Nitric Oxide: News from Stem Cells to Platelets

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Abstract: Nitric oxide (NO) is a diffusible, short-lived, diatomic free radical ubiquitously produced by mammalian cells. The generation of NO from L-arginine is enzymatically regulated by three different isoforms of NO synthases. The NO signaling pathway involves mainly the activation of soluble guanylyl cyclase to produce cyclic GMP (cGMP) as a second messenger and downstream mediator. In addition, the free radical activity of NO can cause cellular damage through a phenomenon known as nitrosative stress. NO is a pleiotropic biomodulator in several systems, including the cardiovascular, nervous and immune systems. In the hematopoietic system, NO is thought to be an autocrine or paracrine messenger but also an intracellular effector molecule. Megakaryopoiesis and subsequent thrombopoiesis occur through complex biologic steps that involve hematopoietic stem cell commitment to megakaryocytic lineage, megakaryocyte maturation and finally, platelet release. Here, we summarize the current knowledge regarding the role of exogenous and endogenous NO in hematopoietic stem cell biology, megakaryocyte development and platelet biogenesis as well as relevance of platelet-derived NO generation on platelet function. Dysregulation of NO synthesis has been observed in several diseases, and the evaluation of a series of pharmacological agents with the ability to modulate the NO/cGMP pathway in platelets will also be discussed.

Keywords: Nitric Oxide, cGMP, platelets, megakaryocytes, stem cells, apoptosis, statins, NO-NSAID.

INTRODUCTION

Nitric oxide (NO) is a multifunctional signaling molecule that regulates a wide variety of complex cellular processes, such as platelet function, vasodilation, immunity, and neurogenesis among others [1-5]. NO acts in both autocrine and paracrine signaling modes through a variety of mechanisms, such as soluble guanylyl cyclase activation, binding to iron in the prosthetic groups of proteins, nitrosylation of proteins, inhibition of ribonucleotide reductase, and inhibition of diverse enzymes of the respiratory chain [6-9]. Binding of NO to soluble guanylyl cyclase induces an increase in cyclic GMP (cGMP) levels that leads to activation of cGMP-dependent protein kinase (PKG) [10-13], stimulation or inhibition of phosphodiesterases (PDE) 2, 3 and 5 [14], and activation of cyclic nucleotide-gated (CNG) channels [15] that trigger effector responses in neurons, platelets, endothelial cells and vascular smooth muscle cells [16-18].

This free radical is endogenously synthesized from L-arginine by a group of enzymes known as NO synthases (NOS). There are three NOS genes in the mammalian genome, coding for the neuronal (nNOS or NOS1), endothelial (eNOS or NOS3), and inducible (iNOS or NOS2) isoforms. Expression of each NOS has been reported in many cell types, including human and rodent bone marrow and blood [19]. For instance, iNOS mRNA can be detected in megakaryocytes, eosinophils, unstimulated monocytes, and $CD34^+$ cells [20-22]; eNOS mRNA has been found in platelets, megakaryocytes, and lymphocytes [23-25]; and nNOS mRNA has been detected in neutrophils [21, 26].

The most studied actions of NO are in the cardiovascular system where its powerful inhibitory effect on both platelet aggregation and vascular smooth muscle cell proliferation has been extensively demonstrated *in vitro* and *in vivo* [1, 2]. In addition, since the anti-inflammatory effect of endothelial NO has been well established along with its protective effect in the atherosclerotic disease, NO is now considered as a therapeutic option to slowing progression of atherosclerosis and reducing the risk of thrombosis [27].

It is important to note that the NO concentrations mediating its physiologic or protective effects are extremely low (picomolar to nanomolar). However, at higher concentration, NO can be vastly cytotoxic, a feature that is exploited by inflammatory cells in response to pathogens by upregulation of iNOS expression [28]. Indeed, a large body of evidence support the notion that high concentrations of NO suppress proliferation and/or induce apoptosis of different cell types, such as macrophages [29, 30], thymocytes [31], smooth muscle cells [32], pancreatic B cells [33], cardiomyocytes [34], and endothelial cells [35] among others. The mechanisms involved in the NO cytotoxic effect have been carefully reviewed previously [36].

In the hematopoietic system, NO has a major role in megakaryo/thrombopoiesis by acting on the physiopathology of the different cell types involved in this process, including hematopoietic stem cells (HSC), progenitors, mature megakaryocytes and platelets. The most relevant findings obtained in this field during the last decade and the consensus or controversies they present to the scientific community are discussed in this review.

NO AS A REGULATOR OF HSC BIOLOGY

Development of the hematopoietic system is a stage-specific process where the bone marrow eventually becomes the principal source of hematopoiesis in the adult mammalian organism. Sustained hematopoiesis in the bone marrow, however, depends on the self-renewal of the resident HSC, which give rise to a defined set of committed progenitor cells (HPC) to maintain the homeostasis of hematopoiesis, or to provide a source of precursor cells to repair damaged tissues. Under steady-state conditions, most HSC are in close contact with bone marrow stromal cells in the osteoblastic niche,

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which provide a quiescent microenvironment for HSC maintenance [37, 38]. The regular demand of hematopoietic cells under physiological or stress conditions, such as bone marrow ablation, stimulates a sequence of events where HSC are recruited to a permissive (vascular) niche where they can proliferate, differentiate, and reconstitute hematopoiesis [39].

The first studies examining the effect of NO on hematopoietic stem and progenitor cells were published in the mid 90's, and they demonstrated that NO can change the extent of hematopoietic maturation through the inhibition of erythroid and myeloid colony formation [20, 40]. In a similar *in vitro* model, the anti-proliferative action of Interferon-γ (IFN-γ) and Tumor Necrosis Factor-α (TNF-α), as well as lipopolysaccharide (LPS), on both murine and human hematopoietic cells was blocked by NOS pan-inhibitors [20, 41, 42], indicating that endogenous NO is one of the mediators involved in the inflammation-mediated hematopoietic suppression. In these studies, the decrease in cell growth was associated with a cytotoxic effect of NO, especially with apoptosis induction, because of the high levels of NO generated by activation of iNOS synthesis. Different growth factors present in the bone marrow, such as Thrombopoietin (TPO), Granulocyte-colony stimulating factor (G-CSF) and Stem cell factor (SCF) as well as cAMP analogs have shown to rescue hematopoietic progenitors from cell death mediated by NO [43] indicating that the concerted action of all these molecules is critical for the protection of HSC against the stress-induced demands for hematopoiesis.

