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Nitric oxide signalling in the regulation of cardiovascular and platelet function

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1. ABSTRACT

Nitric oxide (NO) exerts important protective actions on the cardiovascular system. Generated from L-arginine by the action of endothelial (or type 3) nitric oxide synthase (NOS3), NO regulates vascular tone in humans and causes endothelium-dependent vasodilation. Additionally endothelium-derived NO exerts antioxidant, antiproliferative and anti-inflammatory properties, thus playing an important role in inhibiting the atherosclerotic process. With regard to effects on platelet function, NO produced by both endothelial cells and platelets has important antithrombotic effects by decreasing platelet activation, a phenomenon which contributes importantly to the thrombotic tendency which accompanies a variety of cardiovascular disease states. Additionally, by inhibiting platelet activation, NO prevents heterotypic aggregation between platelets and monocytes, thereby reducing monocyte-platelet aggregates in the circulation which are believed to play an important pathophysiological role in the initiation and progression of atherosclerosis. New therapeutic interventions aimed at improving NO availability have been investigated in animal as well as *in vitro* studies and show considerable promise, but it remains to be seen whether such therapies will be equally efficacious in humans clinically.

2. INTRODUCTION-VASCULAR BIOLOGY OF NITRIC OXIDE

Nitric oxide (NO) is a colourless gas that in the presence of oxygen is very unstable and converts within seconds to nitrogen dioxide. It has one unpaired electron, and is therefore a free radical (1,2). Uncharged NO diffuses across cell membranes to its intracellular targets. It has a very short half-life of about five seconds in biological fluids, limiting the distance from its synthesis site over which it is active.

NO in whole blood is oxidized to nitrite (NO_2^-), which is then rapidly oxidized further to nitrate (NO_3^-) (3). NO can react within seconds with superoxide anion to form peroxynitrite, a reaction which is more important at low NO concentrations (4). It also reacts with haem iron in seconds to form the charge transfer complex required to activate soluble guanylyl cyclase (sGC) (5), and with thiols to form S-nitrosothiols (6). The reaction with thiols serves to stabilise, store and transport NO in non-toxic form. Binding of NO with the haem of haemoglobin leads to its inactivation (7). On the other hand, S-nitrosylation of specific cysteine residues in haemoglobin gives rise to stable pools of NO in red blood cells (8); thus, binding of NO by haemoglobin can serve either to stabilize and

transport it or to inactivate it, depending on the precise site of binding. Furthermore, S-nitrosylation of the cysteine residue Cys-34 of serum albumin leads to the formation of S-nitroso-albumin, which functions as one of the most important reservoirs of circulating NO (9).

NO is formed from the amino acid L-arginine (10) by a family of enzymes, nitric oxide synthase (NOS), and plays an important role in many physiological functions. Among these, one of the most important is endothelium-dependent vasorelaxation, as first described by Furchgott and Zawadski in 1980 (11). They demonstrated that the relaxation of isolated rabbit aorta exposed to acetylcholine (ACh) requires the presence of an intact endothelium. The substance that was produced in response to ACh, causing relaxation, was named endothelium derived relaxing factor (EDRF). A few years later it was demonstrated that the biological actions of EDRF are due to the endogenous release of NO (12, 13). Ignarro and colleagues showed in intrapulmonary artery and vein that the vascular effects of released EDRF were the same as those of superfused NO. They were both labile, inactivated by pyrogallol and superoxide anion (O^{-}_2), stabilised by superoxide dismutase (SOD), and inhibited by oxyhaemoglobin. Furthermore they both produced similar concentrations of cyclic guanosine-3',5'-monophopsphate (cGMP) (12). A year before, it had already been demonstrated that EDRF activates sGC (5). Palmer and colleagues, around the same time, showed that porcine aortic endothelial cells release NO, which has the same biological activity as EDRF, its action being inhibited by haemoglobin and enhanced by SOD (13). Furthermore, the effects of EDRF and NO were found to be the same as regards inhibition of platelet aggregation and adhesion (14). These discoveries led to the elucidation of the endothelial L-arginine/ NO pathway (15).

2.1. The NOS family

NOS enzymes catalyse the biosynthesis of NO via a reaction involving the conversion of L-arginine to L-citrulline. There are three distinct isoforms of NOS which differ in function and structure.

NOS1 (NOS I, neuronal NOS, or nNOS), is a Ca^{2+}-calmodulin (CaM) dependent enzyme, and requires intracellular Ca^{2+} levels to increase in order to become activated. It is constitutively expressed in central nervous system, spinal cord, sympathetic ganglia and adrenal glands (16). NOS1 is also highly expressed in peripheral "nitroxidergic" neurones (nonadrenergic and noncholinergic nerves), thus non-adrenergic non-cholinergic vascular relaxation occurs due to neuronal release of NO (17, 18). Furthermore, NOS1 is also expressed in non-neuronal tissues such as cardiac myocytes (19), skeletal myocytes (20), and vascular smooth muscle cells (21). It may contribute significantly to vascular function through smooth muscle cell production of NO (21, 22). More specifically, it has been shown recently that in humans, NOS1 plays an important role in the physiological regulation of human microvascular tone, as a specific inhibitor of the enzyme causes a dose-dependent reduction in forearm blood flow and in coronary flow, an effect which is independent of NOS3- induced vasodilatation. The same investigators showed, on the other hand, that the effect of shear stress on endothelial production of NO is via NOS3 and not NOS1 activation (23, 24).

NOS2 (NOSII, inducible NOS, or iNOS) is expressed at high levels after induction by inflammatory cytokines or lipopolysacharide (LPS), and its activity does not require any increase in intracellular Ca^{2+}, due to its high affinity irreversible binding with CaM (25). Due to its low expression under basal conditions in the vasculature, it is unlikely to have an important role in cardiovascular regulation. NOS2 can be induced in macrophages (26), neutrophils (27), smooth muscle cells (28, 29), endothelial cells (30), cardiac myocytes (31), hepatocytes, chondrocytes, and human adenocarcinoma cell line (32). It has also been suggested to be expressed in small amounts in platelets (33), although more recent studies have documented only expression of NOS3 in platelets (34).

NOS3 (NOSIII, endothelial NOS, or eNOS) is expressed constitutively in endothelial cells, and is the main source of endothelial NO under physiological conditions (35, 36). Other sources relevant to the cardiovascular system include cardiac myocytes (37), platelets and red blood cells (33, 38). Although in the past red blood cells have been proposed to carry a non-functional NOS, it has recently been shown that they express a functional and active NOS similar to (or the same as) the endothelial type, which regulates deformability of the red cell membrane and inhibits platelet activation (39).

2.1.1. Structure and function of NOS3

The enzyme functions as a dimer consisting of two identical monomers, which can each be functionally divided into two major domains: a C-terminal reductase and an N-terminal oxygenase domain. The former contains binding sites for one molecule of reduced nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), and the latter binds haem, L-arginine and tetrahydrobiopterin (BH_4). Between these two regions lies the CaM binding domain, which plays a key role in regulating both the structure and function of the enzyme (40). In the reductase domain, close to the FMN binding site, an auto-inhibitory loop exists which impedes CaM binding to NOS3 at low Ca^{2+} concentrations and inhibits electron transfer (41). The FAD and FMN accept two electrons each from NADPH, and allow one electron to flow to the haem domain at a time. Haem binds the NADPH-derived electrons so that the haem iron can bind O_2 and catalyse NO biosynthesis, and is also important for the dimerisation of the enzyme which renders it functional (42). BH_4 promotes NOS3 dimer formation and increases its stability (42). A proposed model of NOS3 dimer function is shown in Figure 1.

