# PATHOPHYSIOLOGY AND CLINICAL APPLICATIONS OF NITRIC OXIDE

Edited by
GABOR M. RUBANYI


## Part B

Endothelial Cell Research Series
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# Pathophysiology and Clinical Applications of Nitric Oxide

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This edition published in the Taylor & Francis e-Library, 2005.



Amsteldijk 166
1st Floor
1079 LH Amsterdam
The Netherlands

**British Library Cataloguing in Publication Data**
A catalogue record for this book is available from the British Library.

ISBN 0-203-30372-5 Master e-book ISBN

ISBN 0-203-34370-0 (Adobe eReader Format)
ISBN: 90-5702-415-2 (Print Edition)
ISSN: 1384-1270

**DEDICATION**

During the printing of this book we received the news of the award of the 1998 Nobel Prize in Medicine to Dr Robert Furchgott, Dr Louis Ignarro and Dr Ferid Murad for their pioneering work in the discovery of nitric oxide as a novel mediator of biological functions. In the name of all contributors to this book and numerous other scientists in the field we congratulate Bob, Lou and Ferid on this great personal accomplishment and we would like to dedicate this volume to them. The Nobel Prize also reflects a well-deserved appreciation of the entire field, some important recent progress of which is presented here.

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# PREFACE

The identification that nitric oxide (NO) mediates endothelium-dependent vasodilation and that NO is synthesized by nitric oxide synthase (NOS) from L-arginine represented key discoveries of the 1980s. These observations opened the way to unprecedented research activity in the early 1990s which led to new insights into the physiology and pathophysiology of numerous biological and disease processes.

Research on NO has continued at a high pace in the past 5 years and it is by now clear that in addition to the cardiovascular system, NO is implicated in the function and disease of several other organs and systems as well (e.g., inflammatory diseases in the brain, joints, lung and gut; immunological disorders, cancer, diseases of the reproductive organs, etc.). It is also evident that we are entering into a new phase of NO research: several of the basic research observations are turning into novel therapeutic principles, and these are being pursued in clinical trials. Selective inhibitors of nitric oxide synthase isoforms are being discovered, novel NO-donors are being developed and the first reports on gene therapy approaches have been published—just to name a few examples which illustrate the point.

Athough some excellent books have been published in the past about NO research, this work is the first that summarizes the quantum leap from basic sciences to clinical applications of novel therapeutic principles emerging from this decade-long research activity.

The book is divided into two parts. Part A starts with a historical perspective written by Dr Robert Furchgott. His cornerstone observation in 1980 about the essential role of the endothelium in acetylcholine-induced vasorelaxation undoubtedly represented the start of this whole field. The rest of this volume is dedicated to the theme Generation and Biological Actions of Nitric Oxide. Leading experts give state-of-the-art overviews of various aspects of this theme, including the description of NOS function and regulation, the biological actions of NO, and the functional consequences of NOS gene knock-out in mice.

Part B has two sections. The first of these describes the known role of NO (its deficiency and/or excess) in the pathophysiology of diseases, such as those of the cardiovascular, kidney, reproductive, nervous and skeletal systems. The second section of Part B summarizes the recent progress achieved with therapeutic applications of NO. This section describes, among other topics, the discovery and therapeutic application of new NO donors, the therapeutic use of NO inhalation, selective inhibitors of NOS isoforms, and gene therapy approaches with both the constitutive and inducible forms of NOS.

Based on the scope, the excellent contributions by the leading experts, and the very efficient and professional work of the publisher, I believe this book on the pathophysiology and clinical application of nitric oxide is a one-of-a-kind work which will be of interest and benefit to both the experts working in the field and interested professionals of a wide variety of disciplines.

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# Nitric Oxide and Pathophysiology of Diseases

# 13
# Nitric Oxide and Its Role in Hemostasis

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Nitric oxide (NO) is a short-lived, free radical gas that is found in a variety of cell types and organ systems. In vascular hemostasis, NO is important for preventing platelet activation, adhesion, and aggregation. NO may play a role in the development of hemostatic disorders that occur when NO availability is altered. In this chapter, we discuss the importance of NO in the hemostatic response with particular emphasis on the pathogenesis of thrombotic and hemorrhagic diatheses.

**Key words:** Fibrinolysis, hemostasis, nitric oxide, thrombosis

## INTRODUCTION

Hemostasis depends, in part, on the ability to form a protective platelet plug in the event of vascular injury and thereby prevent excessive blood loss. A poorly controlled or excessive hemostatic response, however, can be manifested as hemorrhage or thrombosis, respectively. The primary hemostatic response is dependent upon platelets and products of the endothelium that modulate platelet function. One mediator that has been shown to have antiplatelet activity is nitric oxide (NO). Under resting conditions the endothelium is stimulated by flow to produce NO, which can regulate platelet adhesion and aggregation (Pohl and Busse, 1989; Cooke *et al.,* 1991; de Graaf *et al.,* 1992). The production of NO leads to activation of soluble guanylyl cyclase with a concomitant increase in cGMP, which is a principal mediator of the effects of NO. NO can inhibit platelet adhesion and aggregation, and can also induce disaggregation of previously aggregated platelets (Radomski and Moncada, 1991a,b; Radomski *et al.,* 1987a,b,c). Thus, in different

disease states in which less or more nitric oxide is being produced by the vasculature, platelet function may be adversely altered. The platelet response may also be modulated in patients with cardiovascular disease who use organic nitrates, such as nitroglycerin, which act as NO donors. While lack of NO may lead to thrombosis, an excess of NO may be detrimental such that platelet aggregation is impaired and a bleeding diathesis results. In this chapter we will provide evidence for the importance of NO in regulating platelet function and hemostasis, and illustrate hemostatic disorders that arise when NO production or bioactivity is altered.

## PATHOGENESIS OF THROMBOSIS

The process of thrombus formation involves the interaction of elements in the vessel wall with platelets and activated coagulation factors (Figure 13–1). In arteries, a thrombus may occlude the vessel resulting in decreased or absent blood flow to tissues, potentially leading to ischemia or infarction.

One of the most important rheologie properties of flowing blood that regulates hemostasis is the blood's tendency to move in a pattern of concentric, cylindrical laminae, termed laminar flow (Goldsmith *et al.,* 1986). These laminae are arranged such that a gradient exists within the vessel, with the greatest velocities found in the laminae closest to the center of the vessel and the lowest velocities found near the vessel wall. Because of cell size and charge properties, the laminae in the center of the vessel are enriched in red blood cells while the laminae closest to the vessel wall are enriched in platelets. Since the blood velocity is relatively low in the vicinity of the vessel wall, platelets have a long residence time in that domain, thereby allowing for rapid activation should the vessel become damaged.

