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REVIEW ARTICLE

Nitric oxide and its relationship to thrombotic disorders

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Summary. Nitric oxide (NO) is released by the endothelium preventing platelet adhesion to the vessel wall. When released by platelets, NO inhibits further recruitment of platelets to a growing thrombus. Modulation of endogenous NO release may be a mechanism by which the thrombotic response can be regulated as suggested by several clinical diseases associated with impaired bioactive NO. Diseases including atrial fibrillation and coronary atherothrombotic disease have been associated with impaired NO release or decrease in NO bioavailability.

Keywords: antioxidants, cardia, nitric oxide, platelets, thrombosis, superoxide.

Introduction

Superficial and intimal injury caused by endothelial denudation and deep intimal injury caused by plaque rupture expose collagen and von Willebrand factor (VWF) to platelets [1]. Platelets adhere directly to collagen or indirectly via the binding of VWF to glycoprotein Ib/IX and to matrix. Local platelet activation by tissue factor-mediated thrombin generation or by collagen stimulates further thrombus formation and additional platelet recruitment by supporting cell-surface thrombin formation and releasing platelet-derived ADP, serotonin, and thromboxane A_2 [2]. Thrombus forms as platelets aggregate via the binding of bivalent fibrinogen to glycoprotein IIb/IIIa. In addition to releasing substances that stimulate recruitment, endothelial cells and activated platelets release the platelet inhibitor nitric oxide (NO). Nitric oxide released by the platelets and endothelium prevents platelet adhesion to the vessel wall as well and provides a negative feedback mechanism for the propagation of thrombus formation. In this review, the role of NO in experimental and clinical thrombotic disorders will be explored.

Activation and recruitment of platelets is tightly regulated. Adhesion of platelets to the endothelium is prevented by several mechanisms, including endothelial cell production of prostacyclin and NO [3,4]. Nitric oxide inhibits platelet activation [5,6] and prevents thrombosis [7]. Exogenous NO has been shown to inhibit the normal activation-dependent increase in the expression of platelet surface glycoproteins, including P-selectin and the integrin glycoprotein IIb/IIIa complex [8]. Nitric oxide inhibits platelet function by stimulating soluble guanylyl cyclase to produce cyclic GMP (cGMP). This action results in the stimulation of cGMP-dependent protein kinase that leads to a reduction in fibrinogen binding to glycoprotein IIb/IIIa and modulation of phospholipase A2- and C-mediated responses [9]. In addition, NO attenuates the oxidation of arachidonate [10], inhibits the agonist-dependent increase in platelet cytosolic free calcium in a cGMP-dependent manner [11], and inhibits platelet phosphoinositide 3-kinase leading to enhanced dissociation of fibrinogen from glycoprotein IIb-IIIa [12].

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The effect of endothelium-derived nitric oxide on thrombosis

The vascular endothelium, which mediates vasomotor tone, in part, through NO release, has been extensively characterized. Endothelium-dependent dilation is impaired in animal models of atherosclerosis and in isolated human atherosclerotic coronary arteries [13]. Endothelium-dependent dilation of systemic arteries is also impaired in patients with cardiovascular disease (for a more detailed discussion see [14]), as well as adults and children with risk factors for atherosclerosis [15,16]. Although correlative changes in NO production and thrombotic propensity were not directly measured in these studies, the reported abnormalities in vascular reactivity imply that the normal NO-dependent antithrombotic properties of the vessel are attenuated, as well.

Experimental models of NO insufficiency primarily utilize inhibitors of NO synthase (NOS). Because these inhibitors have non-specific effects on the three NOS isoforms and affect both platelets and the vessel wall, many of the pathophysiological findings are also non-specific. Thus, in studies utilizing NOS inhibitors and the NOS substrate L-arginine to examine the effect of NO production on thrombosis, it may be difficult to determine whether findings are due to altered vascular reactivity, platelet activation, or a combination of both. Despite these limitations, the role of NO insufficiency has been examined in many experimental models in an attempt to characterize the atherothrombotic effects of NO.