Recent studies using transgenic mice revealed that NO that is constitutively produced in the bone marrow by the eNOS isoform, has also a great impact in the regulation of HSC. It has been shown that $eNOS^{-/-}$ mice have an impaired hematopoietic reconstitution, a reduced Vascular Endothelial Growth Factor (VEGF)-induced mobilization of HSC and endothelial progenitor cells (EPC), and a significantly increased mortality after 5-fluorouracil-induced myelosuppression compared with wild-type mice, suggesting that eNOS is essential for recruitment of stem cells and hematopoietic recovery. Moreover, a paracrine action of vascular stromal cell-derived NO appears to account for the regulation of the HSC functionality [44]. Bone marrow ablation or chemokine/cytokine administration induces upregulation of metalloprotease-9 (MMP-9) resulting in the release of sKitL, a membrane bound cytokine, governing growth, survival, and/or differentiation of hematopoietic cells [39]. Because MMP-9 is a major target for NO and $eNOS^{-/-}$ mice have a blunted MMP-9 activity, a deficient NO/MMP-9/sKit axis appears to be the underlying mechanism in the mobilization defect of HSC in $eNOS^{-/-}$ mice.

Among other studies, that used NOS inhibitors to examine mice's HSC functionality *in vivo*, Michurina *et al*. reported that the exposure of mice to NOS inhibitors, either directly or after irradiation and bone marrow transplantation, increases the number of stem and progenitor cells in the bone marrow and this introduces the notion that NO regulates the quiescence of HSC [45]. Indeed, further studies from the same group have shown that nNOS expressed in stromal cells were responsible for the NO-mediated paracrine control of HSC quiescence [46]. However, in contrast to these findings, Ozuyaman *et al.* demonstrated that the bone marrow from mice with deficient eNOS increased EPC ($CD34^+$Flk-1^+) but not HSC. Moreover, while G-CSF effectively mobilized HSC in eNOS mutant mice, EPC response to this cytokine was null, suggesting that eNOS derived-NO effects would be restricted to this EPC subpopulation [47]. On the other hand, HSC damage induced by ionizing irradiation was linked to the nNOS-derived NO apoptosis induction [48], highlighting that there is not only an autocrine role for nNOS in HSC but that it is also opposite to the protective function of the NO released from the stromal nNOS. Fig. (**1**) summarizes the putative roles of NO in HSC biology.

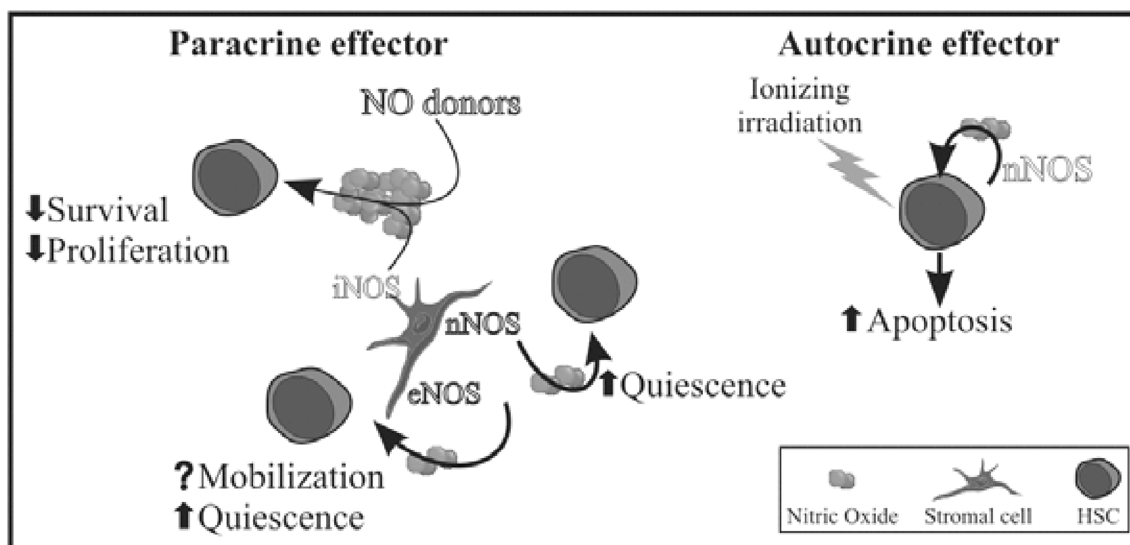

Fig. (1). NO as a paracrine and/or autocrine effector on hematopoietic stem cells. HSC are in close contact with bone marrow stromal cells which regulate quiescence of HSC through the release of eNOS-derived NO [44]. However, Krasnov *et al*. have attributed this role to NO derived from nNOS [46]. Whether NO is involved on HSC mobilization remains unclear. High levels of NO generated by iNOS or NO donors promote a decrease in cell growth and induction of apoptosis. HSC damage mediated by ionizing irradiation has been linked to cell death induced by nNOS-derived NO [48].

ROLE OF NO IN MEGAKARYOPOIESIS

Megakaryopoiesis is the process leading to the development and production of megakaryocytes and platelets from progenitor cells. Megakaryocytes are the largest cells of the bone marrow characterized by a single polylobulated nucleus and represent less than 0.05% of the marrow cells. After the discovery that platelets produce NO and that platelets are enucleated cells with a limited capacity of protein synthesis, the presence of NOS isoforms was searched for in both platelets and their parent cells. Initial studies showed that the human megakaryoblastic cell line, Meg-01, possessed constitutive NOS (cNOS) and that cell stimulation by cytokines like Interleukin-1β (IL-1β) and TNF-α resulted in expression of the iNOS isoform. Moreover, like in other cell types, a reciprocal interaction between iNOS and cNOS appeared to regulate their activity [49]. The expression of both isoforms was further confirmed in primary megakaryocytes from patients with atherosclerosis or with normal arteries. Interestingly, the ratio of iNOS/cNOS expression and activity was higher in patients with severe coronary atherosclerosis than in the control group [50]. The prevalence of iNOS function in megakaryocytes correlated with the notion of the inflammatory nature of atherosclerosis disease. However, as iNOS was not detected in the platelets of these patients, it is not clear whether this increased iNOS expression in megakaryocytes has any relationship with the atheroma development or with the process of megakaryopoiesis [50]. Further studies focused on the identification of the NOS isoforms in the bone marrow revealed that cNOS in megakaryocytes corresponded to the eNOS isoform, iNOS was observed only in a subpopulation and nNOS was not detected in megakaryocytes [21].