Another hemodynamic factor that is important for understanding the hemostatic function of platelets is shear rate (defined as the product of blood viscosity and shear stress), which is determined by the velocity gradient in the vessel. According to Poiseuille's Law, velocity is inversely proportional to the crossectional area of a vessel or a vascular bed. Thus, blood velocity is highest in those vessels that have the smallest crossectional area, such as the aorta. Capillaries have the largest crossectional area, and, therefore, the lowest velocities. Large vessels with high velocities have higher rates of shear than do small vessels with low velocities. Increases in the shear rate lead to an increase in platelet deposition, while low shear rates are associated with preferential fibrin deposition (Loscalzo, 1994).

Under normal circumstances, platelet activation is suppressed. There are three known endothelial products that inhibit platelet activation: cyclooxygenase and lipooxygenase metabolites, such as prostacyclin and 13-hydroxyoctadecadienoic acid (13-HODE) respectively; ecto-nucleotidase ADP diphosphohydrolase (ecto-ADPase); and nitric oxide (Radomski *et al.,* 1987b; Moncada, 1982; Buchanan *et al.,* 1991). Prostacyclin has been shown to inhibit platelet aggregation (Radomski *et al.,* 1987a), while 13-HODE regulates the adhesiveness of the vessel wall, modulating integrin receptor expression (Buchanan *et al.,* 1991). The ecto-nucleotidases metabolize ADP to AMP and adenosine, resulting in a decrease in platelet recruitment and activity by the dinucleotide agonist (Marcus *et al.,* 1993); this molecule has recently been identified as CD39 (Marcus *et al.,* 1997). The role of NO as an inhibitor of platelet aggregation will be elucidated next.

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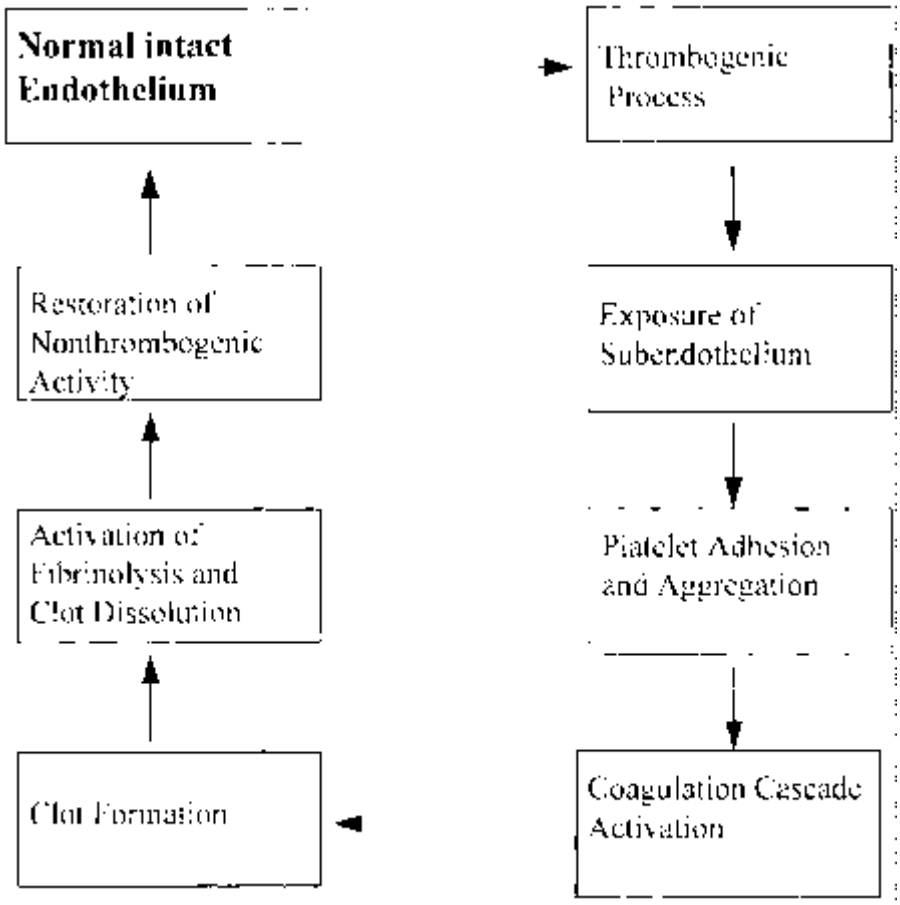


**Figure 13-1.** A simplified scheme of the hemostatic response.

## Platelet Adhesion

Adhesion of platelets to the subendothelial matrix following vascular injury (or contact with foreign surfaces in the blood) is the initial event in the process of thrombosis. Binding interactions occur between glycoproteins found on the platelet surface and the connective tissue of the subendothelium. Some of these glycoproteins include: GPIb/IX/V, GPIa/IIa, and GPIIb/GPIIIa. GPIb, which noncovalently complexes with GPIX and GPV, is the main surface receptor for the adhesion of platelets to the subendothelium. Adhesion is mediated by von Willebrand factor (vWF), which links the platelet integrin to collagen in the subendothelium (Vaughan, 1996). After these components come into contact, vWF undergoes a conformational change that is essential for the platelet and subendothelium to remain in contact. Direct binding of the platelet to collagen is mediated by GPIa/IIa.