NO biosynthesis by NOS3 involves a two step oxidation of L-arginine to L-citrulline, with concomitant production of NO. L-arginine is the physiological precursor for NO biosynthesis (10), in a reaction that consumes 1.5 mol of NADPH and 2 mol of oxygen per mol of citrulline formed. Two mol of water are presumptive co-products. In

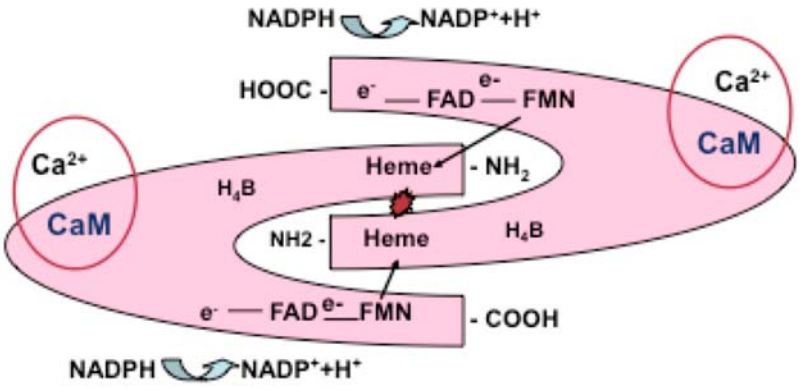

Figure 1. Schematic of NOS3 dimer function. CaM: calmodulin; H_4B: tetrahydrobiopterin; NADPH: Reduced nicotinamide adenine dinucleotide phosphate; FMN: Flavin mononucleotide; FAD: Flavin adenine dinucleotide.

step one, two electrons are contributed by NADPH, and a guanidino nitrogen of arginine undergoes a two electron oxidation. In the second step, one electron is contributed by NADPH, and N-hydroxy-L-arginine (NOH-ARG) undergoes a three electron oxidation to form citrulline and NO (43).

Apart from producing NO, NOS3 can generate O^-_2 in a Ca^{2+}-CaM-dependent manner, under conditions of limited supply or absence of BH_4, and this can be inhibited by the administration of BH_4 (44, 45). In cultured endothelial cells, chronic exposure to low-density lipoprotein (LDL) increases the production of O^-_2 by NOS3, an effect that is inhibited by treatment with L-arginine (46). In apoE-deficient mice crossed with NOS3-over-expressing mice, generation of O^-_2 is increased, due to uncoupling of NOS3, and the administration of BH_4 inhibits O^-_2 generation (47). Inhibition of dihydrofolate reductase (DHFR) by downregulation of its expression due to angiotensin II-induced production of O^-_2, results in BH_4 deficiency and uncoupling of NOS3(48).

In the presence of increased vascular oxidative stress, which may accompany diseases such as atherosclerosis, hypertension and diabetes, BH_4 can be oxidised by peroxynitrite ($ONOO^-$) to dihydrobiopterin, leading to depletion of this essential cofactor of NOS3 (49, 50). In the absence of BH_4, NOS3 can become uncoupled and generate O^-_2 instead of NO (44). In accordance with this, studies have shown that the resultant reduced availability of NO can be corrected by restoring BH_4 depletion in several disease states such as atherosclerosis (51), hypertension (50), hypercholesterolemia (52), congestive cardiac failure (53), and diabetes (54). Moreover it has been shown that uncoupling of NOS3 can be observed even in the absence of overt vascular oxidative stress in mice over-expressing NOS3, due to discordance between the amount of the enzyme and that of the cofactor BH_4 (55).

2.1.2. NOS3 activation

The Ca^{2+}-CaM complex is essential for activating NOS3 (56). An increase in intracellular Ca^{2+}, resulting either from an influx of extracellular Ca^{2+}, or from release of Ca^{2+} from intracellular stores, allows CaM binding and activation of NOS3 (57). The Ca^{2+}-sensitive activation of NOS3 is mediated by CaM binding to the enzyme (57). CaM, the first protein shown to regulate NOS3 activity, was identified in the primary structure of NOS3 (58), and proteins that bind CaM inhibit NOS3 activity (57). CaM is essential for the electron transfer within the reductase domain and also from the reductase to the oxygenase domain (59). Stimuli that increase intracellular Ca^{2+} activate NOS3. These include hormones such as catecholamines and vasopressin (60, 61), autacoids such as bradykinin and histamine (62), and platelet-derived mediators such as ADP, thrombin and serotonin (63-65).

NOS3 can also be phosphorylated and thus activated by certain stimuli without a sustained increase in intracellular Ca^{2+} being necessary. For instance, physiological stimuli such as shear stress and vascular endothelial growth factor (VEGF) result in the activation of phosphatidylinositol 3-kinase (PI3K) and thus of protein kinase B (Akt) downstream, which in turn phosphorylates NOS3 on Ser^{1177} (human)/ Ser^{1179} (bovine) thus increasing its activity (66, 67). However, especially for shear stress, it has been shown that although a sustained increase in intracellular Ca^{2+} is not necessary, its chelation abolishes shear stress-induced NO production by endothelial cells. It is now clear that shear stress activation of NOS3 is related to phosphorylation of the enzyme and at the same time to an increase in the sensitivity of the enzyme to basal levels of intracellular Ca^{2+} (this is a result of its phosphorylation), rendering it able to undergo activation. Other stimuli which activate NOS3 through the Akt pathway include oestrogen (69), bradykinin (70), insulin (71), and hydrogen peroxide (72). Recently, activation of NOS3 by Akt phosphorylation on Ser^{617} has also been identified (73), but its precise physiological significance is unclear.

NOS3 can also be activated by phosphorylation on Ser^{1177} (human)/ Ser^{1179} (bovine) by protein kinase A (PKA), cGMP-dependent protein kinase (PKG) II, AMP-activated protein kinase (AMPK), and CaM-dependent kinase II (CaMKII) (74), (75), (76). PKA can also activate NOS3 by phosphorylation on Ser^{633} (human)/ Ser^{635} (bovine) after application of shear stress or stimulation with

VEGF or bradykinin (77), and on Ser^{617} after stimulation with bradykinin or adenosine trisphosphate (ATP) (73). $beta_2$ adrenergic agonists, through both PKA and PI3K/Akt phosphorylation on Ser^{1177}, activate NOS3 in human umbilical vein endothelial cells (HUVEC) (78).

Conversely, both protein kinase C (PKC) and AMPK decrease NOS3 activity by increasing its phosphorylation on Thr ^{495} (human) /Thr ^{497} (bovine) (74). Similarly, angiotensin II and reactive oxygen species activate proline-rich tyrosine kinase 2 (PYK2), which in turn leads to phosphorylation of NOS3 on Tyr657, attenuating NO production and endothelium-dependent vasodilatation(79).

2.1.3. Regulation of NOS3 activity

NOS3 activity is controlled by both chronic and acute mechanisms. In response to chronic exposure to a stimulus such as shear stress, NOS3 expression increases both by transcriptional induction and by stabilising its mRNA. In response to acute stimuli, NOS3 is regulated by associated proteins, posttranslational modifications (including phosphorylation, as discussed above), and the availability of substrates and co-factors.

NOS3 is localised both to the endothelial plasma membrane (36, 56) and to intracellular membranes such as the Golgi apparatus (82). A unique property of the enzyme, which distinguishes it from the other NOS isoforms, is its post-translational modifications. NOS3 can be myristoylated (35) and palmitoylated (83) at the N-terminal site. This property is considered essential for its association with the endothelial cell membrane.

In the plasmalemma NOS3 is localised to the caveolae, which are micro-domains of the membrane, whose main structural components are cholesterol and proteins, including caveolin-1 (84). Caveolin-1 binding to the NOS3 oxygenase domain attenuates enzyme activity in a Ca^{2+}-CaM sensitive manner. This inhibitory effect on NOS3 is released after stimulation with Ca^{2+}-elevating agonists such as ACh, by displacement of caveolin-1 by CaM (85).

Heat shock protein 90 (Hsp90) is the most well established protein that regulates NOS3 activity. It is associated with NOS3 in resting endothelial cells and, upon stimulation of NOS3 with VEGF, histamine, fluid shear stress or oestrogen, this association is enhanced, resulting in activation of NOS3 and in increased NO production (87). More recently it has been shown that, after stimulation of endothelial cells with VEGF, Hsp90 recruits NOS3 and Akt to adjacent regions of the same domain, thereby facilitating NOS3 phosphorylation and activation by Akt (88).

NOS3 interacting protein (NOSIP) and NOS3 traffic inducer (NOSTRIN) inhibit NOS3 activity by modulating the uncoupling of NOS3 from the plasma membrane to intracellular compartments (89), (90).

Dynamin-2, which is a guanosine triphosphate (GTP)-protein, and porin, which is a voltage-dependent anion channel, both co-localise and bind with NOS3, after stimulation by intracellular Ca^{2+}, resulting in an increase in NOS3 activity (91, 92). Similarly, more recently, it has been demonstrated that overexpression of Kruppel-like transcription factor 2 (KLF2), whose expression is induced by laminar shear stress in HUVEC, induces not only the expression but also the enzymatic activity of NOS3 (93).