The role of NO in megakaryopoiesis has been investigated in several studies. NO donors induce apoptosis in Meg-01 and HEL megakaryocytic cell lines as well as in primary cells including mature megakaryocytes and its progenitors [42, 51]. Moreover, the induction of endogenous NO following up-regulation of iNOS by inflammatory substances like TNF-α, IL-1β IFN-γand LPS results in inhibiting megakaryocyte proliferation, suggesting that both exogenous and endogenous sources of NO can regulate megakaryocyte growth [42]. The downstream signaling pathways participating in NO-mediated megakaryocyte apoptosis involve cGMP dependent and independent mechanisms. Intracellular cGMP accumulation *via* permeable analogues, activators of guanylyl cyclase or inhibitors of cGMP-specific PDE enhances apoptosis [52]. However, a direct effect of peroxynitrite could also account for the cytotoxic effect of NO. The cell response to NO has been linked to the cell's ability to simultaneously release oxygen radicals, specifically superoxide that produce peroxynitrite, removing free NO from the cellular environment in the process. In fact, treatment of megakaryocytic cells with factors that reduce the formation of superoxide partially abrogates NO-induced apoptosis [51].

Further dissection of the molecular basis involved in NO-mediated apoptosis of megakaryocytic cells shows regulation of the balance between pro- or anti-apoptotic members of the Bcl-2 family. In this sense, NO promotes the decrease of the anti-apoptotic bcl-2 and bcl-xl proteins with a concomitant enhanced expression of the pro-apoptotic molecule Bax [51, 52]. The role of caspase-3 activation in apoptosis triggered by NO is still not clear. Although in Meg-01 and HEL cell lines the NO-induced apoptosis appears to be caspase independent, in primary cells, NO promotes the cleavage and activation of caspase-3 [52]. As NO kills not only mature but also megakaryocyte progenitors, NO apoptotic activity in the bone marrow should be effectively controlled to avoid thrombocytopenia. Several molecules present in the bone marrow microenvironment play an important role in megakaryocyte survival. TPO, the main megakaryocyte growth factor, promotes proliferation, differentiation, and survival of megakaryocyte precursors. In megakaryocytic cell lines, TPO inhibits apoptosis mediated by NO [51]. In addition, prostacyclin (PGI_2), the main metabolite of arachidonic acid in endothelial cells, appears to be another relevant regulatory molecule of the bone marrow milieu that protects megakaryocyte from NO-mediated death. The anti-apoptotic activity exerted by PGI_2 is associated with the elevation of intracellular cAMP levels and the suppression of cGMP accumulation [52]. Interestingly, this opposite interplay between cyclic nucleotides in megakaryocytes apoptosis is in contrast to the well-known synergistic inhibitory effect of these second messengers on platelet activation [53] reflecting possible downstream signals in megakaryocytes, not present in the enucleated platelets.

Terminal stages of megakaryopoiesis include proplatelet formation, platelet release and the eventual megakaryocyte death [54]. Thromobopoiesis, the mechanism by which platelets are produced from megakaryocytes, is not completely understood. In recent years a new hypothesis for platelet formation has emerged in which activation of the apoptotic cell machinery of megakaryocytes is directly involved in platelet shedding. In fact, the process of platelet assembly in megakaryocytes exhibits some characteristics associated with apoptosis, including cytoskeletal reorganization, membrane condensation and ruffling [55]. How would a dying megakaryocyte lead to platelet assembly and release? Interestingly, Batinelli *et al.* show that NO induces apoptosis of Meg-01 cells and, at the same time together with TPO, triggers the release of functional platelet-sized particles [56]. They suggest that in the thrombopoiesis process, NO performs a dual function, promoting apoptosis *via* a cGMP-dependent mechanism and enhancing terminal differentiation and platelet release through a yet unidentified mechanism Fig. (**2**).

It has been reported that the inhibition of NOS by L-N(G)-monomethyl arginine (L-NMMA) or L-N(G)-nitroarginine methyl ester hydrochloride (L-NAME) in rats resulted in a significant reduction in platelet count [57, 58]. In addition, transgenic mice lacking iNOS were reported to have one-half the platelet count of either the wild-type or eNOS null mice [56]. Nevertheless, the decreased platelet count in these mice could be a consequence of either impaired megakaryocyte maturation in the bone marrow, a defect in the platelet formation/release process itself or a reduction in circulating platelets due to the adhesion/aggregation of cells associated with the NO deficiency in these animals.

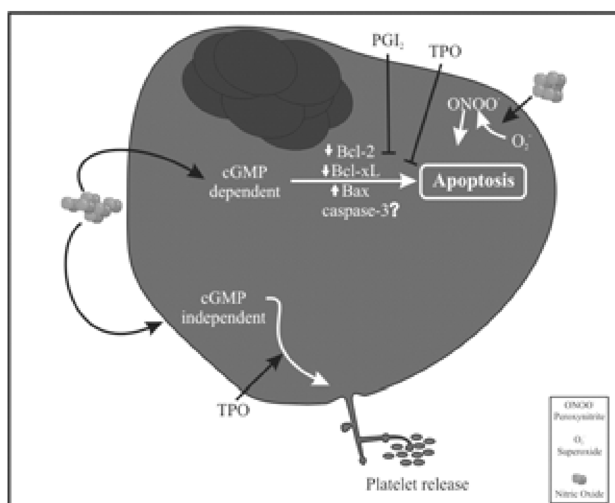

Fig. (2). Role of NO in megakaryocyte survival and thrombopoiesis. NO induces megakaryocyte apoptosis through a cGMP-dependent mechanism that downregulates Bcl-2 and Bcl-xl anti-apoptotic members of the Bcl-2 family, and enhances pro-apoptotic molecule Bax. Although NO-mediated apoptosis of megakaryocytic cell lines is caspase-3 independent, apoptosis of megakaryocyte primary cells appears to involve caspase-3 activation [42, 51]. Alternatively, NO induces apoptosis by reacting with oxygen radicals producing peroxynitrite. TPO and PGI_2 protect megakaryocytes from NO-induced apoptosis. NO also triggers platelet release through a cGMP-independent pathway, a process that is enhanced by TPO [52, 56].