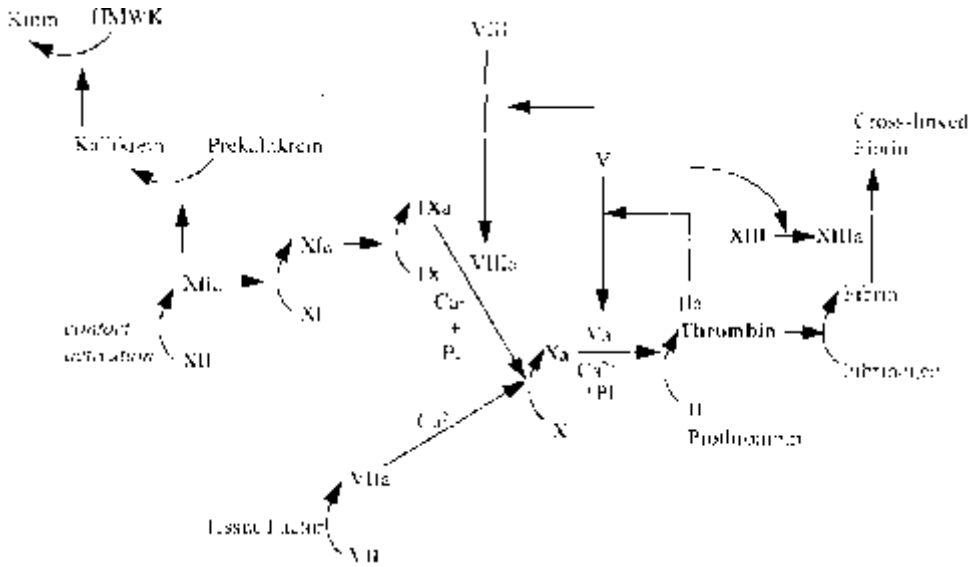


**Figure 13-2.** Representation of the normal pathways of coagulation.

### Platelet Recruitment and Aggregation

After adhesion to the subendothelial matrix, the platelet initiates a series of reactions that include release of adenosine 5'-diphosphate (ADP) and serotonin (5-HT) from densegranules; and release of a-granule constituents, including fibrinogen, vWF, vitronectin, fibronectin, thrombospondin, platelet factor 4, and platelet-derived growth factor (PDGF) (Vaughan, 1996). At the same time, the eicosanoid thromboxane $A_2$ ($TxA_2$) is formed from arachidonic acid via membrane-bound phospholipase C. Through the release of ADP, 5-HT, and $TXA_2$, more platelets are recruited to the area. Platelet recruitment occurs through the GPIIb/GPIIIa complex, also known as the fibrinogen receptor, which undergoes a calcium-dependent conformational change to become active (George *et al.,* 1984; Ginsberg *et al.,* 1988). Its ligand, fibrinogen, has two different sequences that can directly interact with platelets, including two RGD sequences in the a chain and a dodecapeptide sequence near the carboxyterminus of the chain (Hawiger *et al.,* 1982). Platelet-to-platelet linkages thereby form with fibrinogen as the bridge between GPIIb/IIIa molecules on adjacent platelets.

Simultaneously, the platelet aggregate activates the coagulation cascade via the assembly of prothrombinase on the platelet surface. This series of reactions leads to further platelet aggregation by the production of yet another platelet agonist, thrombin (Loscalzo, 1994). Thrombin is produced by cleavage of its inactive precursor prothrombin in the common pathway of the coagulation cascade (Figure 13–2). This reaction occurs through the action of activated factor X (Xa) and activated factor V (Va) along with calcium and phospholipid cofactors. Factor X activation can occur through either the intrinsic or extrinsic pathways. The intrinsic pathway is activated by contact of the blood with subendothelial structures like collagen or basement membrane components in areas of vessel damage. Factor VII undergoes a conformational change to expose its active site, which converts the zymogen prekallikrein to kallikrein and converts factor XI to activated factor XI (XIa). Next, factor XIa converts the zymogen factor IX into activated factor IX (IXa) that, in turn, activates factor X (Colman *et al.,* 1984). The extrinsic pathway is activated when the

membrane proteolipid, tissue factor, is either exposed in the subendothelium to flowing blood or induced to form on the surface of endothelial cells or vascular smooth muscle cells by inflammatory mediators such as cytokines and endotoxin. Tissue factor activates factor VII directly, which then activates factor X of the common pathway as described above (Wilcox *et al.,* 1989; Marmur *et al.,* 1991). In recent years, the exclusivity of the intrinsic and extrinsic coagulation pathways has been questioned. Crosstalk between early events in these pathways has been identified that both complicates our understanding of clotting mechanisms and adds yet another level of redundancy to hemostatic defense.

The platelet plug that forms in response to vascular injury is not stable. The fibrin meshwork formed by the action of thrombin on fibrinogen is required in order to add stability. Fibrin is produced when the serine protease thrombin cleaves the Aα and B β chains of fibrinogen. Fibrin can then be further stabilized through transamidation reactions by factor XIIIa, which itself is generated by the action of thrombin on factor XIII (Loscalzo, 1994).

## Endothelial Defense Mechanisms

If the hemostatic response were to occur inappropriately or without proper regulation, pathological thrombosis could result. The endothelium and the platelet itself release mediators that attenuate the hemostatic response, providing a mechanism of defense against unbridled hemostasis and thrombus formation. Two such mediators are prostacyclin and nitric oxide. Prostacyclin has been shown to inhibit platelet activation through cAMP-dependent mechanisms, while NO impairs platelet adhesion and activation, in part, by an increase in cGMP (Moncada *et al.,* 1982; Radomski *et al.,* 1990). The endothelium itself is also able to regulate thrombosis by degrading any prothrombotic vasoactive amines present in the blood, inactivating thrombin, and inducing expression of thrombomodulin, a thrombinbinding surface protein that facilitates thrombin-dependent activation of protein C, a naturally occurring anticoagulant that degrades factors Va and VIIIa (Esmon *et al.,* 1993). The fluidity of the blood is also maintained by endothelial surface glycosaminoglycans that catalyze the binding of the anticoagulant serine protease inhibitors (serpins), antithrombin III and heparin cofactor II, to specific coagulation proteins, such as thrombin, thereby attenuating coagulation and platelet activation (Rosenberg *et al.,* 1994). The endothelium also contributes to thrombus dissolution by producing molecules essential for fibrinolysis, including plasminogen activators.

## Fibrinolysis

Plasmin, the fibrinolytic counterpart to thrombin, is the principal mediator of fibrinolysis. This enzyme is converted from its plasma zymogen plasminogen by plasminogen activators, including tissue-type plasminogen activator (t-PA) and the urokinase-type plasminogen activators, all of which are serine proteases. Plasmin, once formed, cleaves fibrin leading to the dissolution of the fibrin clot or the thrombus.

The mechanism by which platelets regulate fibrinolysis has not been established. Platelets can directly bind plasminogen and t-PA, leading to enhanced activity of plasminogen (Adelman *et al.,* 1988). Platelet α-granules contain a myriad of substances that can regulate the fibrinolytic response, including plasminogen activator inhibitor-1 (PAI-1), $\beta_2$-antiplasmin, Cl esterase inhibitor, and $\beta_2$-macroglobulin, all of which are inhibitors of fibrinolysis (Ouimet and Loscalzo, 1994) (Figure 13–3). PAI-1 is one of the most important modulators of fibrinolysis, representing 60% of the plasminogen activator inhibitory activity in plasma (Ouimet and Loscalzo, 1994). Other roles for platelets in fibrinolysis include facilitation of clot retraction,

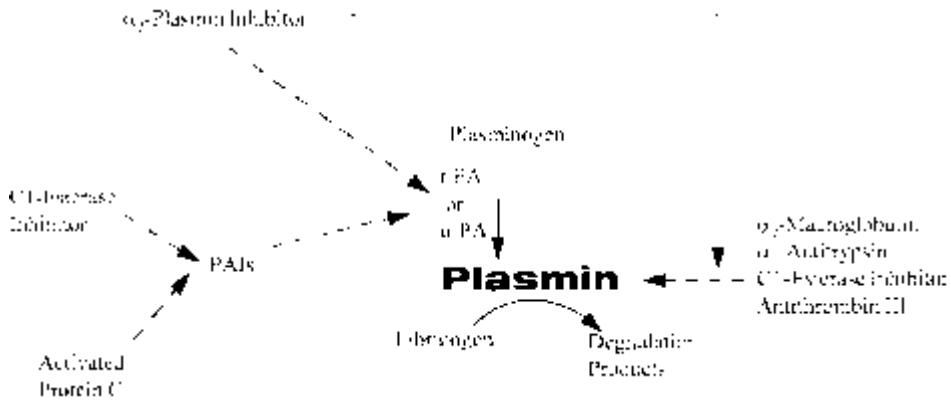


**Figure 13-3.** Molecules involved in plasminogen activation and its subsequent conversion to plasmin. Solid lines indicate activation; dashed lines indicate inhibition.

potentiation of plasminogen activation, modulation of fibrinolysis in the microenvironment by secretion of a-granule contents, and protection from factor XIIIa inactivation.