The effect of NOS inhibitors on intracoronary thrombosis and reocclusion has been characterized in a dog model of coronary occlusion and in which thrombus formation in the coronary artery could be delayed by an L-arginine infusion [17]. Lysis of

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thrombus in the coronary arteries could also be hastened by infusion of L–arginine [17]. In addition, *ex vivo* platelet aggregations were also inhibited after animals were treated with L-arginine, suggesting that increasing NO production inhibits platelet function and attenuates thrombus formation.

Thrombosis also appears to be regulated by the bioactivity of NO in the cerebrovascular system. In a rat model of thromboembolic stroke, infusion of the NOS inhibitor L-NAME caused both an increase in platelet deposition and a reduction in global flow [18], suggesting that both hemodynamic and thrombotic determinants contributed to the enhanced cerebral deficits.

Inhibition of NO appears to affect glomerular thrombosis. The role of endogenous NO production in the development of glomerular thrombosis associated with septic shock was studied an endotoxin-induced model of renal thrombosis [7]. Nitric oxide production was increased by endotoxin injection, and this effect was prevented by infusion of the NO synthase inhibitor L-NAME. Close examination of kidneys from rats given endotoxin and L-NAME revealed thrombosis in 55% of glomeruli as compared to less than 5% of the glomeruli from rats given either endotoxin or L-NAME alone. These investigators concluded that NO is critical in preventing renal thrombosis resulting from septic shock. As with septic shock, NO synthesis is enhanced in glomerulonephritis. In a model of nephrotoxic nephritis, rats depleted of plasma L-arginine developed systemic hypertension and glomerular thrombosis, suggesting that the enhanced production of NO in this condition prevents further acute glomerular injury [19].

Although most studies have focused on the impact of endogenous NO release in arterial patency, there has also been limited examination of thrombosis in the venous system. In a study of rabbit mesenteric arterioles and venules, inhibition of NO synthase with a NOS inhibitor increased the duration of embolization and the number of emboli in venules but not the arterioles [20]. Infusion of L-arginine, but not D-arginine, reversed the increase in venous embolization in this model.

In addition to studies of endothelial NO, the effect of exogenous sources of NO on thrombosis has also been evaluated. Using the Folts model, which manifests cyclic reductions in coronary flow as a consequence of transient platelet occlusion [21], the NO donor S-nitroso-bovine serum albumin dramatically reduced the frequency of flow cycles. The IC_{50} for inhibiting cyclic platelet-dependent occlusion was $1\,nmol\,kg^{-1}$ [21]. Importantly, these effects on coronary flow occurred at concentrations of the NO donor that had no or minimal effects on mean arterial blood pressure: at $1\,nmol\,kg^{-1}$ S-nitroso-serum albumin no effect on mean arterial pressure, and at $100\,nmol\,kg^{-1}$ not more than a 10% decrease in mean arterial pressure was observed. These observations suggest that the antiplatelet, antithrombotic effects of this NO donor are comparatively selective in this model of platelet-mediated coronary thrombosis.

Platelet-derived nitric oxide and thrombosis

In addition to the effects of endothelium-derived NO, constitutive nitric oxide synthase (cNOS) has been identified in both human platelets and megakaryoblastic cells [22,23] (Fig. 1). Platelet aggregation is modestly enhanced by incubation with inhibitors of cNOS and inhibited by incubation with the cNOS substrate L-arginine [24]. *In vivo*, systemic infusion of the cNOS inhibitor L-N^G -monoethyl arginine citrate (L-NMMA) causes a reduction in bleeding time without a change in vessel tone [25] and enhanced platelet reactivity to various agonists [26]. Several groups have reported NO release from resting [27] and aggregating platelets [28,29]. Nitric oxide release from activated human platelets has been indirectly measured and estimated to be 11.2 pmol nitric oxide $min^{-1}/10^8$ cells, indicating that the amount of NO released by platelets may be comparable to that of endothelial cells [27]. In addition, platelet NO release inhibits platelet recruitment to the growing thrombus [30] (Fig. 2).