NITRIDERGIC PATHWAY IN PLATELETS

Platelet NO Synthesis

The endogenous synthesis of NO in platelets was first described in 1990 when Radomski *et al.* demonstrated that a L-arginine/NO pathway negatively regulates collagen-induced platelet aggregation [59]. Since then, the NO release by platelets has been reported by several groups using different methods, including assays of the conversion of oxy-hemoglobin to met-hemoglobin or generation of nitrite and nitrate [60], electrochemical analyses [61], and assays of the NOS-dependent conversion of L-arginine to L-citruline [23, 62].

While most of the studies have predominantly identified eNOS as the NOS isoform present within platelets [21, 23], iNOS expression is still controversial. Although iNOS mRNA expression has been shown in platelets [63], the majority of studies have failed to detect iNOS protein [50, 64]. Nevertheless, it is possible that under certain inflammatory conditions, platelet express iNOS either by new synthesis or in a form inherited from megakaryocytes [21]. In this regard, as was already mentioned, megakaryocytes but not platelets from patients with atherosclerosis were shown to express the inducible NOS isoform [50]. However, *in vitro* studies showed that iNOS protein has been immunoprecipitated from platelets stimulated with LPS [63]. Therefore, more studies are required to further elucidate platelet iNOS expression during inflammation.

Regarding nNOS, there is consensus that this isoform is not expressed in platelets [21, 23, 63].

Platelet eNOS activation is regulated by Ca^{2+}-dependent and independent mechanisms. Among the former type, von Willebrand Factor (vWF), thrombin or collagen trigger eNOS activation; however, the pathways leading to eNOS activation are specific for each agonist. Although vWF binds to glycoprotein (GP) Ib-V-IX and GPIIbIIIa, only the interaction with GPIb-V-IX triggers platelet eNOS activation [65]. Similarly GPVI and GPIaIIa are the platelet receptors for collagen, but only GPVI is involved in collagen-induced NO synthesis [66]. Mechanistic approaches have identified that while Src family kinases are required for eNOS activation promoted by either collagen, vWF or thrombin, only vWF and collagen further involve Phosphoinositide 3-kinase (PI3K) [65, 66].

Interestingly, Ser^{1177}, the only serine phosphorylation site that has been demonstrated in platelet eNOS, is critical for Ca^{2+}-independent eNOS activation [67]. While β_2-adrenoreceptors stimulation [62] and adenosine [68] activate platelet eNOS by increasing cAMP levels and protein kinase A (PKA) activation [69], insulin promotes PI3K and AMP-activated protein kinase (AMPK) activation [70] and both pathways converge on eNOS serine phosphorylation. So far, the protein kinase(s) that directly phosphorylate human platelet eNOS Ser^{1177} has not been identified. Phosphorylation of platelet NOS appears to exert a dual effect on enzyme activity depending on the phosphorylated residue. In fact, while phosphorylated Ser positively regulates eNOS, tyrosine phosphorylation is associated with reduced NOS activity and NO bioavailability [71]. Moreover, dephosphorylation of platelet eNOS by association with SHP-1 tyrosine phosphatase increases enzyme activity, suggesting that phosphatases also modulate eNOS activation [72]. The tyrosine residues that are phosphorylated in platelet eNOS as well as the other phosphatases that regulate its activity are not yet characterized.

Physiological Function of NO Produced by Platelets

The role of endogenous NO generation in platelet function is still not completely understood and the studies that tried to clarify this point gave controversial and conflicting results. Initially, the L-arginine/NO pathway in platelets was inferred by the use of drugs that regulate NO synthesis, including L-arginine and L-NAME [59]. However, other studies using these drugs were not able to find any effects in platelet aggregation [73, 74] and questioned the role of endogenous NO as a negative regulator. The first studies on transgenic mice, showed that thrombocytopenic eNOS deficient mice transfused with $eNOS^{-/-}$ platelets have shorter bleeding times compared with when they are transfused with platelets from wild-type mice suggesting that platelet-derived NO *in vivo* might participate in the haemostatic response by decreasing platelet recruitment [75].

More recent data suggested that NO synthesis rather than inhibition of platelet responses by itself, would enhance the effect of platelet inhibitory molecules, such as insulin or adenosine [68, 70]. In the same line of evidence, studies with $eNOS^{-/-}$ mice showed that although platelet aggregation did not differ from that in wild-type mice, the expression of eNOS was required for the platelet disaggregation caused by blocking of PI3K [76]. Therefore, these studies introduced

the concept that platelet NO might contribute to the effect of anti-aggregating agents instead of playing a direct role on agonist-stimulated aggregation. Interestingly and contrary to what it was expected, Li *et al*. conducted a series of elegant experiments and found that the aggregation response mediated by subthreshold concentrations of thrombin or vWF were potentiated by either PKG agonists [77] or low amounts of NO donors [78]. Moreover, $PKG^{-/-}$ or $eNOS^{-/-}$ mice have an impaired platelet aggregation and ATP secretion when exposed to low concentrations of several agonists in addition to a prolonged bleeding time ($PKG^{-/-}$mice) or tendency to rebleeding ($eNOS^{-/-}$ mice) [77, 78] and reduced abilities to form arterial thrombus [78]. Contradicting the classical paradigms that the activation of eNOS and the downstream signaling pathway of NO/cGMP/PKG is a negative regulatory axis of platelet aggregation, they proposed that NO/cGMP could exert a dual effect. While low and early NO-mediated cGMP levels have an effect of stimulating platelets (through ERK phosphorylation and PI3K activation), higher and delayed cGMP levels trigger other signals (e.g., VASP phosphorylation) that turn off platelet activation and limit the size of platelet aggregates [77, 78]. Adding more confusion to the role of eNOS derived NO in platelet physiology and thrombus formation, two studies simultaneously suggested no role of eNOS in platelet functionality. *In vitro* experiments show that the aggregation and P-selectin expression is unchanged in $eNOS^{-/-}$ platelets compared with platelets from wild-type animals [79] and that no eNOS protein was detected in platelets from wild-type mice. In addition, no significant differences were observed in the bleeding time or in arterial thrombus formation in mice lacking eNOS compared with their wild-type littermates [79, 80]. Furthermore, Gambaryan *et al*. have recently reported some provocative data that suggested that highly purified murine or human platelets do not express either eNOS or iNOS. Using appropriate controls, they demonstrated that pharmacological inhibition or the deficiency of platelet eNOS had no effect in platelet aggregation, and that erroneous eNOS identification in platelets was caused by both leukocyte contaminating cells and the use of commercial antibodies that recognize other proteins than eNOS [81]. Moreover, they found that cGMP levels can be augmented in platelets from $eNOS^{-/-}$ mice, revealing that the levels of cGMP could be regulated by NO-independent mechanisms [81]. The current notions regarding the role of endogenous NO on platelet function are represented in Fig. (**3**).