Plasmin has been shown to have the ability both to activate and inhibit platelets (Niewiarowski *et al.,* 1973; Guccione *et al.,* 1985). High concentrations of plasmin can act as a platelet agonist, yet low concentrations of plasmin lead to inhibition of platelet aggregation. At very high concentrations, plasmin modifies the platelet GPIIIa fibrinogenbinding domain leading to impaired fibrinogen binding and platelet aggregation, thus providing an explanation for the dichotomous actions of plasmin on platelet functional responses (Pasche *et al.,* 1991).

In addition to the endothelial and fibrinolytic systems discussed above, other systems also exist to limit the extent of thrombosis. Anithrombin III is a serpin that inhibits the formation of fibrin by inhibiting thrombin as well as other coagulation factors, including factors IXa, Xa, XIa, and XIIa (Rosenberg *et al.,* 1973). Heparin and endothelial heparan sulfate potentiate the anticoagulant actions of antithrombin III by catalyzing the binding of this serpin to these serine proteases (*Bjork et al.,* 1976). Proteins C and S provide another mechanism of anticoagulation. Protein C is activated by the thrombomodulin-thrombin complex, and in its active form is able to inactivate factors Va and VIIIa (Clouse *et al.,* 1986). Protein S is a cofactor for activated protein C, which has the added ability to potentiate fibrinolysis by complexing to plasminogen activator inhibitor -1 and enhancing plasminogen activation by t-PA.

## NITRIC OXIDE SYNTHESIS

Nitric oxide synthesis occurs in both the vascular endothelium and the platelet. NO is produced when the terminal guanidino nitrogen of L-arginine undergoes a 5-electron oxidation to form L-citrulline and NO (Palmer *et al.,* 1988; Nathan, 1992) (Figure 13–4). This reaction is catalyzed by the nitric oxide synthase (NOS) family of enzymes. There are two main isoform classes of NOS, a constitutive NOS (cNOS) and inducible NOS (iNOS, *Nos2* gene product), although the distinction between these isoform classes has been recently blurred by the finding that the "constitutive" endothelial isoform (eNOS or *Nos3* gene product) can be upregulated by exercise and the "inducible" isoform is constitutively expressed in the kidney (Green *et al.,* 1996; Markewitz *et al.,* 1993). The differences between these isoforms are shown in Table 13–1.

Constititutive NOS is found in vascular endothelial cells (eNOS) as well as in neuronal cells (nNOS, *No si* gene product) and is regulated by $Ca^{2+}$ and calmodulin. $Ca^{2+}$ is released from intracellular stores when acetylcholine or bradykinin stimulates inositol 1,4,5-triphosphate ($IP_3$) binding to endoplasmic reticulum receptors with the subsequent release of intracellular $Ca^{2+}$. This increase in $Ca^{2+}$ leads to its binding to calmodulin, which then binds to and activates cNOS (Dinerman *et al.,* 1993; Bredt *et al.,* 1990; Busse *et al.,*

**Table 13±1.** Characterization of inducible a nd constitutive nitric acid synthases.

| NOS isoform | iNOS | cNOS |
|---|---|---|
| Human Genome Nomenclature | | |
| Commutee denotation | [illegible] | [illegible] |
| Calcium dependence | no | yes |
| Calmodulin dependence | yes | yes |
| Cell genotype | macrophage | endothelial cells |
| Subcellular localization | cytosolic | membrane associated |
| Expression | inducible | constitutive |
| Human chromosomal localization | 17q11.2–q12 | 7q35–q36 |

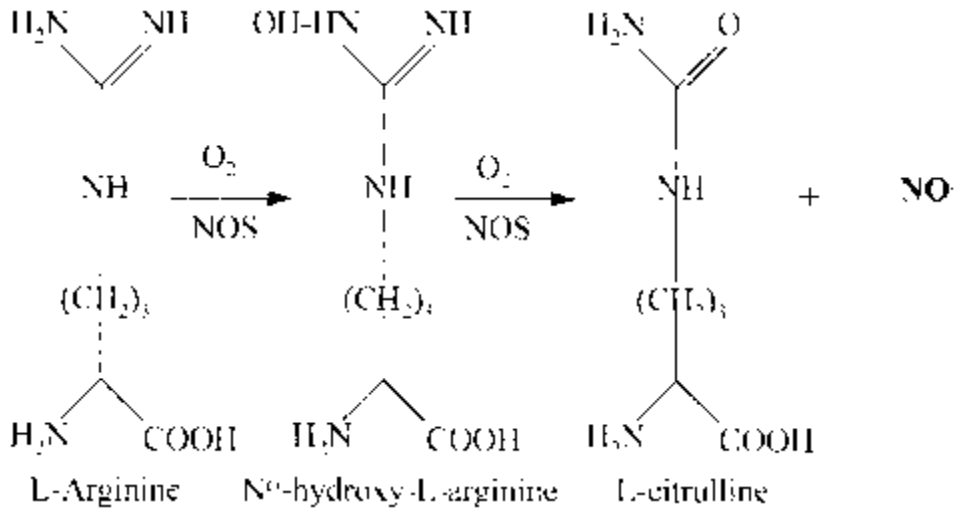


**Figure 13±4.** Diagram of nitric oxide synthesis by nitric oxide synthase (NOS).

1990; Mayer *et al.,* 1989). The production of NO by cNOS is, therefore, rapid, transient, and continuous until the $Ca^{2+}$ level returns to baseline. Inducible NOS has been found in many cell types including the macrophage, and the neutrophil (Marietta *et al.,* 1988; Yui *et al.,* 1991). Inducible NOS is stimulated by exposure to bacterial endotoxin or cytokines (Drapier *et al.,* 1988; Stuehr *et al.,* 1987; Ding *et al.,* 1988), and its activity is not dependent upon $Ca^{2+}$ concentration, but, rather, is regulated at the transcriptional level (Xie *et al.,* 1992). The production of NO by iNOS is delayed in comparison to production by cNOS, but the amounts of NO generated and the duration of its production by this isoform far exceed those of cNOS.