As discussed, the endothelial isoform of nitric oxide synthase (eNOS) has been identified in human platelets and is the source of nitric oxide (NO) released following platelet activation. Because inhibitors of NOS have non-specific effects on the three NOS isoforms and affect both platelets and the vessel wall, many of the pathophysiological findings from studies utilizing these agents are also non-specific. To specifically define the role of eNOS in platelet function and hemostasis, we studied mice lacking a functional eNOS gene (NOS3 null mice) [31]. ADP-stimulated platelets from eNOS-deficient mice released no measurable NO in contrast to platelets from wild-type mice (0.0 vs. 15.9 pmol/10^8 platelets, respectively) [31]. To

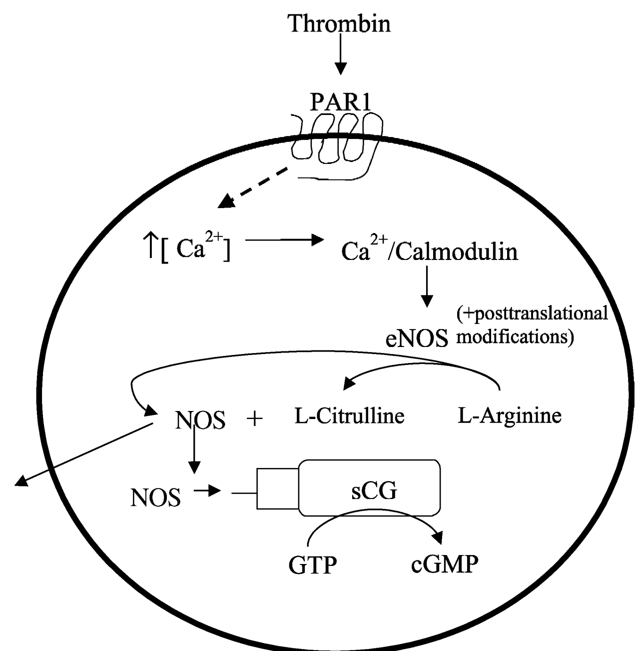

Fig. 1. Platelet-derived nitric oxide. Thrombin stimulation of the PAR1 receptor increases intracellular Ca^{2+} concentration both by releasing Ca^{2+} from intracellular stores and allowing external Ca^{2+} to enter the cytosol. The binding of the Ca^{2+}-calmodulin complex in addition to post-translational modifications (including phosphorylation, dephosphorylation, myristoilation, and palmitoylation) of cNOS activates the enzyme to catalyze the conversion of of L-arginine to L-citrulline and NO. Nitric oxide subsequently binds the heme moiety of sGC activity of this enzyme to increase cGMP production.

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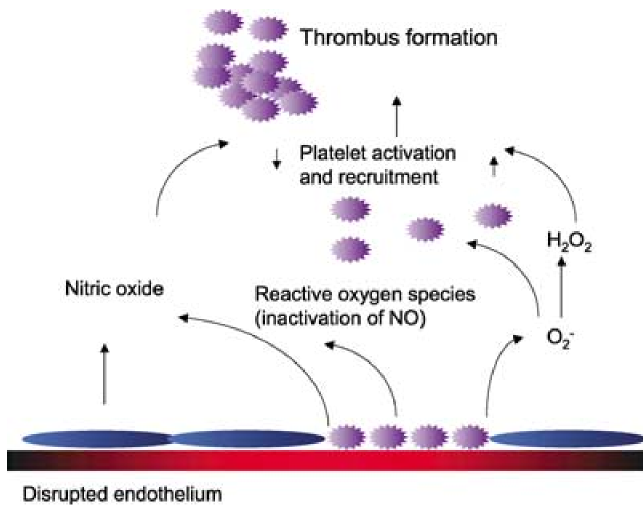