Beta-actin, apart from being a structural protein that organizes and maintains the shape of non-muscle cells, is also a signalling molecule which in its globular form (G-actin) has been shown to be directly associated with NOS3 in pulmonary artery endothelial cells and platelets (94). This interaction results in an increase of NOS3 activity and enhancement of the affinity of NOS3 for Hsp90 (95).

Platelet endothelial cell adhesion molecule-1 (PECAM-1) and NOS3 are co-localised at the endothelial cell plasma membrane and associate physically. PECAM-1 plays an important role in cell signalling, and it has been shown that shear stress induces its tyrosine phosphorylation and subsequent phosphorylation of both Akt and NOS3. This effect is abolished by using the tyrosine kinase inhibitor PP1, indicating a role of PECAM-1 in NOS3 shear stress-induced activation (96).

Gab1 is a scaffolding protein which, in response to flow, translocates from cytoplasm to endothelial cell junctions and binds to the tyrosine phosphatase SHP2, to the 85 subunit of PI3K, and to PECAM-1. Gab1 and SHP2 play a role in shear stress-induced activation of NOS3 as, in animal models where the complex Gab1 and SHP2 is not functional, both phosphorylation and activation of NOS3 by shear stress are abrogated (98).

Figure 2 shows schematically NOS3 function in both the coupled and uncoupled states, together with physiological factors regulating coupled NOS3 function.

3. CARDIOVASCULAR EFFECTS OF NO

3.1. Vasodilator properties of NO

Following its synthesis in the endothelial cell, NO diffuses to the subjacent vascular smooth muscle cells (VSMC) and activates haem-containing sGC (5). Stimulation of sGC leads to the conversion of GTP to cGMP. cGMP decreases intracellular Ca^{2+} causing vasorelaxation (99). cGMP exert its effects through protein kinase G (PKG), which is its principal mediator, and the most abundant isoform in the cardiovascular system is PKG I, being present in both smooth muscle cells and platelets (100). Substrates for PKG I are phospholamban (101) and the IP_3 receptor-associated cGMP kinase substrate (IRAG) (102).

PKG I phosphorylates phospholamban, which in its phosphorylated state dissociates from sarcoplasmic reticulum ATPase (SERCA) resulting in activation of the enzyme (101). SERCA leads to increased sequestration of Ca^{2+} into intracellular stores, thereby decreasing $[Ca^{2+}]_i$ (103) .The refilling of intracellular stores inhibits Ca^{2+} influx, which in turn maintains low levels of $[Ca^{2+}]_i$ and

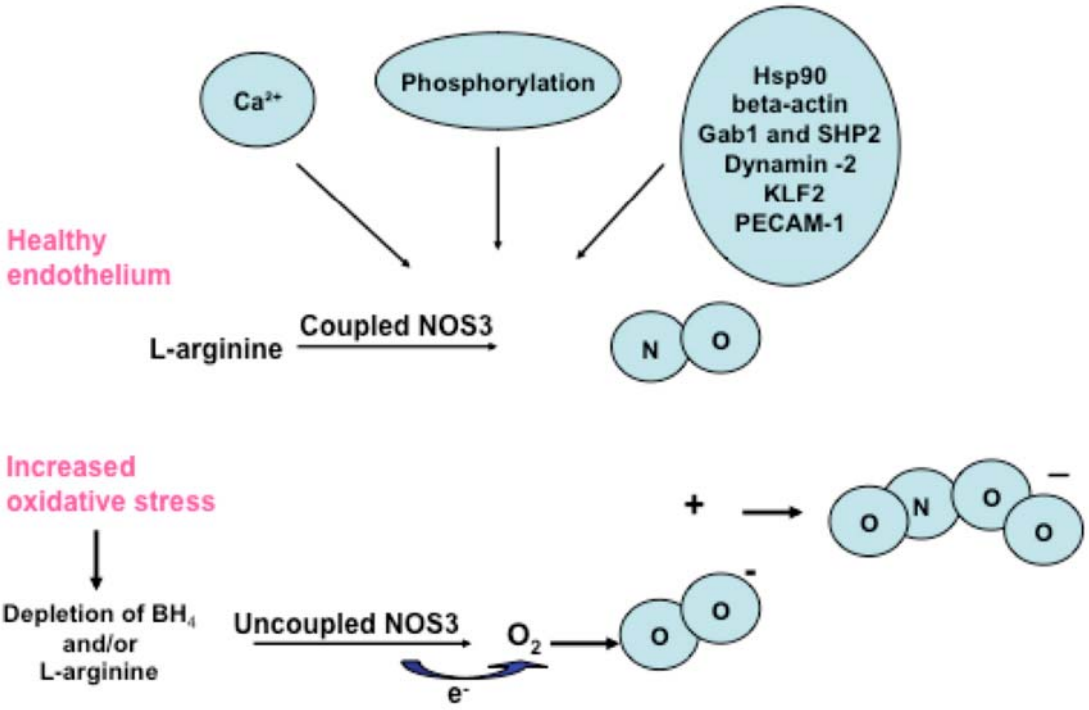

Figure 2. Coupled and uncoupled NOS3 function, and factors regulating coupled NOS 3. In healthy endothelium, NOS3 upon activation with stimuli through the Ca^{2+}-sensitive pathway or via phosphorylation will generate NO from L-arginine. Hsp90, beta-actin, Gab1 / SHP2, dynamin-1, KLF2 and PECAM-1 enhance NOS3 activity under conditions of health. Under conditions of increased oxidative stress, NOS3 can become uncoupled and generate O^-_2 instead. NO and O^-_2 react rapidly to form peroxynitrite ($ONOO^-$). Hsp90: heat shock protein 90; KLF2: Kruppel-like transcription factor 2; PECAM-1: platelet-endothelial cell adhesion molecule-1.

leads to vasorelaxation (103). PKG I also phosphorylates IRAG; this results in a strong inhibition of IP_3-induced Ca^{2+} release from the sarcoplasmic reticulum, and once again a decrease in $[Ca^{2+}]_i$ (102). NO can activate Ca^{2+}-dependent potassium channels, resulting in hyperpolarisation of the cell membrane and decreased Ca^{2+} entry. This action is both independent (104) and dependent on activation of PKG I (105). Addittionally, there is evidence for inhibition of voltage-gated Ca^{2+} channels, leading again to a decrease in $[Ca^{2+}]_i$, by PKG I (106).

Basal release of NO by the endothelium plays an important role in maintenance of basal tone in resistance arteries, and in tonic regulation of blood pressure (BP) and distribution of blood flow. It has been shown in rabbits that intravenous infusion of N^G-monomethyl-L-arginine (L-NMMA), an endogenous methylated L-arginine analogue which inhibits NOS, increases mean arterial BP in a dose-dependent manner, whereas ACh infusion causes hypotension. The effect of ACh was found to be reversed by L-NMMA, and supplementation with L-arginine to inhibit the effect of L-NMMA (107). In another study performed in healthy humans, brachial artery infusion of L-NMMA was found to cause a dose-dependent decrease in forearm blood flow (FBF), which was prevented by co-infusion of L-arginine. In these experiments, ACh increased FBF and this effect was attenuated by L-NMMA. The study investigators concluded that endothelium-derived NO is continuously released in the forearm arterial vasculature, and that it plays a significant role in determining basal blood flow and mediating the vasodilating effect of ACh (108). Confirming these early findings, more recent studies have shown that acute intravenous infusion of L-NMMA increases mean arterial pressure in healthy humans (109, 110). In longer-term animal studies, it has been demonstrated that chronic infusion of the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) in rats leads to time- and dose-dependent hypertension (111), and that aortic rings from mice lacking the gene for NOS3 do not relax to ACh, develop hypertension (112), and exhibit an increase in neointimal proliferation in response to vascular injury (113).

In recent years, it has become clear that, apart from the cGMP-dependent signalling pathways, NO may exert certain cellular effects in a cGMP-independent manner involving the S-nitrosylation of protein cysteine residues, leading to the generation of S-nitrosothiols (SNOs). S-nitrosylation of several proteins, many of which regulate cardiovascular physiology, allows NO to exert multiple protective cellular effects. On the other hand, both hypo- and hyper-S-nitrosylation of proteins have been implicated in the pathophysiology of cardiovascular diseases.