The mechanism of activation of NO synthase in the endothelial cell has not been elucidated. Since the constitutive form of NO synthase is $Ca^{2+}$-dependent, it has been postulated that this cation is responsible for the activation of NO synthase *in vivo* (Xie *et al.,* 1992). The process of endothelial activation may lead to activation of $Ca^{2+}$ channels leading to an increase in intracellular calcium, thereby regulating expression of NO synthase and NO production.

## NITRIC OXIDE AND GUANYLYL CYCLASE

The enzyme guanylyl cyclase has been shown to mediate, in part, the platelet inhibitory actions of NO. NO binds to the heme moiety of guanyly cyclase and induces a conformational change that displaces the iron out of the plane of the porphyrin ring (Ignarro, 1986). This effect results in the enzymatic conversion of guanosine 5'-triphosphate to cyclic guanosine 3',5'-monophosphate (cGMP) with concomitant stimulation of cGMP-dependent protein kinase. Cyclic GMP-dependent protein kinase phosphorylates intracellular enzyme targets that are responsible for regulation of intracellular calcium levels. Regulation of calcium level in platelets has been suggested as one mechanism of action of guanylyl cyclase. The mechanisms of action of cGMP-dependent protein kinase are many, including inhibition of platelet fibrinogen binding to the GPIIb/IIIa receptor, inhibition of phosphorylation of myosin light chains and of protein kinase C, and stimulation of phospholipase A2- and C-mediated responses (Radomski *et al.,* 1991a,b).

Cyclic GMP itself inhibits receptor-mediated calcium influx in platelets. An increase in soluble guanylyl cyclase leads to a reduction of intracellular $Ca^{2+}$ in platelets through cGMP-mediated inhibition of $Ca^{2+}$ release from intracellular stores, an increased rate of $Ca^{2+}$ extrusion, and decreased $Ca^{2+}$ entry from extracellular stores (Nakashima *et al.,* 1986; Matsuoki *et al.,* 1989; Morgan and Newby, 1989; Geiger *et al.,* 1992; Johansson and Hayness, 1992). An inhibitor of guanylyl cyclase, 1H-(1,2,4) oxadiazolo (4,3-a) quinoxalin1-one, blocks these cGMP-dependent effects resulting in an increase in platelet aggregation (Moro *et al.,* 1996). Inhibition of cGMP-mediated platelet responses can also occur through cyclic nucleotide phosphodiesterases, which can degrade cGMP. Radomski and colleagues showed that M+B 22984, a cGMP phosphodiesterase inhibitor, can potentiate L-arginine mediated anti-platelet effects (Radomski *et al.,* 1990).

## PLATELETS AND NITRIC OXIDE

Nitric oxide synthase isoforms exist in human platelets (Muruganandam *et al.,* 1994). An eNOS-like isoform has been isolated from platelets and appears to require similar co-factors as does eNOS itself. The molecular size of this constitutively expressed NOS was found to be 80 kDa in contrast to the 130-kDa protein found in endothelial cells, which could represent either differential splicing of the *Nos*3-like transcript or be the result of post-translational processing. An isoform of NOS homologous with iNOS has also been characterized in platelets. Since the platelet is anucleate with a minimal pool of mRNA (Djaffar *et al.,* 1991), and thus able to synthesize only modest amounts of protein, most platelet proteins are derived from its precursor cell, the megakaryocyte. The megakaryocyte expresses both constitutive and inducible NOSs (Lelchuk *et al.,* 1992).

Release of NO from the platelet has recently been established (Zhou *et al.,* 1995). Stimulation of NO synthase, dependent upon L-arginine, leads to activation of soluble guanylyl cyclase and, thereby, increases cGMP, with concomitant inhibition of platelet aggregation (Radomski *et al.,* 1990). Freedman and colleagues have recently demonstrated that NO can also inhibit platelet recruitment to a growing platelet thrombus (Freedman *et al.,* 1997). L-arginine has been shown to inhibit platelet aggregation *in vivo* and *in vitro*. A single, saturable, sodium-independent transporter system for movement of L-arginine into human platelets has recently been postulated (Vasta *et al.,* 1995). Radomski (1990) showed that L-arginine inhibited platelet aggregation induced by ADP and arachidonic acid. Yet, inhibition of thrombin-induced aggregation occurred only when other platelet antagonists were present, including prostacyclin and cGMP phosphodiesterase inhibitors.

The mechanism underlying NO's ability to regulate platelet aggregation is currently under investigation. Platelet agonists induce aggregation by increasing intracellular calcium levels via stimulation of the inositol

3-phosphate pathway. NO's ability to regulate intracellular calcium levels through cGMP and cGMP-dependent protein kinase has, therefore, been postulated as its mechanism of action, as described above. Recently, a molecular mechanism of how NO may modulate platelet aggregability via cGMP has been proposed. Reep and colleagues have suggested that phosphorylation of the signaling molecule rap 1 β in platelets is induced by NO stimulation of guanylyl cyclase and activation of cGMP-dependent protein kinase (Reep *et al.,* 1996). They showed that incubation with S-nitroso serum albumin leads to inhibition of collagen-stimulated platelet aggregation that was correlated with phosphorylation of rap 1 β in a dose-dependent manner. These data suggest that cGMP leads to activation of a signaling cascade within the platelet that results in modulation of platelet aggregation.

It appears that NO released from both endothelial cells and platelets regulates the platelet response and prevents thrombus formation. NO from endothelial cells suppresses platelet adhesion and aggregation. When stimulated with bradykinin, endothelial cells release NO in amounts sufficient to inhibit platelet adhesion, as is the case when NO is released under constant flow conditions in coronary and pulmonary vessels (Venturini *et al.,* 1989; Pohl *et al.,* 1989). Endothelial NO decreases aggregation of platelets immediately downstream from where it is released, suggesting that the endothelium is responsible for regulating platelets in its vicinity as opposed to platelets at a distance from the endothelial source of NO (De *Graaf et al.,* 1992). Release of endothelial NO *in vivo* by cholinergic stimulation and by substance P leads to inhibition of platelet aggregation induced by collagen or ADP (Humphries *et al.,* 1990). NO from the endothelial cell has also been shown to have the ability to disaggregate preformed platelet aggregates (Radomski *et al.,* 1987a).