Fig. 2. In the intact vessel, production of prostacyclin and nitric oxide by the endothelium prevents adherence and activation of platelets to the vessel surface. In the disrupted endothelium, platelets adhere to the subendothelial matrix, become activated, and release the prothrombotic substances thromboxane A_2, serotonin, and ADP that support the recruitment of additional platelets to the growing thrombus. This process may by limited by the release of nitric oxide by the activated platelet.

determine the relative contribution of endothelial- and platelet-derived NO to the bleeding time in these mice, platelets from eNOS-deficient or wild-type mice were isolated, transfused into thrombocytopenic eNOS-deficient mice, and the bleeding time measured. Compared with the thrombocytopenic controls, bleeding times in mice transfused with eNOS-deficient platelets were decreased to a significantly greater extent than those transfused with wild-type platelets (Δ bleeding time $= 24.6 \pm 9$ s vs. 3.4 ± 5 s, $P < 0.04$). Because the bleeding time was significantly shortened even after controlling for endothelial NO production, these findings support a role for platelet-derived NO in *in vivo* hemostasis and platelet-dependent thrombus formation.

Nitric oxide insufficiency and clinical thrombotic disorders

Clinical disorders have been reported in which an insufficiency of endogenous NO production is believed to contribute to a thrombotic event (Table 1). It is well established that thrombosis is the usual cause of unstable angina and myocardial infarction [32,33], and, importantly, activated platelets from patients with these acute coronary syndromes produce significantly less NO as compared to patients with stable coronary artery disease [34]. This decrease in NO production is significant even after controlling for cardiovascular risk factors and the extent of atherosclerotic disease. This observation suggests that impaired platelet-derived NO may contribute to the development of acute coronary syndromes by influencing platelet function or recruitment and consequent thrombus formation.

These observations are supported by a study demonstrating that platelets from patients with acute myocardial infarction and unstable angina, despite aspirin treatment, are still partially activated as measured by platelet surface expression of P-selectin and active glycoprotein IIb/IIIa [35]. In this study of patients with either unstable angina or acute myocardial infarction, surface expression of P-selectin and active glycoprotein GPIIb/IIIa was reduced by treatment with NO donors, including nitroglycerin or S-nitrosoglutathione.

Patients with atrial fibrillation, a condition associated with increased intracardiac thrombosis and cerebral embolism, have decreased plasma levels of nitrite and nitrate as well as lower levels of platelet cGMP [36], suggesting that there may be decreased levels of bioavailable NO in this setting. The precise mechanism for the decreased NO levels associated with atrial fibrillation is currently unknown; however, turbulent flow conditions have been associated with decreased NO synthase activity [37] perhaps owing to endothelial injury.

Thrombosis has also been attributed to NO deficiency in non-cardiac clinical disorders. In women with preeclampsia, a disease state association with hypertension, intrarenal thrombosis, and vasospasm, cGMP levels were depressed [38]. In patients with recurrent forms of thrombotic microangiopathy, including hemolytic uremic syndrome and thrombotic thrombocytopenic purpura, there is evidence that endothelial damage is a crucial feature in the development of microvascular thrombosis. Patients with these disease states manifest elevated plasma concentrations of NO metabolites, and serum from these patients enhances NO release when incubated with cultured endothelial cells. Importantly, superoxide production and lipid peroxidation are also enhanced [39], suggesting that the interaction of these reactive oxygen species with NO reduces its bioactivity by increasing its oxidative state, potentially leading to enhanced thrombus formation.