Platelet Derived NO Production in Diseases

Accumulated evidence suggests that there is a strong correlation between NO synthesis impairment in platelets and vascular diseases. For instance, the platelets from patients with acute coronary syndromes such as unstable angina or myocardial infarction generate less NO and a lower level of platelet NO production was found to be an independent predictor of an acute coronary syndrome [82]. Moreover, coronary risk factors such as aging, smoking, hypertension, obesity and hypertriglyceridemia are associated with an impairment of platelet-derived NO release [83, 84].

Platelet eNOS activity is decreased in patients with type I and type II diabetes, a disease characterized by enhanced platelet activation [85]. Furthermore, high levels of plasma glucose and glycosylated hemoglobin A1c show a negative correlation with platelet NOS activity [86, 87]. Interestingly, platelets from type II diabetes patients have an enhanced intracellular Ca^{2+} release in response to ADP which may be explained by an increase in superoxide anion production that might be scavenging the platelet-derived NO and therefore reducing its bioavailability [88]. The decreased levels of platelet NO reported in hypertensive patients are also associated with higher concentrations of platelet cytosolic Ca^{2+} [89], but rather than being caused by reduced NO bioavailability, it may be caused by the reduced intraplatelet NO from the diminished transport of L-arginine from plasma to platelets in hypertensive patients [90]. The inhibition of L-arginine transport as well as a 50% reduction in the expression of platelet NOS has also been demonstrated in platelets treated with oxidized LDL. In addition, this reduction in protein expression has been observed in healthy hypercholesterolemic donors [91].

Plasma L-arginine levels are reduced in chronic renal failure (CRF) patients, especially in those with malnutrition; such patients also have an enhanced occurrence of throm-

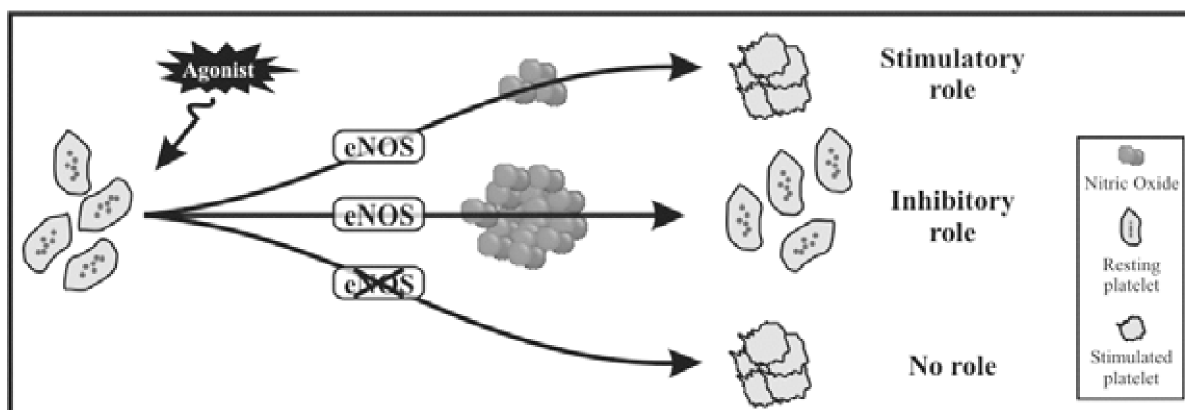

Fig. (3). Effect of eNOS-derived NO on platelet functional responses. Two mutually exclusive points of view: i) platelets contain functionally active eNOS and ii) eNOS is not expressed in platelets. Consistent with the first statement, while low levels of NO stimulate platelets through PKG activation [77, 78], high NO levels inhibit platelet function [75, 78]. Opposite to these statements, two independent groups claim that eNOS is not expressed either in mouse [79, 81] or human platelets [81] and therefore can not play a role in platelet function.

botic events. An analysis of the relationship between the nutritional status and the L-arginine/NO pathway in platelets shows that while mal-nourished patients have decreased platelet eNOS activity and normal platelet aggregation, well-nourished patients have impaired aggregation due to an increase in L-arginine transport and NOS activity [92]. The absence of this adaptive response in the L-arginine/NO pathway in platelets from mal-nourished patients may account for the higher incidence of thrombotic episodes. Nevertheless, this hypothesis requires further investigation, since the study was performed in a small number of patients.

While a reduction of NO production from platelets is associated with development of cardiovascular events, an increase in platelet NO release has been related to Alzheimer disease [84], cirrhosis [93] and pregnant women with small for gestational age (SGA) fetuses [94]. The brain of Alzheimer disease patients is characterized by the presence of amyloid plaques that are made of a core of insoluble beta-amyloid (Aβ), which derive from a larger amyloid precursor protein (APP) [95]. It has been reported that platelets contain both APP and Aβ stored in α-granules which are released during activation [96]. Platelets from patients with Alzheimer disease have elevated levels of NO and peroxynitrite and it is proposed that this alteration may contribute to the increased production of superoxide anion and NO observed in the brain during the acute phase of the disease [84]. Cirrhosis is characterized by variceal bleeding due to abnormal platelet function [97] and platelets from patients with cirrhosis show not only a defective intracellular Ca^{2+} release but also show increased NO levels and NOS activity [93]. Platelets from pregnant women with SGA fetuses produce high levels of NO and peroxynitrite that may play a role in the pathophysiology of intrauterine growth restriction [94].