One of the mechanisms by which endothelial NO can regulate platelet function has recently been elucidated by Murohara and colleagues (1995). P selectin is normally present in the Weibel-Palade bodies found in endothelial cells and also in the α-granules of platelets. Once the endothelial cell is activated, the granules fuse with the plasma membrane and P selectin is rapidly translocated to the extracellular surface. NO synthase inhibitors, such as L-NAME, induce endothelial P-selectin expression on the endothelial cell surface in the rat mesenteric circulation *in vivo*. This suggests that NO regulates adhesion of platelets to the endothelium by decreasing expression of the P selectin needed for initial attachment of the platelet to the endothelium. Michelson and colleagues have shown that S-nitroso-N-acetylcysteine can markedly inhibit upregulation of P-selectin on the platelet surface (Michelson *et al.,* 1996). Murohara and colleagues elucidated the mechanism of P-selectin expression by suggesting that thrombin stimulation leads to an increase in P-selectin on the surface of platelets through the activation of phosphoinositol-specific phospholipase C (PLC), which leads to an increase in intracellular $Ca^{2+}$ and activation of phosphorylation cascades within the platelet. They have shown that phorbol myristylacetate (PMA), a PKC activator, stimulates rapid P-selectin expression. Also, N,N,N-trimethylsphingosine, an inhibitor of PKC, has been shown to inhibit P-selectin expression on thrombin-stimulated cells. Since it has also been shown that NO and cGMP inhibit PKC activity, the authors postulated that NO may regulate PKC through phosphorylation by a cGMP-dependent kinase and, therefore, leads to down regulation of P-selectin expression on the surface of both platelets and endothelial cells.

Other blood-borne cell types have also been found to release NO, possibly augmenting the vascular NO pool provided by the platelet and the endothelial cell. The contribution of NO released from neutrophils in the regulation of platelet function *in vivo* has not been established; however, it has been shown that human neutrophils and mononuclear cells release a factor that has a similar pharmacological profile as NO with the ability to inhibit platelet aggregation (Salvemini *et al.,* 1989). The neutrophil itself can be regulated by NO, with NO inhibiting adhesion and chemotaxis of stimulated cells. (Kubes, 1991; Moilanen, 1993).

## Effect of S-Nitrosothiols on Platelet Aggregation

NO reacts with thiols in the presence of oxygen to produce S-nitrosothiols. In plasma, S-nitrosothiols are the primary redox state of NO (Stamler *et al.,* 1992). S-nitrosothiols have been suggested to be a more stable source of NO in plasma, in contrast to free NO, and, therefore, S-nitrosothiols have been postulated to be a storage pool of NO. S-nitrosothiols activate platelet soluble guanylyl cyclase and elevate cGMP levels, leading to inhibition of platelet aggregation (Mellion *et al.,* 1985). Loscalzo and colleagues showed that nitroglycerin and nitroprusside can inhibit platelet aggregation at pharmacologically achievable concentrations *in vivo* and this effect is potentiated by thiols (Loscalzo and Welch, 1995). Mendelsohn and colleagues showed that S-nitroso-N-acetylcysteine, an S-nitrosothiol compound, decreased the intracellular calcium flux in response to ADP stimulation leading to inhibition of fibrinogen binding in activated platelets, an effect accompanied by a rise in intracellular cGMP (Mendelsohn *et al.,* 1990). Keaney and colleagues showed that endogenous NO can react with serum albumin to form S-nitroso-albumin, which was shown to have antiplatelet properties (Keaney *et al.,* 1993). Simon and colleagues bolstered the importance of S-nitrosothiols in platelet function by showing that the platelet surface is able to facilitate the release of NO from S-nitrosothiols (Simon *et al.,* 1993).

## Synergy of NO with other Platelet Inhibitors

Other factors produced by the endothelial cell and the platelet work in concert with NO to potentiate inhibition of platelet activation and aggregation. Some of these factors include $PGI_2$, $PGD_2$, and t-PA (Radomski and Moncada, 1991a,b; Loscalzo, and Vaughn, 1987). It appears, however, that no single factor is able to limit the activated-platelet response; rather the synergy of several factors is essential for limiting platelet aggregation optimally. Prostacyclin synthesis by endothelial cells is stimulated by mechanical injury, PDGF, and bradykinin (Gerrard and White, 1982). Prostacyclin, a potent inhibitor of aggregation, is a very weak inhibitor of platelet adhesion, and NO does not synergize with prostacyclin as an inhibitor of adhesion. Prostacyclin's ability to limit platelet adhesion appears to be regulated by cGMP-dependent responses as opposed to its usual mechanism of action through cAMP-dependent responses. Radomski and colleagues have shown that NO and PGI2 do not act synergistically to inhibit adhesion since there is no anti-adhesive actions of PGI2 (Radomski *et* al., 1987a). In the clinical realm, inhibition of NO production *in vivo* decreases the ability of aspirin to attenuate platelet aggregation (Golino *et al.,* 1992). NO and prostacyclin appear to synergize with each other through an NO-induced increase in cGMP with subsequent inhibition of cGMP-inhibited cAMP phosphodiesterase (PDE), resulting in potentiation of prostacyclin's cAMP-mediated antiplatelet effects (Maurice and Haslam, 1990).

The actions of NO can also be potentiated by protective mechanisms within the platelet. Superoxide anions produced by damaged endothelial cells, leukocytes, and smooth muscle cells can inactivate NO through the formation of peroxynitrite, which is further metabolized to nitrite and nitrate. Peroxynitrite is very highly reactive and able to cause cell toxicity through lipid peroxidation and nitrosation of protein tyrosyl residues. Moro has demonstrated that peroxynitrite stimulates platelet aggregation and limits NO and prostacyclin's ability to impair aggregation (1994). Superoxide dismutase (SOD), an enzyme which catalyzes the dismutation of Superoxide into water and hydrogen peroxide, can protect NO from oxidative inactivation by superoxide. SOD potentiates the ability of NO to prevent adhesion and aggregation of platelets induced by thrombin *in vivo*. Administration of superoxide dismutase to damaged and stenotic carotid arteries leads to a decrease in thrombus formation at the site of vascular damage (Meng *et al,* 1995).

## THROMBOSIS AND NO DEFICIENCY

Decreased production of NO can lead to thrombosis. Clinical evidence substantiates the antiplatelet role of NO. Endothelial damage induced by laser injury leads to inhibition of NO release and, consequently, platelet aggregation (Rosenblum *et al.,* 1987). Nishimura and colleagues showed that nitric oxide synthase blockade *in vivo* enhances the accumulation of activated platelets in areas of endothelial damage within brain pial arterioles (Nishimura *et al.,* 1991). L-Nitroarginine methyl ester (L-NAME), an inhibitor of NO synthesis, potentiates the pulmonary accumulation and prolongs the disaggregation of [$^{111}$In]-labeled platelets induced by ADP, PAF, and thrombin *in vivo* (Radomski and Moncada, 1991a,b). In experimental coronary stenosis in rabbits, massive platelet adhesion and formation of aggregates on the surface of the damaged endothelium was observed in those animals treated with $N^G$-monomethyl-L-arginine (L-NMMA), another inhibitor of nitric oxide synthesis, prior to the production of the stenosis (Herbaczynska-Cedro *et al.,* 1991). Folts and colleagues used a canine model of coronary artery stenosis to demonstrate that intravenous nitroglycerin, an organic nitrate, can improve ischemia associated with acute coronary artery disease by inhibition of platelet thrombus formation (Folts *et al.,* 1991).