Although peripheral vasodilatation mediated by NO is regulated by cGMP, there is recent evidence to suggest that cGMP-independent pathways may also be involved (104). In this regard, S-nitrosylation of proteins by NO appears to be of importance. For example, deletion of S-nitrosoglutathione reductase (GSNOR), an enzyme which catalyses the reduction of one of the SNOs, S-nitrosoglutathione (GSNO), leads to lowering of systemic vascular resistance (114), and inhibition of this enzyme has been reported to cause vasodilatation (115). In addition, GSNOR null mice are prone to develop hypotension (114). The above data demonstrate that GSNO plays a major role in NO-dependent vasodilatation. Furthermore, there are studies which imply that both Ca^{2+}-insensitive and Ca^{2+}-sensitive mechanisms of endothelium-dependent vasodilatation involve not only NO generation but also NO release from SNOs. In particular, it has been demonstrated that activation of endothelial cells by shear stress leads to S-nitrosylation of more than 100 proteins (116), and that ACh stimulates both NOS3 activity and NO release from SNO pools (117). Furthermore, S-nitrosylation of the p47phox subunit of NADPH oxidase, one of the most important sources of endothelial O^-_2, prevents uncoupling of NOS3 by inhibiting the production of O^-_2 in healthy endothelium, thereby leading to preservation of NO availability (118). It is clear, therefore, that S-nitrosylation of a number of proteins provides another physiological mechanism through which NO exerts its effects.

On the other hand, either excessive or impaired S-nitrosylation may have deleterious cardiovascular effects. For example, increased S-nitrosylation of arginase, which uses L-arginine as a substrate and therefore may cause NOS3 uncoupling through depletion of its substrate, increases its activity and thereby enhances NOS3 uncoupling (119). This will cause increased O^-_2 production by the endothelium. Furthermore, although albumin serves as a major pool of plasma NO, excessive sequestration of generated NO as S-nitroso-albumin may have a negative impact in regulating vascular tone. More precisely, it has been shown that albumin raises BP not only through its oncotic effect but also through redistribution of NO from plasma to the hydrophobic albumin core.The formation of the S-nitroso-albumin complex renders NO less available to regulate vascular smooth muscle tone (120). Indeed, enhanced sequestration of NO by albumin has been implicated in the pathogenesis of hypertension in pre-eclampsia (121), and also has been related to adverse cardiovascular outcomes and hypertension in patients with end-stage kidney disease(122). Likewise, impaired S-nitrosylation of proteins involved in the regulation of circadian rhythm has been related to hypertension (123). Additionally, it has recently been demonstrated *in vitro* that S-nitrosylation of sGC by S-nitrosocysteine (CSNO), results in its desensitization, a phenomenon which has been implicated in the pathophysiology of NO tolerance (124).

3.2. Antiplatelet properties of NO

NO produced by the vascular endothelium inhibits platelet adhesion to endothelium (125, 126) and platelet aggregation (14), and stimulates disaggregation of activated platelets (127). NOS inhibition shortens bleeding time in humans (128); and NO produced by endothelium and by NO donors diffuses into platelets and, by stimulating cGMP production, inhibits platelet aggregation (129). This is related to SERCA-dependent refilling of intraplatelet Ca^{2+} stores (130) and to inhibition of IP_3-stimulated rise in platelet cytosolic Ca^{2+} (131). cGMP prevents platelet activation through at least three additional mechanisms. Firstly, it indirectly increases intracellular cyclic adenosine monophosphate (cAMP) levels through inhibition of phosphodiesterase type 3 (PDE 3) (132). The resulting increase in intraplatelet cAMP acts synergistically with cGMP to inhibit platelet aggregation (133). Secondly, cGMP inhibits the activation of PI3K (134), which in turn causes activation of glycoprotein (GP) IIb-IIIa fibrinogen receptors (135). Thirdly, cGMP, through the action of its downstream kinase PKG, causes phosphorylation of the thromboxane A_2 (TXA_2) receptor, thereby inhibiting its function (136).

Apart from the cGMP-dependent antiplatelet effects of NO, there is also evidence to indicate that NO regulates platelet function through cGMP-independent pathways. For example, NO donors such as S-nitrosocysteine, *S*-nitrosoglutathione, and diethylamine diazeniumdiolate, or inhaled NO, inhibit platelet aggregation to a degree unaffected by inhibition of sGC (137-139). Furthermore, NO inhibits ATP-dependent Ca^{2+} uptake into platelet membrane vesicles by a mechanism that cannot be attributed to its effector cGMP (140). Moreover, both exogenous and endogenous NO prevent platelet activation by blocking platelet exocytosis of dense granules, lysosomal granules and alpha-granules (141), effects which are not prevented by sGC inhibition but which appear to be mediated by S-nitrosylation of N-ethylmaleimide-sensitive factor (NSF), another target of NO inside platelets (141). Thus NO, by regulating platelet exocytosis, prevents the release of mediators necessary for the interactions between platelets, leucocytes, and endothelial cells. Consequently, platelets from NOS3 knock-out mice exhibit increased P-selectin expression, increased rolling along the vessel wall and increased exocytosis *in vivo*, compared to wild-type (141, 142).

However, a physiological role for NO in inhibiting platelet function remains controversial. Male NOS3 knockout mice, whilst having a reduced lifespan, die not from thrombotic complications but from cardiac failure, whilst female NOS3 knockout mice have a normal lifespan (143). Additionally L-NMMA, when administered into the brachial artery in humans in quantities sufficient to decrease FBF, does not alter platelet aggregation measured *ex vivo* in the blood draining the infused arm (144).

3.3. Anti-inflammatory properties of NO

NO inhibits migration of leucocytes from the blood stream and their adhesion to damaged endothelial cells, which are key steps in atherogenesis (145). Compared to wild type, mice deficient in NOS1, NOS2 or NOS3 exhibit enhanced leucocyte adhesion to the endothelium (146). Furthermore, NO donors inhibit, and L-NMMA enhances, expression on the endothelial cell surface of adhesion molecules, such as monocyte chemoattractant

protein-1 (MCP-1), vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1), which are induced in endothelial cells by the action of cytokines; these actions of NO are likely to be mediated by inhibition of the transcriptional factor nuclear factor kappa B (NF kappaB) and involves its antioxidative properties (147). Indeed, inhibition of NOS in HUVEC increases leucocyte adhesion, an effect that is inhibited by intracellular radical scavengers but not by cGMP analogues (148). It has been shown that in part, inhibitory S-nitrosylation of the p50 subunit of NF kappaB regulates its cellular activity and thereby contributes to the anti-inflammatory properties of NO (149).

Leucocyte adhesion on endothelial cells requires interaction between P-selectin, expressed on the endothelial cell surface, and its receptor P-selectin glycoprotein ligand-1 (PSGL-1), expressed on leucocytes. The translocation of P-selectin from endothelial Weibel-Palade bodies to the cell surface is mediated by NSF, which is a major component of the exocytotic trafficking machinery. NO inhibits NSF-mediated disassembly of soluble NSF attachment protein receptor (SNARE) complexes by nitrosylating critical cysteine residues of NSF (150).

3.4. Proliferation and angiogenesis regulated by NO

Early in the atherosclerotic process, VSMC lose their functional characteristics such as contractile activity, become more susceptible to pro-proliferative stimuli, and migrate into the vessel intima contributing to intima hyperplasia and to luminal stenosis (151). VSMC proliferation is controlled by angiotensin II, tumour necrosis factor-alpha (TNF-a), and growth factors such as platelet-derived growth factor (PDGF) (152). NO inhibits VSMC proliferation and migration to the intima (153). In mice lacking endothelial NOS3, cuff placement around the femoral artery induces more intimal hyperplasia than in wild type mice (154). The precise role of NO has not been fully clarified, but it may be related to indirect activation of PKA consequent on an increase in cAMP levels through the action of cGMP (155). NO also inhibits VSMC proliferation by changing the expression and activity of cell cycle regulatory proteins, independently of effects on cGMP (156).