Platelet NO-cGMP pathway modulating drugs

After the elucidation of the NO pathway in platelets and the discovery of its alteration in many diseases, a great variety of traditional and new drugs used for the treatment of thrombotic diseases are being monitored for their ability to regulate platelet nitridergic pathway. Among the classical anti-thrombotic drugs, acetyl salicylic acid, statins and dipyridamole, all have been shown to positively regulate either platelet NO synthesis or bioavailability. In addition, NO donors with less hypotensive effects and hybrid molecules formed by the combination of non-steroideal anti-inflammatory drugs (NSAID) and NO-releasing molecules are likely the most promising new drugs for the treatment of cardiovascular diseases (Table **1**).

Acetyl Salicylic Acid

Anti-platelet therapy with acetyl salicylic acid (aspirin) has long been the cornerstone for the treatment for cardiovascular disease, particularly ischemic stroke [98]. Aspirin inhibits platelet aggregation through the inhibition of the cyclooxygenase (COX) enzyme [99]. Since important cross talk between NO synthase and COX has been described in different cell types [100, 101], several studies addressed whether COX inhibition by aspirin in platelets regulates NOS activity. However, the results obtained are inconclusive. Aspirin has been found to stimulate NO synthesis in human blood platelets and to increase cellular cGMP levels indicating that NO does contribute to the aspirin mediated inhibition of platelet aggregation [102]. In addition, it was shown that the acute high-dose exposure of platelets to aspirin raises basal NOS activity through a mechanism independent of COX inhibition [103]. By contrast, short- and long-term (non acute), aspirin treatment inhibits β-adrenoreceptor-mediated NOS activation in a COX dependent manner [103-105], suggesting that aspirin exerts two types of effects on platelet NOS depending on the length and dose of treatment. This would imply that despite the well-established inhibitory effects on platelet aggregation, the effect of aspirin on NO biosynthesis could have important long-term implications on other aspects of platelet activation.

NO-Releasing-NSAID

One of the limitations of NSAIDs is the occurrence of gastrointestinal tract damage, ranging from stomach erosions to life-threatening complications, resulting from the inhibition of vasodilator and cytoprotective prostanoids in the gastric mucosa [106]. Even very low doses of aspirin can markedly increase the risk of gastrointestinal bleeding and ulceration and therefore, long-term use of aspirin as an anti-thrombotic agent is limited by these side effects [107]. Thus, it is clear that, despite the effectiveness of prophylactic aspirin treatment in reducing major cardiovascular events, alternative anti-thrombotic drugs with lower risk of gastrointestinal side effects would be desirable. Considering the protective activity of NO on gastric mucosa [106, 108], several NO-NSAIDs have been developed [109] in the past few years. They are synthesized by addition of a radical, nitrobutyl or nitrosothiol, to the molecule of a conventional NSAID by using a short-chain ester linkage [110]. Among them, hybrid molecules of NO and aspirin represent a novel means of inhibiting platelet aggregation by a combination of COX inhibition and NO generation. The incorporation of a NO-releasing moiety in aspirin can overcome the gastric side effects, but it also offers a degree of NO-mediated anti-platelet effects that are complementary to those of the aspirin moiety and therefore, there is a greater degree of platelet inhibition. One of the most promising agents is nitroaspirin (2-acetoxy-benzoate 2-(2-nitroxymethyl)-phenyl ester, NCX4016), which consists of the parent molecule (aspirin) linked to an 'spacer' *via* an ester linkage connected to a NO releasing moiety [111, 112]. Besides having less adverse gastric effects [112], nitroaspirin, appears to be a better anti-aggregating drug than aspirin in terms of platelet inhibition [113, 114] and anti-thrombotic activity [115-117]. The action of nitroaspirin is most probably associated with a combination of the anti-platelet effects of aspirin and the broad-range cardiovascular activities of NO [114]. There is also evidence that unlike aspirin, it also down-regulates Tissue Factor and inhibits IL-6 and monocyte chemoattractant protein-1 expression after chronic treatment. Thus this compound has additional anti-atherogenic properties in humans [118]. Although NO-releasing aspirin seems to be a promising therapeutic strategy against thrombotic diseases, the drug is still in phase II clinical trials and the outcome of ongoing trials needs to be evaluated before any conclusions can be made about the beneficial role of NO-releasing aspirins compared

with its parent compound in the primary and secondary prevention of vascular events.

Statins

Hypercholesterolemia is one of the major risk factors for the development and progression of atherothrombosis. Statin, a type of cholesterol lowering agents (also known as 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase inhibitors), can markedly decrease cardiovascular events in hypercholesterolemic subjects and in patients with coronary heart disease [119]. However, it has been claimed that prevention of atherosclerotic disease by statins is also due to non-lipid or pleiotropic effects, including an improved endothelial function by promoting the synthesis and/or bioavailability of NO [120, 121]. Regarding the effects of statins on platelet function, it was shown that Fluvastatin, Cerivastatin, Simvastatin and Pravastatin inhibit platelet aggregation through upregulation of the L-arginine/NO pathway with the consequent increase in NO and cGMP levels. In most cases, these effects were linked to the antioxidant properties of these drugs [122-125]. Therefore, by increasing endothelial and platelet NO production, statins may improve blood flow, decrease platelet function and protect against ischemia. More recently, a new class of drugs combining a statin with a NO-donating moiety, nitrostatins (nitropravastatin, nitrofluvastatin and nitroatorvastatin), have been described and found to release functional NO and to exert enhanced *in vitro* anti-proliferative and anti-inflammatory properties when compared with the native statins [126, 127]. *In vivo*, nitrostatins exerted greater lipid-lowering, anti-thrombotic and anti-inflammatory effects than statins, due to a large extent on the NO release [128]. These compounds represent an interesting class of drugs having the potential as therapeutic agents for thrombotic disorders.