Recently, the role of eNOS polymorphisms in thrombosis has been examined. A recent report from the Framingham Heart Study demonstrated that heritable factors play a major role in determining platelet aggregation, and measured covariates play a lesser role [40]. In this study, the best characterized platelet polymorphisms (glycoprotein IIIa Pl (A2) and fibrinogen Hind III beta-148) contributed $<1\%$ to the overall variance. The authors conclude that 'future studies are warranted to identify the key genetic variants that regulate platelet function [40]'. One possible candidate gene of the ligand is eNOS, a polymorphic variation in the genotype of which may account for differences in NO production. Endothelial NOS is located on

Table 1 Thrombotic disorders associated with NO deficiency

Thrombotic disorder
Thrombotic microangiopathy [39]
Hemolytic uremic syndrome
Thrombotic thrombocytopenic purpura
Atrial fibrillation [36]
Unstable coronary syndromes [34]
Acute myocardial infarction
Unstable angina
Atherothrombotic vascular disease
Pregnancy and preeclampsia [38]
Pediatric thromboembolic cerebrovascular disease [57]

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chromsome 7q35–36, and estimates suggest that genetic variations contribute to at least 30% of the variance in plasma NO levels in the population [41,42].

Several polymorphic variants of the eNOS gene have been described, and the most extensively characterized variant is the 894-G/T polymorphism in exon 7 of the gene resulting in a glutamate or aspartate at position 298. Healthy individuals with the 894T allele have higher plasma levels of nitrogen oxides [43], and epidemiologic studies have shown an increased risk of hypertension, myocardial infarction, and stroke in patients homozygous for the Glu298Asp variant [44–47]. Another eNOS polymorphism, designated ecNOS4a, has been identified on intron 4 and has 4 tandem 27 bp repeats as compared to the wild-type allele that has 5 tandem repeats (ecNOS4b). The ecNOS4a allele has been associated with premature coronary artery disease [48], and this genotype has also been associated with a history of myocardial infarction [48].

One of us (J.F.) recently studied the occurrence of eNOS variants and found that polymorphisms in the promoter region ($P = 0.002$) or in exon 7 ($P = 0.007$), but not in intron 4 ($P > 0.05$), were associated with lower levels of platelet-derived NO [49]. In addition, increased ($P = 0.047$) release of superoxide was observed with platelets from subjects with the variant in the promoter region, but not with other eNOS genetic variants. The eNOS gene polymorphisms did not affect ADP-induced platelet aggregation, but the exon 7 variant did alter collagen-induced aggregation. These data demonstrate that the eNOS variants in the promoter region and in exon 7 decrease platelet-derived NO, with increased platelet aggregation noted in homozygotes for the exon 7 variant but not in subjects with other genotypes. Taken together, these data suggest that select eNOS variants may influence thrombotic propensity.

The role of antioxidants and reactive oxygen species in NO-dependent thrombosis

A prominent feature of both abnormal platelet function and dysfunctional endothelium-dependent vasodilation in the setting of cardiovascular and thrombotic disease is oxidative stress. Evidence also suggests that oxidative stress normally accompanies platelet activation. Platelet aggregation is associated with a burst of oxygen consumption [50] and a marked rise in glutathione disulfide [51]. While dramatic changes in platelet redox status occur during normal aggregation, conditions that provoke oxidative stress without inducing a florid aggregation response have also been shown to be prothrombotic. Reactive oxygen species contribute causally to many pathophysiologic conditions, and superoxide, in particular, is known to augment platelet aggregation responses [52]. Superoxide and NO readily combine to form peroxynitrite ($OONO^-$), thereby inactivating NO.

Antioxidants may indirectly inhibit platelets through their inference on the metabolism of reactive oxygen species, many of which alter platelet function. Hydroperoxides produced by activated platelets (prostaglandin G_2, 12-hydroperoxy-eicosatetraenioc acid, and phospholipid hydroperoxides) are metabolized by the cytosolic selenium-dependent enzyme cellular glutathione peroxidase. Cellular glutathione peroxidase (cGPx) is tightly coupled to the hexose monophosphate shunt through reduced nicotinamide adenine dinucleotide phosphate (NADPH), which maintains the obligate cosubstrate of cGPx reduced glutathione (GSH) and re-establishes the platelet thiol redox state via glutathione reductase. Glutathione depletion in platelets leads to attenuated cGPx activity and increased lipid peroxidation [53]. Increased lipid peroxides, in turn, lead to an increased likelihood of lipid peroxyl radical formation (LOO), which can react with and inactivate NO by forming lipid peroxynitrites (LOONO). Cellular GPx potentiates the inhibition of platelet function by NO by reducing both LOOH and derivative LOONOs.