VEGF, an important stimulator of endothelial NOS3 (157), is a major stimulus to angiogenesis and vessel growth, through S-nitrosylation of proteins in the vascular endothelium. VEGF-induced activation of NOS3 leads to S-nitrosylation of mitogen-activated protein kinase phosphatase 7 (MKP7), rendering the phosphatase inactive and unable to inhibit the activation of JNK3. Consequently, the chemokine stromal cell-derived factor-1alpha (SDF-1alpha), a key regulator of angiogenesis, activates JNK7, a step which is critical for endothelial migration and endothelial progenitor cell homing to sites of ischaemia (158).

NO has also been shown to support the function of dynamin, a protein which promotes endocytosis and endothelial cell survival signalling, through S-nitrosylation of specific cysteine residues. It has been demonstrated that S-nitrosylation of dynamin regulates the counterbalances of TNF-alpha-induced apoptosis and NO-dependent survival signals, with implications highly relevant to angiogenesis (159).

3.5. Antioxidant properties of NO

Increased vascular oxidative stress contributes to the pathogenesis of atherosclerosis and thereby of cardiovascular diseases. O^-_2 can react rapidly with NO to form the potent oxidant $ONOO^-$ (160). However, in the presence of physiological concentrations of SOD, the formation of $ONOO^-$ from O^-_2 competes with the formation of hydrogen peroxide catalysed by SOD. Thus, physiological concentrations of NO may exert antioxidant effects in the presence of physiological concentrations of SOD (6). The most well established antioxidant effect of NO is the reduction in pro-atherogenic lipid peroxidation (161).

Furthermore, NO induces the expression of extracellular SOD. In aortas of mice lacking NOS3, extracellular SOD was found to be reduced and exercise training did not result in upregulation of its expression, unlike in the wild-type (162). SOD catalyses the dismutation of O^-_2 to hydrogen peroxide (162) which, although itself a strong oxidant, also increases NOS3 expression and activity (163).

NO exerts additional antioxidant effects by increasing the expression of haem-oxygenase 1 (HO-1) (164). HO-1 catalyses the formation of bilirubin and carbon monoxide (CO) (165). Bilirubin is an O^-_2 scavenger (166), and CO activates sGC (167). NO also increases the expression of ferritin (168), which binds free iron ions (generated by the activity of HO-1) and reduces O^-_2 generation (169).

Overall, NO contributes importantly to the anti-atherogenic and vasodilating properties of the endothelium. It has been shown that mice lacking the gene for NOS3 develop hypertension, and that ACh-induced vasodilatation is absent in aortic rings from such mice (112). Genetic deficiency of NOS3 accelerates atherosclerosis in male apo E/NOS3 double knock-out mice, an effect that is independent of the hypertension that developes in these animals (170). However, under basal conditions, it is uncertain whether NO production is essential to suppress endothelial activation. NOS3 knockout endothelial cells do not display characteristics of activated cells, so NOS3 deficiency itself may not necessarily result in the expression of an activated endothelial phenotype (171). It is therefore possible that the L-arginine/NO system is more important in counterbalancing other signals which promote endothelial activation.

4. ENDOTHELIAL DYSFUNCTION

Endothelial dysfunction refers to a loss of the vasodilating, anti-atherosclerotic and anticoagulant properties of the endothelium. It is mainly detected by a loss of endothelium-derived NO availability, and is usually recognized as an impairment of endothelium-dependent

dilatation. In this regard, endothelium-dependent dilatation has been found to be impaired not only in the forearm, but also in the coronary and the renal vasculature of patients with cardiovascular diseases.

Disturbances in the NO pathway may be caused by decreased expression of NOS3, impaired NOS3 activation, insufficient substrate (L-arginine) or cofactors (e.g. BH_4), or the presence of increased endogenous NOS antagonists (asymmetric dimethylarginine [ADMA] and/or L-NMMA), as well as by increased degradation of NO by reactive oxygen species (ROS) leading to diminished NO availability (172). Where there is excess production of ROS, O^-_2 and NO undergo an extremely rapid reaction to form $ONOO^-$. This reaction not only results in a loss of the beneficial effects of NO but also increases the damaging effects of $ONOO^-$, which is one of the strongest oxidants found in biological systems.

The presence of endothelial dysfunction in cardiovascular disease states has been investigated predominantly by the use of two methods which investigate two distinct signalling pathways of endothelial NO generation in the forearm vasculature: FBF as measured using venous occlusion plethysmography, and flow-mediated dilatation (FMD). The former is generally used to investigate the forearm blood flow responses to agonists such as ACh, bradykinin, or albuterol. The latter method, which is based on the effect of shear stress on endothelial cells, investigates Ca^{2+}-insensitive NOS3. In recent times, brachial FMD has been more widely used as it is entirely non-invasive and, when a strict protocol is used, is considered relatively easy to perform and reproducible. In a recent study FMD was compared with two other non-invasive methods of measuring endothelium-dependent dilatation - vascular responses to inhaled albuterol by pulse wave analysis and pulse contour analysis - and was found to be the most reproducible (173). However, critical elements of FMD methodology have yet to be standardised, for example cuff occlusion time, site of cuff placement and control of environmental conditions.

FMD has established itself as an independent predictor of future cardiovascular events in patients with a history of cardiovascular disease (174). More recently, it has also been found to predict cardiovascular events in older adults (175), in subjects with no history of cardiovascular disease (176), and in individuals without clinically detected atherosclerosis (177). Nevertheless, the addition of FMD to traditional risk factors does not improve risk stratification in all individuals. A recent meta-analysis demonstrated that FMD is related to the principal cardiovascular risk factors and to the estimated 10-year risk of coronary heart disease predominantly in populations who are at low baseline risk (178). Furthermore, another population-based study recently showed that, although FMD predicts incident cardiovascular events, its prognostic value is not independent of traditional risk factors (179). FMD appears to represent the most physiologically relevant method for estimating NO-mediated endothelial vasodilatation. Therefore, a diminished brachial FMD response reflects low systemic NO availability and is consequently associated with a loss of all the protective effects of NO on the vasculature. Presently it is considered a valuable research tool for population studies; however, its additive and independent clinical value over and above established cardiovascular risk markers remains to be proven.

Endothelial dysfunction has been found to be associated with cardiovascular risk factors as well as with overt clinical cardiovascular disease. Smokers who are otherwise healthy and passive smokers exhibit a reversible impairment of endothelium-dependent dilatation (180, 181). These abnormalities are associated also with impaired fibrinolysis, providing a mechanism through which endothelial dysfunction promotes thrombosis (182). Impaired FMD in apparently healthy subjects is associated with smoking, older age and male gender, but not with serum cholesterol, blood pressure or family history of coronary heart disease (183). In addition, hypercholesterolemia has been consistently related to endothelial dysfunction (184, 185), and oral L-arginine supplementation improves this (186). There is also a direct association between high-density lipoprotein (HDL)-cholesterol and endothelial function (187). Children with familial hypercholesterolemia, without any clinically apparent sign of atherosclerosis, also have impaired endothelial function (184). Other cardiovascular risk factors such as hyperhomocysteinaemia (188), ageing (189) and diabetes mellitus (190, 191) are also characterized by endothelial dysfunction. For type 1 diabetes mellitus, apart from the effect of other risk factors, endothelial dysfunction appears to be related marginally to the degree of glycaemic control, duration of disease, and the presence of complications (192, 193). Thus patients with newly diagnosed type 1 diabetes mellitus and young patients may have preserved endothelial function. By contrast, in type 2 diabetes mellitus, the majority of studies have shown impaired endothelial function. This may be partly due to clustering of this condition with other components of the metabolic syndrome, such as high blood pressure, obesity, high triglycerides and low HDL-cholesterol levels (194), all of which may contribute to endothelial dysfunction.

In patients with coronary heart disease, ACh induces a paradoxical vasoconstriction in atherosclerotic coronary arteries (195), and endothelial dysfunction of the coronary arteries in response to ACh is related, although weakly, to endothelial dysfunction in the forearm circulation in response to shear stress (196). However, in a study where the same stimulus was used, a stronger correlation was documented between flow-mediated dilatation in the coronary and brachial arteries, indicating that the latter noninvasive assessment can be used as a surrogate measure for coronary artery endothelial function (197). Furthermore, the degree of impairment of endothelial vasodilatation in patients with mild coronary heart disease is associated with the magnitude of increase in incidence of subsequent cardiac events (198).

