.⁷⁷ We sequenced those regions that showed differences by SSCP analysis and identified a novel polymorphism in the promoter region that confers an independent risk for stroke in these individuals (relative risk=2.5; confidence interval, 1.31 to 4.81). The possible role of this polymorphism in the regulation of GPx-3 gene expression has yet to be characterized.

The importance of vascular NO in preventing thromboembolic strokes is additionally supported by recent animal data showing that inhibition of NO synthase leads to platelet accumulation in the cerebral vasculature.⁷⁸ The importance of (cellular) GPx (ie, GPx-1) activity in endothelial antiplatelet actions has been shown by the dependence of an aspirin-sensitive

inhibitory activity (prostacyclin synthesis and stability) on selenide content of the endothelial cell.⁷⁹

Reactive Oxygen Species, Inactivation of NO, and Arterial Thrombosis

NO is a reactive free radical that can participate in several types of redox reactions, some that mediate its biological effects and others that limit its activity (Figure 4). Examples of the first type of reaction include the reaction of NO with heme iron (ie, nitrosylation), which is responsible for the activation of guanylyl cyclase, and the reaction of NO with thiol groups in the presence of oxygen (or via peroxynitrite intermediates) to form S-nitrosothiols, which stabilize bioactivity. Inactivation of NO occurs largely through oxidative reactions mediated by reactive oxygen intermediates, including superoxide, hydrogen peroxide, and lipid peroxy radicals generated from lipid peroxides¹⁷ as well as the F₂-isoprostane 8-epi-prostaglandin F₂.⁸⁰ These reactive oxygen intermediates are found in a variety of vascular disorders, including hypertension, hypercholesterolemia, diabetes mellitus, atherothrombosis, and the endothelial dysfunction that underlies or accompanies these disorders.⁸¹

Platelets are themselves a rich source of reactive oxygen species, including superoxide and 12(S)-HpETE.^{71,73} The consumption of oxygen by activated platelets is robust and serves as a mechanism to enhance the aggregation response through the formation of proaggregatory prostanoid derivatives of arachidonic acid. This oxygen-dependent autoamplification of the activation of platelets can, in the theoretical extreme, proceed without restraint. For this reason, antioxidant mechanisms have evolved to limit unbridled expansion of the platelet aggregate. Endothelium- and platelet-derived NO represent one class of regulatory molecule that impairs platelet activation and recruitment to the growing thrombus. In addition, the antioxidant enzyme GPx-3 represents another regulatory molecule that, in conjunction with extracellular superoxide dismutase found on the endothelial surface, serves to inactivate those reactive oxygen intermediates that can inactivate NO either directly or via other radical derivatives. The formation of peroxynitrite by the reaction of either endothelial- or platelet-derived NO with either endothelial- or platelet-derived superoxide represents yet another mechanism for limiting the reactive oxygen species-dependent thrombotic response, both by limiting available superoxide and by impairing thromboxane A₂ generation via the 3-nitrotyrosine-dependent inactivation of cyclooxygenase-1⁴¹ and by S-nitrosothiol-dependent inhibition of thromboxane A₂ synthesis.⁸² Platelet-dependent arterial thrombotic responses, then, are both a cause and a consequence of excessive oxidant stress in the vasculature, and arterial thrombotic disorders are their clinical counterpart. NO, derived both from the endothelial cell and the platelet, modulates platelet activation, adhesion, and aggregate formation, thereby serving as an important deterrent to platelet-mediated arterial thrombosis. The studies reviewed here clearly show that vascular oxidant stress produced by an excess of oxidants or an acquired or genetically determined deficiency of antioxidant enzymes is a risk factor for arterial thrombosis. Efforts to restore the normal vascular redox balance may provide one therapeutic

avenue for reducing platelet-dependent arterial thrombosis in these individuals.

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