NO Donors

Exogenous NO released by nitro-vasodilators or ‘NO donor drugs’, such as nitrates, have been used to restore NO function. However, there is only limited evidence from large clinical trials that NO delivery can reduce cardiovascular morbidity and mortality. In addition, the anti-aggregating properties of these drugs are usually detected at supratherapeutic dosage, which can induce hypotensive side effects. Thus, NO donors are recommended only in particular clinical situations, such as when a well-established treatment is contra-indicated or has an insufficient effect. Nevertheless, there is growing insight into the anti-platelet properties of new NO donors that casts a new light to nitrate therapy [129]. An example of this type of drug is the NO donor LA419, a neutral sugar organic nitrate with a protected thiol group that has been tested *in vivo* in pigs [130, 131] and in humans *ex vivo*. Used along or in combination with clopidrogel, LA419 exhibits strong anti-platelet action. It has been shown to significantly inhibit the interaction of platelets with highly thrombogenic collagen surfaces or under high shear rate conditions [129, 132]. Other examples of this type of drugs are LA810, a platelet selective S-nitrosothiol compound, which proved to inhibit platelet function without hypotensive side-effects [133] and the NO donor MAHMA NONOate that has both platelet inhibitory and vasodepressor effects [134, 135].

PDE Inhibitors

Cyclic nucleotide PDEs comprise a superfamily of metallophosphohydrolases that specifically cleave the 3', 5’-cyclic phosphate moiety of cAMP and/or cGMP to produce the corresponding 5'-nucleotide. PDEs are critical determinants for negative feedback control of increased cellular cAMP and/or cGMP levels elicited by different stimuli [136]. Human platelets contain PDEs 2, 3 and high concentrations of PDE5 and thus PDE inhibitors are able to potentiate the inhibitory effects of cGMP/cAMP elevating drugs [137].

Dipyridamole was initially thought to be a PDE3 inhibitor that exerted anti-platelet activity by preventing the conversion of cAMP to AMP and thereby increasing the intracellular levels of cAMP [138]. However, it is now known that this drug modulates other signaling pathways that account for its anti-aggregating effect. It is likely that the inhibition of the cGMP PDE5 enzyme, and the enhancement of the anti-platelet effect of the NO/cGMP pathway is a major effect of this drug [98]. Other effects include increasing plasma adenosine concentrations, promoting the release of prostanoids from the endothelium and suppressing of oxygen free radical formation in platelets and endothelial cells, thereby improving the redox status of the cells extending the half-life and increasing the bioavailability of NO [139, 140].

Sildenafil, Vardenafil and Tadalafil are the PDE5 inhibitors most currently used for the treatment of erectile dysfunction. These drugs act by enhancing the NO-induced cGMP-mediated smooth muscle relaxation in the vasculature of the corpus cavernosum, leading to increased blood influx and penile tumescence [141]. Since type 5 is the major PDE in platelets, it is not surprising that PDE5 inhibitors suppress agonist-induced platelet aggregation to some extent. In fact, it has been demonstrated that although Sildenafil and Zaprinast failed to increase platelet cGMP levels when used alone, they both amplify the increase in platelet intracellular cGMP induced by the NO-donor sodium nitroprusside and impair the aggregation and serotonin release from thrombin-stimulated human platelets [137, 142]. The ability of PDE5 inhibitors to decrease platelet function without increasing global intra-platelet cGMP levels can be explained by the studies of Wilson *et al.* which demonstrated that PKG selectively activates PDE5 within a defined microdomain in platelets and propose that this mechanism allows spatial and temporal regulation of cGMP signaling in platelets [143].

Hormones

Cardiovascular disease is less common in premenopausal women than in men; this benefit progressively disappears after menopause, and the rates equalize after the sixth decade of life. Experimental studies in animals as well as retrospective analysis of clinical studies demonstrated that estrogens could be responsible for this cardiovascular protection. These observations stimulated the execution of several prospective and randomized clinical trials. However, the impact of estrogens on the cardiovascular system and their ability to regulate platelet function are still matters of controversy [144, 145]. Despite an overwhelming amount of literature, in a very short time, our understanding of the effects of hormonal replacement has rapidly and dramatically changed from a potential positive effect on cardiovascular disease to a harm-

ful effect and then to a possible protective effect in some women [146, 147]. Do estrogens have any effect on platelet eNOS? For many years, the rationale for the investigation of the effects of estrogens on human platelets has been questioned because of the supposed absence of specific receptors in these enucleated cells. Interestingly, early studies by Nakano *et al.* demonstrated that 17β-estradiol plays an important role in inhibiting platelet aggregation by promoting Ca^{2+} extrusion or reuptake activity that was dependent on the production of cGMP by increased NO synthesis. Given that platelets are enucleated cells and the fact that between 17- or 17β-estradiol, only the latter had an inhibitory effect, they assumed a specific role of this hormone probably acting on an unidentified platelet membrane receptor. However, it was recently demonstrated that not only megakaryocytes but also platelets contain both α and β estrogen receptors [148]. Studies aimed at investigating the nongenomic effects of estrogens on platelet function have shown controversial results. It has been shown that estrogens can directly sensitize platelets to physiologic agonists by regulation of the GPIIbIIIa through estrogen receptor β and Src kinase [149]. However, platelet activation triggered by the advanced glycation end-products-modified albumin are largely prevented by 17β-estradiol through an increased Ser^{1177} phosphorylation of eNOS; this was consistent with an augmented synthesis of NO and an enhancement of cGMP levels [150, 151]. A comparative study in pigs, between the effect of three clinically used drugs, such as 17β-estradiol, conjugated equine estrogen, or raloxifene showed that although the three different estrogenic treatments prevented the increase on platelet aggregation caused by ovariectomy, only 17β-estradiol increased eNOS platelet mRNA and the release of platelet-derived NO, suggesting the presence of transcriptional and posttranscriptional regulation of protein synthesis in bone marrow megakaryocytes and circulating platelets [152].

Raloxifene is a benzothiopene derivative and a selective estrogen receptor modulator (SERM) that is currently used for the treatment of postmenopausal osteoporosis. This drug should be more appropriate for postmenopausal women than 17β-estradiol because of its estrogen-antagonistic effect on bone and lipid metabolism. After 12 months of treatment, raloxifene users showed a significant increase in platelet NOS activity and NO generation together with a decrease in peroxynitrite levels [94]. So far, the effects of raloxifene on cardiovascular risk are under evaluation in a large trial in postmenopausal women with cardiovascular disease and breast cancer as primary endpoints [153].