In hypertensive patients, early studies demonstrated both a diminished response to ACh in coronary and forearm vasculature (199-201), and a blunted

response to L-NMMA (202), as compared to healthy controls. These results indicate not only impaired endothelium-mediated vasodilatation due to decreased NO generation by the endothelium, but also an impaired contribution of NO produced by endothelial cells to basal vascular tone. By contrast, other investigators have found no difference in the FBF dilator response to nitroprusside, ACh, carbachol or isoproterenol, nor in the FBF constrictor response to L-NMMA, between healthy controls and hypertensive patients (203, 204). On the other hand, in patients with untreated essential hypertension, FMD has been found to be impaired (205, 206), while therapeutic interventions with angiotensin converting enzyme (ACE) inhibitors, angiotensin receptor blockers, calcium-channel blockers (207, 208), or with L-arginine improve it (209). The heterogeneity of essential hypertension may, in part, explain the differences observed in studies investigating endothelial function (210). In accordance with this, an early study demonstrated that, in apparently healthy subjects, FMD is independently associated with smoking, older age and male gender, but not with serum cholesterol, blood pressure or family history of coronary heart disease (183).

5. PHARMACOLOGICAL INTERVENTIONS THAT ENHANCE ENDOTHELIAL NO BIOSYNTHESIS

In recent years, the use of transcription enhancers of endothelial NOS3 has emerged as a promising therapeutic approach in order to improve NO bioavailability. In an animal model of experimental myocardial infarction, the transcription enhancer AVE9488 was found to improve cardiac structural and functional parameters as well as to increase NOS3 expression, Ca^{+2}-sensitive NOS3 activity and endothelium-dependent NO-mediated vasorelaxation. The beneficial effects of AVE9488 on left ventricular dysfunction and remodelling after myocardial infarction were abrogated in NOS3 knock-out mice, indicating the importance of such pharmacological interventions specifically designed to increase NOS3-derived NO (211).

Visfatin is a NAD biosynthetic enzyme, which regulates the activity of the cellular survival factor Sirt1. In both HUVEC and coronary artery endothelial cells, recombinant human visfatin has been reported to upregulate and stabilize NOS3 mRNA, as well as to increase production of NO and cGMP through phosphorylation of NOS3 by Akt and mitogen-activated protein kinases (212).

Hydroxy-methylglutaryl-coenzyme A reductase inhibitors (statins) improve endothelial NO availability predominantly by influencing post-transcriptional mechanisms related to NOS3 expression. More specifically, *in vitro*, both simvastatin and lovastatin have been shown to stabilize NOS3 mRNA, upregulate NOS3 expression and enhance NOS3 activity (213). In another study, atorvastatin was found to reduce caveolin-1 expression, decrease the inhibitory interaction between caveolin-1 and NOS3, and enhance basal and agonist-stimulated NOS3 activity (214). Moreover, cerivastatin has been reported to induce a time-dependent release of NO in endothelial cells, similar to that of traditional agonists, and to scavenge O^{-}_{2}, leading to preservation of bioactive NO (215). Similarly, atorvastatin reduces the half-life of angiotensin II type 1 (AT_1) receptor mRNA, leading to down-regulation of AT_1 receptor gene expression and to decreased ROS production, thereby preventing NOS3 uncoupling and consumption of bioactive NO (216). The above studies suggest that statins may exert multiple beneficial effects on the endothelium beyond simple lowering of cholesterol.

Pharmacological blockade of the renin-angiotensin system has been shown to have beneficial effects on NOS3 activity and NO availability. In an animal model of experimental hypertension, ACE inhibition and AT_1 blockade have been found to reverse the reduction in NOS3 mRNA levels as well as to improve endothelial-dependent vasodilatation (217). These effects were mediated through bradykinin $Beta_2$ receptor activation, as the use of a $Beta_2$ antagonist blunted the beneficial effects of both drugs (218). In addition, ACE inhibition either alone or in combination with aldosterone antagonism improves NO release and endothelial-dependent vasodilatation, as well as reducing $ONOO^{-}$ generation (219).

6. PLATELET L-ARGININE/NO PATHWAY

NOS3 is the predominant isoform in platelets (220), although the presence of NOS2 in relatively small amounts has also been reported by some investigators, as discussed above.

Radomski and colleagues first reported that, in platelets activated by collagen, L-arginine inhibited aggregation, stimulated sGC and increased cGMP levels (221). Platelet NOS3 was shown to be Ca^{2+}/CaM-regulated and required NADPH for its activity (221), as is the case with endothelial NOS3. The same workers further showed that other pro-aggregatory agents such as adenosine diphosphate (ADP) and arachidonic acid (AA) could increase platelet NOS3 activity. Whilst L-arginine was found to inhibit platelet aggregation, L-NMMA enhanced the aggregatory response and inhibited cGMP production in activated platelets (222). It was concluded that an increase in intracellular Ca^{2+} due to stimulation of platelets by pro-aggregatory agents activates platelet NOS3 and leads to NO production. It was further proposed that NO generated from platelets in this way plays an inhibitory role in platelet aggregation (222).

Platelets appear to produce NO not only upon stimulation but also, to some degree, under resting conditions. That resting platelets can produce NO was shown in studies using two different methods, namely the conversion of oxyhaemoglobin to methaemoglobin, and the generation of nitrite and nitrate (223). Basal platelet NOS activity has also been detected by measuring the conversion of L-arginine to L-citrulline (220, 224). NOS3 mRNA and protein have both been detected in platelets, both in health and in disease states (220, 225, 226).

6.1. Regulation of platelet NOS3 activity

Platelet NOS3, similar to endothelial NOS3, can be activated in both Ca^{2+}-sensitive and insensitive manners.

Aggregating agents such as ADP and thrombin increase intracellular Ca^{2+}, thereby activating platelet NOS3 and inducing NO generation (222, 227), which in turn acts as a negative feedback mechanism to limit platelet activation. By contrast, $beta_2$ –adrenoceptors (AR), which are present on platelets and couple to adenylyl cyclase (228), stimulate NOS3 activity and platelet NO production through cAMP and an increase in PKA activity, with no detectable increase in intraplatelet Ca^{2+} (224). beta-AR stimulation inhibits platelet adhesion to cultured HUVEC monolayers, and this effect is abolished by NOS inhibition. In platelets, $beta_2$AR activation of NOS is entirely dependent on PKA and completely abolished by the adenylyl cyclase inhibitor SQ22536 (224), whereas in HUVEC both PKA and Akt contribute to $beta_2$AR activation of NOS3 via an increase in its Ser^{1177} phosphorylation (78, 229). PKA plays a role in regulating platelet NOS3 activity in response to catecholamines, at concentrations that occur physiologically in the human circulation, and to adenosine (230, 231). This effect of PKA on NOS3 has again been linked to phosphorylation of the Ser^{1177} residue (232).

Insulin also activates NOS3 in platelets in a Ca^{2+}-insensitive manner, this time through phosphorylation on Ser^{1177} by Akt, an effect which is inhibited by the PI3-K inhibitor wortmannin (233). Insulin leads to an increase in intraplatelet cGMP of two- to four-fold, considerably greater than the increase in platelet cGMP induced by collagen; this in turn attenuates thrombin-induced platelet aggregation, an effect which is prevented by NOS inhibition (233, 234). The stimulatory effect of insulin on platelet NOS3 is also dependent on cAMP-dependent protein kinase activation, through phosphorylation of NOS3 on Ser^{1177}, and on Hsp90. Inhibition of the association of Hsp90 with platelet NOS3 by geldanamycin attenuates activation of the enzyme, cGMP production, and the inhibitory effect of insulin on platelet aggregation (233). By contrast, in endothelial cells, although insulin acutely induces phosphorylation of NOS3 through the action of Akt, this is not associated with a short-term increase in NO production, nor with relaxation of endothelium-intact arterial segments (235). On the other hand, application of insulin to native endothelial cells leads, after 18-24 hours, to a significant increase in NOS3 mRNA and in intracellular cGMP (235).

Similar to endothelial NOS3, platelet NOS3 binds to the globular, but not to the filamentous, form of beta-actin, and the affinity of this binding is substantially increased by Hsp90. Binding results in the formation of a ternary complex (NOS3/globular beta-actin/Hsp90), localised in platelet caveolae, and subsequent activation of platelet NOS3, as documented by an increase both in NOS3 activity and in cGMP levels in platelets. This phenomenon occurs independently of changes in intracellular Ca^{2+}. The formation of the ternary complex gives rise to an increase in Hsp90 degradation, which acts as a negative feedback to platelet NOS3 activation (94).