In addition, the antithrombotic effects of nitrates are potentiated by pretreatment of the animals with reduced thiols such as N-acetylcysteine. Impaired NO synthesis has been demonstrated in hypertension (Cadwgan and Benjamin, 1991) and in diabetes mellitus (Calver *et al.,* 1992), and this deficiency could be involved in the thrombotic complications that occur in these disease processes. In addition, studies with inhibitors of NOS have shown that decreased generation of NO *in vivo* can lead to platelet and polymorphonuclear leukocyte activation and thrombosis (May *et al.,* 1991; Herbaczynska-Cedro *et al.,* 1991; Golino *et al.,* 1992; Yao *et al.,* 1992). When L-NMMA was given to healthy subjects and platelet function monitored *in vivo,* hemostasis was disturbed and platelets became activated as measured by the increase in plasma levels of markers of platelet activation, including β-thromboglobulin and platelet factor 4 (Bodzenta-Lukaszyk,1994).

We recently studied two brothers with a cerebral thrombotic disorder and found that their platelets could not be inhibited by NO. The activity of the antioxidant enzyme, plasma glutathione peroxidase, was found to be decreased in the patients' plasma, accounting for their insensitivity to NO. Addition of exogenous glutathione peroxidase led to restoration of platelet inhibition by NO. The mechanism of action of glutathione peroxidase involves reduction of lipid hydroperoxides to their corresponding alcohols. Granule secretion following platelet activation results in release of eicosanoids, including lipid hydroperoxides, which are then reduced by glutathione peroxidase (Freedman *et al.,* 1995). Impaired metabolism of lipid hydroperoxides, as occurs when glutathione peroxidase activity is decreased, can lead to an increase in hydroperoxyl radical concentration which can, in turn, react with and reduce bioactive NO. These reactions thus impair the ability of NO to inhibit platelet activation, leading to a thrombotic propensity (Freedman *et al.,* 1996). Previously, Freedman and colleagues had shown that glutathione peroxidase can potentiate the ability of the naturally occurring S-nitrosothiol, S-nitroso-glutathione, to inhibit platelets (Freedman *et al.,* 1995).

## NO AND ATHEROTHROMBOSIS

Thrombosis is a major determinant of atherosclerosis. In humans and animals with atherosclerosis, the ability of the endothelium to synthesize bioactive NO is greatly reduced (Chester *et al.,* 1990). Impaired NO production may promote atherosclerosis by several mechanisms. Mitogens, like PDGF, have been postulated to be responsible for the proliferation of smooth muscle cells within atherosclerotic lesions (Radomski and Moncada, 1991a,b). Mitogen activity can be reduced by suppressing their release from

stimulated platelets, an effect supported by NO. Administration of L-arginine, the substrate for NOS, was found to restore impaired production of NO, decrease activation of platelets and leukocytes, and limit the extent of the vascular lesions associated with this disease in an animal model of hypercholesterolemia (Dexter *et al.,* 1991; Cooke and Tsao, 1992).

Vascular injury occurs when balloon angioplasty is performed to treat occlusive arterial lesions. Angioplasty results in endothelial denudation and varying degrees of smooth muscle cell injury. Normally, endothelial injury exposes the procoagulant molecules located in the subendothelium, leading to platelet adhesion, aggregation, and thrombosis. Marks and colleagues found that local administration of a relatively stable S-nitrosothiol, polyS-nitroso-bovine serum albumin, can inhibit intimai proliferation and platelet deposition after denuding arterial injury (Marks *et al.,* 1995). Another group used balloon injury to denude the endothelium of rat carotid arteries, thereby limiting the ability of the endothe lium to produce NO (von der Leyen *et al.,* 1995). They restored NO production by using gene transfer techniques to deliver the *Nos3* gene to the site of vascular injury, leading to inhibition of neointimal lesion formation.

In coronary bypass grafting, either an artery or a vein may be used as a new conduit. It has been suggested that more effective release of NO by arterial vessels may be responsible for greater patency of arterial grafts than venous bypass grafts (Luscher *et al.,* 1989). Production of NO is stimulated by ADP and other agents in the internal mammary artery but not in the saphenous vein. Thus, the lower NO synthesis rate in the saphenous vein coronary bypass grafts may explain the patency differences.

Atherosclerotic plaques have been demonstrated to contain the cytokines, IL-1$\beta$ and TNF- (Arbustini *et al.,* 1991). These cytokines can induce iNOS in cells within the plaque, so that they may be partly involved in the balance between prothrombotic and antithrombotic processes by this mechanism. In injured arteries, vascular smooth muscle cell iNOS is rapidly induced within one day of injury. This leads to inhibition of platelet adhesion and maintenance of blood flow in the injured vessel (Yan *et al.,* 1996). Yet, production of NO via iNOS has also been shown to produce cellular toxicity via peroxynitrite generation. Peroxynitrite, which decreases the bioavailability of NO, has been observed to produce nitrostyrosine at sites of endothelial injury in atherosclerotic lesions (Kooy *et al.,* 1994).

Lipoproteins that may be present in atherosclerotic lesions can also regulate NO activity. Low-density lipoprotein (LDL) can decrease the bioactivity of NO by an unknown mechanism (Flavahan *et al.,* 1992). Possible explanations for this decreased activity include inhibition of NOS activity by LDL, inactivation of NO itself, or changes in NO metabolism. In addition, LDL may decrease the uptake of L-arginine into platelets, and this may lead to a decrease in the NOS activity, thereby promoting thrombosis (Chen *et al.,* 1994). The presence of high-density lipoproteins (HDL), however, is beneficial since HDL decreases platelet activitity by increasing NOS activity in platelets.