Dehydroepiandrosterone (DHEA) is a natural steroid prohormone produced from cholesterol by the adrenal glands, the gonads, adipose tissue, brain and in the skin. DHEA is the precursor of androstenedione, which can undergo further conversion to produce the androgen testoster-

Table 1. Drugs Modulating Platelet NO-Pathway

Type of Drug	Evaluated in Humans	Mechanism of Action	Examples
NO-donors	*In vitro*	Activation of Guanylyl ciclase and cGMP	LA419 LA810 MAHMA NONOate
Acetyl Salicylic acid	*In vivo*	COX inhibitor Activation/Inhibition of NOS depending on the dose and length of treatment	Aspirin
NO-NSAID	*In vivo*	Intracellular delivery of NO and inhibition of COX enzyme	NCX4016
Statins	*In vivo*	HMGCoA inhibitors Increased NO activity Antioxidant	Fluvastatin Cerivastatin Simvastatin Pravastatin
Nitrostatins	*In vitro*	Combined action of NO donors and statins	Nitropravastatin Nitrofluvastatin Nitroatorvastatin
Phospho diesterase inhibitors	*In vitro* *In vivo* (Dipyridamole and Sildenafil)	PDE-inhibition Elevation of cGMP levels	Dipyridamole Sildenafil Zaprinast Tadalafil Vardenafil
Estrogens	*In vivo*	Enhancement of eNOS activity	17β-estradiol
SERMs	*In vivo*	Enhancement of eNOS activity	Raloxifene
DHEA	*In vivo*	Increase in NO production	DHEA DHEA-S (sulphate)
Resveratrol	*In vitro*	Inhibition of MAPK phosphorylation Increase in NO/cGMP formation	Resveratrol

one and the estrogens estrone and estradiol. Several clinical and population-based studies suggested that DHEA and its sulphate (DHEA-S) may protect against atherosclerosis and coronary artery disease in humans. In a study of male subjects, it was shown that short-term treatment with DHEA increased platelet cGMP, suggesting that chronic DHEA supplementation would exert anti-atherogenic effects particularly in elderly subjects who display low circulating levels of this hormone [154] .

Polyphenols

Many studies have reported promising health benefits from red wine consumption. Evidence from different experimental studies has suggested that these beneficial effects are due to polyphenols found in red wine, especially resveratrol in grape skins. Beyond its antioxidant [155], superoxide-scavenging, ischemic-preconditioning and anti-angiogenic properties [156], resveratrol inhibits platelet aggregation [157-159]. Studies of the mechanistic action of this drug revealed that resveratrol downregulates platelet function by inhibition of phospholipase (PL) PLA_2, PLC, PKC activation and Thromboxane B_2 generation linked to an increase in NO/cGMP formation and VASP Ser^{157} phosphorylation [160]. Some of these properties of polyphenols may explain their protective effects on the cardiovascular system, as well as in other organs of the body.

CONCLUDING REMARKS

Since the L-arginine/NO pathway was described in platelets, the understanding of its role in the pathophysiology of haemostasis has changed from the pathway serving as a relevant negative modulator of thrombus growth, to the pathway having a stimulatory effect, or even more recently, to the challenging notion that eNOS detection in platelets may be an experimental artifact. Certainly, before platelet eNOS is no longer accepted by the scientific community, the absence/presence of platelet NOS as well as the detection of NO release by highly purified platelet preparations should be confirmed. Although platelet NOS expression is still a matter of controversy, the notion that endothelial derived bioavailability of NO represents a central feature in maintaining homeostasis, regulating platelet activity, and preventing the onset of thrombosis and vascular events is widely accepted. In this respect, there is a clear need to develop and test new drugs with the capacity of modulating the NO/cGMP pathway. Clearly, much still needs to be learnt about the compounds mentioned in this review to bring these new therapeutic options to the clinical forefront. Further investigation must be focused on their long-term effects, efficacy, dosage and tolerance in clinical trials. Also because we should continue to deepen our understanding of platelet activation pathophysiology, even more importantly, the study of these compounds is likely to provide useful tools that help to establish further biological roles of NO within the body. NO research in the last ten years has revealed another attractive biological role of NO as a potential mediator of platelet biogenesis and megakaryocyte survival. A deeper insight into the molecular basis regulating these processes is required to clearly understand the pathophysiology implications of NO modulation in thrombopoiesis.

Finally, there is increasing evidence suggesting that NO is an important regulator of HSC biology. However, several questions still remain unanswered. For example, what is the net contribution of the NOS isoforms to the quiescence maintenance, recruitment and mobilization of HSC? How would the bone marrow differentiate between the signals elicited by NO generated from nNOS and eNOS? Are NOS isoforms differentially expressed and/or regulated in the osteoblastic and vascular niche? Is NO-mediated HSC regulation restricted to some subpopulations or is it a more generalized effect? Answers to these questions are essential for a greater understanding of the role of this versatile molecule in hematopoiesis.

ABBREVIATIONS

cAMP = Cyclic adenosine monophosphate
cGMP = Cyclic guanosine monophosphate
COX = Cyclooxigenase
DHEA = Dehydroepiandrosterone
EPC = Endothelial progenitor cell
G-CSF = Granulocyte-colony stimulating factor
GP = Glycoprotein
HPC = Hematopoietic progenitor cells
HSC = Hematopoietic stem cells
IFN-γ = Interferon gamma
IL = Interleukin
L-NMMA = L-N(G)-monomethyl arginine
L-NAME = L-N(G)-nitroarginine methyl ester hydrochloride
LPS = Lipopolysaccharide
MMP = Metalloprotease
NO = Nitric oxide
NOS = Nitric oxide synthase
NSAIDs = Non-steroidal anti-inflammatory drugs
PDE = Phosphodiesterase
PGI_2 = Prostacyclin
PI3K = Phosphoinositide 3-kinase
PK = Protein kinase
PL = Phospholipase
TNF-α = Tumor necrosis factor alpha
TPO = Thrombopoietin
vWF = von Willebrand factor

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