Collagen activates platelet NOS through its receptor GPVI and via activation of both PI3-K/Akt and PKC pathways, in a Ca^{2+}-sensitive manner (236). By contrast, some groups have found no change in intraplatelet Ca^{2+} following stimulation with collagen and subsequent activation of the NO pathway (237, 238). These discrepancies may be explained by methodological differences in measuring intraplatelet Ca^{2+}.

Pyridoxine increases PI3-K activity and thereby Akt phosphorylation which, in turn, enhances platelet-derived NO generation via phosphorylation of the Ser^{1177} residue of platelet NOS3 (239). Aspirin acetylates NOS-3 acutely in platelets, thereby increasing its activity, and this is not associated with any measurable change in intraplatelet Ca^{2+} concentration (240). These effects have not to date been documented in the endothelium.

Other stimuli that activate platelet NOS3 include shear stress (241) and □lpha-tocopherol (242). In a system where platelet adhesion and aggregation on a collagen type III surface was investigated, it was found that high shear stress itself reduced, and NOS3 inhibition enhanced, platelet coverage of the surface (241). alpha-tocopherol, *in vitro* and *in vivo*, increases platelet NO release during ADP-induced platelet aggregation, and this occurs partially through O^{-}_{2} scavenging but also by enabling NOS3 activation via inhibition of the effect of PKC on the enzyme (74, 242).

Other endogenous factors that may be involved in the regulation of platelet NOS3 activity include von Willebrand factor (vWF) and glucose (243, 244). vWF activates platelet NOS3, cGMP production, and induces vasodilator-stimulated phosphoprotein (VASP) phosphorylation (which is a PKG substrate), through its receptor glycoprotein (GP) Ib in a Ca^{2+}-sensitive manner which requires PI3-K and phospholipase C (243). High glucose activates NOS3 in resting platelets by an osmotic mechanism that probably involves PKC_{β}, an isoform of PKC, activation of which causes an increase in intraplatelet Ca^{2+} concentration (244, 245).

Although the importance of phosphorylation of platelet NOS3 in regulation of its activity is now well accepted, only one phosphorylation site, the Ser^{1177} residue, is currently established as of physiological relevance. By contrast, in endothelial cells, as described above, NOS3 can undergo phopshorylation on several other residues. Additionally, although for endothelial NOS3 various regulatory proteins have been identified to date, such as Hsp90, beta-actin, NOSIP and NOSTRIN, dynamin-2 and porin, for platelet NOS3 only Hsp90 and beta-actin are known to affect its function. In particular, Hsp90 associates with the enzyme and enhances its activity in response to insulin (233) and after binding to the globular form of beta-actin (94).

6.2. The role of platelet-derived NO in health

Platelet-derived NO stimulates sGC and cGMP production in platelets, leading to activation of its principal mediator PKG, thereby inhibiting platelet activation through a number of pathways. These pathways involve the SERCA-dependent refilling of intraplatelet Ca^{2+} stores (130), and also the inhibition of inositol 1,4,5-trisphosphate

(IP_3)-stimulated Ca^{2+} release from the sarcoplasmic reticulum, which results in a decrease in cytosolic Ca^{2+} (102) . cGMP prevents platelet activation through three additional mechanisms. Firstly, cGMP increases intracellular cAMP levels through inhibition of PDE 3 (132). cAMP and cGMP act synergistically to inhibit platelet aggregation (127, 133). Secondly, cGMP inhibits the activation of PI3-K (134) and hence activation of GP IIb-IIIa fibrinogen receptors (135). And thirdly, cGMP activates PKG which in turn phosphorylates the TXA_2 receptor inhibiting its function (136).

Although cGMP-independent NO effects have been suggested as alternative signalling pathways regulating platelet activation, and the nitration of platelet proteins by NO has been documented, whether such pathways are relevant to the action of platelet-derived NO has not been fully elucidated. A recent study showed that tyrosine nitration of alpha-actinin by exogenous NO inhibits platelet adhesion to fibrinogen-coated plates, whilst inhibition of the cGMP pathway has no such effect (246). However, the specific role of platelet-derived NO was not investigated. Subsequently, another study similarly demonstrated that exogenous NO induces inhibition of platelet adhesion to immobilized collagen, and this effect is not completely abolished by inhibition of sGC, confirming that cGMP-independent signalling events contribute to the regulation of platelet adhesion by NO. Exogenous NO was found to lead to hyper-nitrosylation of basally S-nitrosylated platelet proteins. Interestingly, platelet activation in the absence of exogenous NO failed to increase S-nitrosylation beyond basal levels, indicating that platelet-derived NO was unable to induce this type of protein modification (247). It is therefore uncertain, and remains to be established, whether platelet-derived NO exerts its effects not only through cGMP-dependent pathways but also through S-nitrosylation of platelet proteins.

Early experiments by Radomski and colleagues suggested that platelet-derived NO importantly regulates primary platelet aggregation (221, 222). In these experiments, with prostacyclin-washed platelets, L-arginine inhibited, and L-NMMA enhanced, platelet aggregation induced by agonists such as collagen, ADP and arachidonic acid. However, later experiments by other groups failed to reproduce these results and suggested that platelet NO may not substantially modulate primary platelet aggregation (248). Moreover, after inhibition of platelet NOS by L-NMMA, platelet aggregation induced by ADP is not altered, and the inhibitory effect of the βAR agonist isoproterenol on platelet aggregation is not affected (224, 227). The optical aggregometry method, which was used in these studies, is not sensitive enough to detect early or subtle changes in platelet function (249), and this may have contributed to the discrepancies found in these studies.

On the other hand, there are data suggesting that, while platelet-derived NO does not consistently inhibit platelet aggregation in response to pro-aggregants, it does enhance the anti-platelet effects of anti-aggregatory mediators. For example, although NOS inhibition appears to have no consistent effect on thrombin-induced platelet aggregation, it reduces the inhibitory effect of insulin on platelet aggregation (233). Similarly, the inhibitory effect of adenosine on platelet aggregation can be partially prevented by NOS inhibition (230).

While the role of platelet-derived NO in regulating platelet aggregation remains controversial, its importance for other aspects of platelet activation is more clearly established. Platelet-derived NO inhibits platelet recruitment to the growing thrombus (227). This process is initiated by activated platelets at the site of vascular injury by secretion of ADP, serotonin, and TXA_2, and further promotes thrombin deposition and thrombus organisation on the platelet surface. In accordance with these results, the same investigators found that bleeding times in NOS3-deficient mice are less than in wild-type mice, due to increased platelet recruitment in the NOS3-deficient mice as a result of impaired platelet NO production (142). Furthermore, it has been shown that $beta_2AR$ stimulation and platelet NO production from activated platelets inhibit platelet adhesion on HUVEC, although a possible contribution of endothelial NO cannot be excluded (224). In addition, platelet-derived NO modulates the rate of thrombus growth on a collagen type III surface, through altering platelet adhesion, after stimulation with insulin, isoproterenol or high shear stress (241).

More recently it has been shown *in vitro* that platelet-derived NO inhibits heterotypic aggregation between leucocytes and platelets, and in particular between monocytes and platelets (250); formation of such aggregates represents an early and robust marker of platelet activation (251), and is implicated in the mechanism of atherogenesis and thrombosis (252). Inhibition of platelet NOS3 increases the expression of P-selectin on the platelet surface after stimulation with ADP (227), which is essential for the interaction between leucocytes and platelets (253), and enhances the formation of monocyte-platelet aggregates, whilst exogenous NO decreases the platelet expression of P-selectin and the extent of monocyte-platelet complex formation (250, 254).

Collectively, these data support a physiological role of platelet-derived NO in the modulation of platelet function and hence thrombus formation. Furthermore, it is well established that, apart from regulating haemostasis and thrombosis, platelets play an important role in the inflammatory and thrombotic pathophysiology of atherosclerosis, in part due to their interactions with leucocytes (255). Platelet-derived NO, by inhibiting the formation of monocyte-platelet aggregates, may contribute to the prevention of atherosclerosis and its complications.