Experimental evidence suggests that antioxidant status is important in normal platelet function and the prevention of thrombosis. Studying cyclic flow variations in rabbit carotid arteries, Meng and colleagues showed that platelet-mediated thrombosis can be attenuated by the intravenous infusion of superoxide dismutase [54]. Consistent with these observations, in a model of endothelium-injured canine coronary arteries, Ikeda and colleagues demonstrated that cyclic flow variation was attenuated by intravenous infusion of superoxide dismutase and catalase [55]. Infusion of xanthine and hypoxanthine or hydrogen peroxide, however, significantly increased cyclical flow variation. These data suggest that reactive oxygen species contribute to platelet activation and thrombosis.

The plasma isoform of glutathione peroxidase pGPx also potentiates the inhibition of platelet function by NO by decreasing LOOH concentrations [56]. Impairment of this process can lead to a clinical thrombotic disorder as shown in two brothers with thrombotic strokes in childhood [57]. In these children, aggregometry and flow cytometry studies showed that their platelets were hyperreactive owing to an abnormality in their plasma. In the presence of their plasma, NO failed to inhibit aggregation or surface expression of P-selectin on normal platelets. The decrease in plasma cGMP levels in these patients was secondary to reduced bioactive NO as pGPx activity was decreased by approximately one-half in the probands. Importantly, sensitivity of the platelets to inhibition by NO was restored by adding exogenous glutathione peroxidase to their plasma. A similar deficiency has been reported in five other families with childhood stroke [58]. This important antioxidant role of pGPx may be of broader relevance than this rare thrombotic disorder in children. Glutathione peroxidase is a selenium-containing enzyme, and selenium deficiency has been reported in patients with acute myocardial infarction and coronary atherothrombotic disease [59].

Supporting the role of antioxidant status in thrombosis are clinical studies indicating that vitamin E (α-tocopherol) exerts a beneficial effect on cardiovascular disease [60]. By examining plasma α-tocopherol concentration in 87 consecutive patients undergoing coronary angiography, we also determined that α-tocopherol is associated with platelet release of NO [61]. α-Tocopherol levels were $21.6 \pm 3.2\ \mu\text{M}$ and $12.2 \pm 12\ \mu\text{M}$ in

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patients in the highest quartile and lower three quartiles of platelet NO production, respectively ($P < 0.01$). Platelet NO production correlated with plasma α-tocopherol concentration ($R = 0.5$; $P < 0.01$) and this effect was independent of aspirin and nitrate treatment. Thus, in patients with unstable coronary syndromes, α-tocopherol levels are associated with platelet-derived NO release. These observations suggest a potential mechanism for the beneficial effect of α-tocopherol in patients with cardiovascular disease. We have also found that α-tocopherol inhibits platelet function both *in vitro* and *in vivo* through a PKC-dependent mechanism [62]. In this study, the platelet α-tocopherol levels achieved with *in vitro* loading (117.6 ± 15.3 pmol/10^8 platelets) were comparable to the levels measured after *in vivo* α-tocopherol supplementation (160.5 ± 70.5 pmol/10^8 platelets). This finding suggests that *in vitro* loading of platelets using supraphysiological levels of α-tocopherol is a reasonable means of increasing platelet α-tocopherol levels to that occurring *in vivo*.

Conclusions

Nitric oxide is an important endogenous inhibitor of platelet activation and hemostasis. A deficiency of nitric oxide supports and sustains platelet-mediated thrombotic responses, and this mechanism may underlie a broad range of thrombotic disorders from childhood stroke to coronary atherothrombotic disease. Modulation of endogenous NO levels may be a mechanism by which the clinical thrombotic response can be altered; however, more careful clinical and basic studies are needed to determine the utility of such methods for regulating platelet function and thrombotic risk.

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