## NO AND BLEEDING DIATHESES

Lipopolysaccharide (LPS or endotoxin) is responsible for the development of septic shock associated with bacterial infection. Severe hypotension and disturbances in the hemostatic balance are among the major clinical symptoms of septicemic reactions. The bleeding diathesis in sepsis is believed to be caused by disseminated intravascular coagulation with consumption coagulopathy, fibrinolysis, and thrombocytopenia (Radomski and Moncada, 1991a,b). Stimulation of endothelial cells with LPS and interferon-$\gamma$, a cytokine produced during inflammatory reactions, results in expression of iNOS. This enzyme is responsible for producing large amounts of NO which are sufficient to inhibit platelet aggregation. Shultz and Raij (1992) reported that LPS can induce production of endogenous NO, which is critical for preventing LPS-induced platelet-dependent renal thrombosis. Another consequence of septic shock that is due to upregulation of

NOS enzymes is hypotension. Keaney and colleagues used methylene blue—an inhibitor of guanylyl cyclase and of NOS, and also a direct NO oxidant—to reduce NO production and restore mean arterial pressure in a rabbit model of LPS-induced hypotension (Keaney *et al.,* 1994). Bleeding complications that accompany endotoxemia may, thus, result from upregulated expression of iNOS in vascular cells and leukocytes.

Hemostatic defects, including prolonged bleeding times and decreased platelet adhesion and aggregation, also occur in uremia. In uremia, metabolites of the urea cycle accumulate, including L-arginine, which may account for the increased NO production in these patients (Radomski and Salas, 1995). Bode-Boger and colleagues showed that L-arginine increases the excretion of NO byproducts, including nitrate. They demonstrated that the addition of L-arginine to the diets of hypercholesterolemic rabbits increases the ratio of L-arginine to the endogenous NO synthase inhibitor assymetric dimethylarginine (ADMA) thereby restoring urinary nitrate excretion (Bode-Boger *et al.,* 1996). It has been shown that the prolonged bleeding times in uremie rats can be normalized by systemic administration of L-NMMA.

NO donors activate the plasma fibrinolytic system in peripheral vascular disease patients by inhibiting the release of PAI-1 (Gryglewski, 1993). Gryglewski reported that PGI2 synergizes with the NO donor molsidomine to increase the activity of the fibrinolytic system in patients with peripheral vascular disease. This cooperative effect is attributed to PGl2's ability to release t-PA from cells coupled with the NO donor's ability to inhibit release of the antifibrinolytic molecule, PAI-1. This work has been substantiated by the results of Korbut and colleagues, who showed that SIN-1, an NO donor, can also inhibit release of PAI-1 from platelets (Korbut *et al.,* 1993).

Inhalation of NO results in an increase in the template bleeding time (Hogman *et al.,* 1993). Simon and colleagues (Simon *et al.,* 1995) found that inhibition of NO production by L-NMMA shortens the bleeding time modestly. *In vitro,* the effects of NO were significantly different, resulting in a significant decrease in platelet activation or aggregation.

## PHARMACOLOGICAL ROLE OF NO DONORS IN VASCULAR THROMBOSIS

Organic nitrates have been used for over a century in the treatment of cardiovascular disease. The potential benefit of nitroglycerin as an inhibitor of platelet aggregation was first observed in 1967 by Hampton (Loscalzo and Welch, 1995). Organic nitrates by themselves are weak inhibitors of aggregation *in vitro;* however, following ADP stimulation, nitroglycerin has been shown to be a potent inhibitor of aggregation *ex vivo* (Chirkov *et al.,* 1993). Intravenous administration of nitroglycerin and isosorbide mononitrates can lead to inhibition of the platelet activation (Wallen *et al.,* 1994); these agents have also been shown to inhibit thrombosis in animals (Werns *et al.,* 1994; Plotkine *et al.,* 1991). Patients suffering from myocardial infarction and treated with nitroglycerin show a decrease in platelet adhesion and aggregation in comparison to patients not given this agent (Diodati *et al.,* 1990). Kuritzky showed that nitroglycerin can disaggregate platelet-rich thrombi in the retinal circulation (Kuritzky *et al.,* 1984). The effectiveness of the organic nitrates correlates with the extent of vascular injury. An animal model in which arterial injury is induced in pigs by balloon angioplasty showed increased inhibition of platelet deposition in the injured area after treatment with nitroglycerin in comparison to pigs with milder injury treated with nitroglycerin (Lam *et al.,* 1988).

Loscalzo showed that nitroglycerin requires intracellular reduced thiols in its mechanism of platelet inhibition (Loscalzo, 1985). The requirement of thiols as potentiators of organic nitrates may also be beneficial for limiting or targetting the effects of NO. It would be useful to inhibit platelet aggregation using organic nitrates without evoking the deleterious effects that NO has on blood pressure. Radomski and

colleagues have shown that S-nitrosoglutathione is able to inhibit platelet aggregation without altering blood pressure in rats (Radomski *et al.,* 1992). Similar results have been obtained by intraarterial injections of S-nitrosoglutathione (de Belder *et al.,* 1994). Organic nitrites may also affect preformed platelet aggregates. Stamler showed that nitrogylcerin can disaggregate platelet aggregates (Stamler *et al.,* 1989). Others have shown that nitroglycerin can reduce platelet adhesion to injured vessels (Lam *et al.,* 1988).

NO donors that do not require metabolic breakdown include sodium nitroprusside, SIN1, molsidomine, and S-nitroso-N-acetylpenicillamine (SNAP). Nitroprusside has been shown to inhibit platelet aggregation *in vivo* and *in vitro* (Levin *et al.,* 1982). Molsidomine and SIN-1 lead to inhibition of thrombosis in jugular and mesenteric venous systems in patients suffering from myocardial infarction and controls (Wautier *et al.,* 1989). Molsidomine and isosorbide dinitrate synergize with prostacyclin and prostaglandin $E_1$ to inhibit platelet activation (Sinzinger *et al.,* 1992). SNAP has been shown to inhibit prostaglandin synthesis by inhibiting lipooxygenase, a key enzyme for arachidonic acid metabolism to oxidized eicosanoid derivatives in human platelets (Maccarrone *et al.,* 1996)

The beneficial properties of aspirin in limiting platelet aggregation and thrombosis have been well established (Patrono, 1994). In an attempt to improve upon the antiplatelet actions of aspirin, while limiting the negative gastric mucosal effects that occur with prolonged treatment, Wallace and colleagues studied the activity of a nitric oxide-releasing, gastricsparing aspirin derivative. The nitroxybutylester derivative of aspirin (NCX 4215) was seven times more potent than aspirin in limiting platelet aggregation *in vitro* without inducing gastric mucosal injury. NCX 4215 was found to release NO in the presence of platelets and to increase platelet levels of cGMP (Wallace *et al.,* 1995).

## CONCLUSIONS

In this chapter we have reviewed the importance of nitric oxide in regulating hemostasis. Our understanding of the role of NO in the hemostatic system has dramatically increased in this past decade. Advances in knowledge of the molecular mechanisms by which NO modulates the hemostatic response will lead to the development of novel therapeutic strategies to prevent the pathological processes of NO-deficient thrombotic disorders and NO-dependent bleeding diatheses.

## ACKNOWLEDGMENTS

The authors wish to thank Stephanie Tribuna for her help in the preparation of this manuscript.

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