However, although the role of platelet-derived NO in the modulation of platelet function is established, it has been shown that male NOS3 knockout mice, whilst having a reduced lifespan, die not from thrombotic complications but from cardiac failure, whilst female NOS3 knockout mice have a normal lifespan (143). Furthermore, although NOS3-mutant mice have increased BP and decreased heart rate, and aortic rings from these animals

exhibit impaired endothelial-dependent vasodilatation to ACh (112), their platelets do not express more P-selectin on their surface and do not produce more thromboxane B_2, both markers of platelet activation (142, 256). Similarly, in another study no difference was found in the expression of GPIIb, and in ADP-induced aggregation, between NOS3-deficient and wild-type mice (256). However, platelets from NOS3-deficient mice, when transfused into thrombocytopenic NOS3-deficient mice, give rise to decreased bleeding time compared with platelets from wild-type mice, an effect which has been attributed to enhanced platelet recruitment in the NOS3-deficient mice, as estimated by serotonin release from activated platelets (142).

It is noteworthy that some recent studies indicate a potential stimulatory role of platelet-derived NO in platelet function through the generation of cGMP. PKG knock-out mice show impaired platelet activation in response to vWF and to low doses of thrombin. This is mediated by impairment in GPIb-IX-induced GPIIb/IIIa activation, which is essential for platelet rolling on immobilised vWF. Similarly another study on human platelets suggests that PKG inhibitors prevent their spreading on vWF and the secondary phase of platelet aggregation. The authors of this study concluded that the platelet response to cGMP effect is biphasic, consisting of an early stimulatory response that promotes thrombosis and a late inhibitory response that limits platelet aggregation (257). This effect may be NO concentration-dependent, such that at low concentrations NO may enhance and at higher concentrations may inhibit platelet activation. In another study, PKG was found to stimulate ADP secretion from platelet dense granules and to enhance the secondary phase of platelet aggregation induced by thrombin and TXA_2 (258). Such a stimulatory effect of NO on platelet function remains the subject of much controversy.

6.3. The role of platelet-derived NO in cardiovascular disease states

The role platelet-derived NO may play in the context of cardiovascular diseases, and in the presence of cardiovascular risk factors without clinically overt disease, has also been investigated. Freedman and colleagues studied the clinical relevance of platelet-derived NO in patients with coronary heart disease (stable angina and acute coronary syndrome). Platelets from patients with unstable angina or acute myocardial infarction were found to produce less NO in response to stimulation, as measured with an NO-selective microelectrode, than platelets from patients with stable angina pectoris (259). Furthermore, platelet-derived NO was found to be an independent predictor of occurrence of acute coronary syndrome. This suggests that impaired platelet NO production may contribute to the development of acute coronary syndromes, due to an increase in platelet activity and hence predisposition to thrombosis.

Queen and colleagues showed that NO generation and cGMP production in response to $beta_2AR$ stimulation are impaired in platelets from patients with type 2 diabetes mellitus. They further showed that NO-attributable cGMP levels were inversely correlated to HbA_{1c} and to fasting glucose levels in these patients. Again, this impairment in platelet NO biosynthesis could contribute to the increased platelet activation seen in these patients (226).

Platelets from long-term smokers release less NO during collagen-induced aggregation as compared with those from non-smokers, with consequently lower levels of intraplatelet cGMP (260). In another study, platelet-derived NO was found to correlate inversely with smoking, age and mean arterial pressure, in a population with multiple risk factors for cardiovascular disease (261).

Oxidised LDL-cholesterol stimulates platelet activation, and this appears to be contributed to by reduced platelet NO production, through a reduction both in L-arginine transport into platelets and in platelet NOS3 expression (262). In accordance with these findings, it has been shown that statin treatment enhances platelet NO release via upregulation of platelet NOS3 mRNA expression, thereby preventing carotid thrombosis in a rat model (263).

In essential hypertension, platelet NO production, as estimated from nitrite levels, has been found to be decreased (264); and other studies in this condition have described reduced platelet L-arginine transport (due to downregulation of the y^+L transport system) (265) and enhanced inhibition of platelet NOS3 by ADMA whose plasma levels are greater in hypertensives compared to normotensives (250). Furthermore, patients with recently diagnosed untreated mild essential hypertension have been found to exhibit impairment in stimulated platelet NOS3 activity; in this study, while albuterol and collagen both increased platelet NOS3 activity in normotensive subjects, they failed increase it in untreated hypertensive subjects (250). As these two agonists stimulate NOS3 through different pathways, it is likely that a generalized defect exists in the ability of platelet NOS3 to undergo stimulation, in patients with essential hypertension.

In accordance with the previously reported decrease in endothelium-derived NO biosynthesis with age (189), platelet NOS activity, as well as intraplatelet cGMP levels, have been found to be reduced in older (>45 years old) compared with younger (<30 years old) subjects (266). Although these subjects were clinically healthy, such an age-related decline in platelet NO-dependent function may predispose to an enhanced thrombotic tendency with age, especially where other cardiovascular risk factors are also present.

6.4. Pharmacological interventions that enhance platelet NOS3 activity

To date, little information is available regarding therapeutic interventions that may enhance platelet NOS activity and NO release. In a recent study, it was demonstrated that inhibition of the GPIIb/IIIa receptor, which is essential for fibrinogen binding and for a sustained platelet aggregation response, leads to an increase in platelet NO release and to attenuation of platelet O_2^-

production upon activation (267). In rats, statin treatment enhances platelet NO release via upregulation of platelet NOS3 mRNA expression, and inhibits P-selectin platelet expression (263). In another study, aspirin was found to activate platelet NOS3 acutely, whilst at the same time inhibiting β-adrenoceptor-mediated NOS3 activation (268). This is explained by acetylation of NOS3 by aspirin, and the effect appears to be insensitive to intracellular Ca^{2+} (240). Finally, as mentioned above, pyridoxine enhances platelet-derived NO generation via phosphorylation of the Ser^{1177} residue of platelet NOS3 (239). However, further work is needed in order to explore the therapeutic utility of such treatments, as well as to develop newer interventions which may target platelet NO biosynthesis more effectively.

7. PERSPECTIVE

Both endothelial and platelet derived NO exert important vasoprotective, anti-atherosclerotic and anti-thrombotic effects. The impairment of endothelial-derived NO observed in several cardiovascular diseases co-exists with decreased platelet-derived NO generation. This generalised decrease in NO availability aggravates the enhanced platelet activation, and thereby the thrombotic tendency, seen in these conditions, but also the atherosclerotic process itself. Although measures which improve NO availability in animal models retard the progression of atherosclerosis, this remains to be shown in man. Long-term studies are now needed in humans, using therapies to improve both endothelial and platelet NO production, to determine their effect on long-term outcome.

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Abbreviations: ACh: Acetylcholine, ADMA: NG, NG-dimethyl-L-arginine, asymmetric dimethylarginine, ADP: : Adenosine diphosphate, ATP: Adenosine triphosphate, AII: Angiotensin II, ßAR: ß-adrenoceptors, BH4: Tetrahydrobiopterin, BP: Blood pressure, Ca2+: Calcium, CaM: Calmodulin, CaMKII: CaM-dependent kinase II, cAMP: Cyclic adenosine monophosphate, cGMP: Cyclic guanosine monophosphate, EDRF: Endothelium-derived relaxing factor, FAD: Flavin adenine dinucleotide, FBF: Forearm blood flow, FMD: Flow-mediated dilatation, FMN : Flavin mononucleotide, GP: Glycoprotein, GTP: Guanosine trisphosphate, Hsp 90: Heat shock protein 90, IP3: inositol 1,4,5-trisphosphate, HUVECs: Human umbilical vein endothelial cell, KLF2: Kruppel like transcription factor-2, L-NMMA: NG, NG-monomethyl-L-arginine , L-NAME: NG-nitro-L-arginine methyl ester, NADH: Nicotinamide adenine dinucleotide , NADPH: Nicotinamide adenine dinucleotide phosphate, NO: Nitric oxide, NOS: Nitric oxide synthase, NOS 3: Endothelial NOS, NOS 2: Inducible NOS, NOS1: Neuronal NOS, NOSIP: NOS 3 interacting protein, NOSTRIN: NOS 3 traffic inducer, O-2: Superoxide anion, ONOO-: Peroxynitrite, PECAM-1: Platelet endothelial cell adhesion molecule 1, PI3K: Phosphatidylinositol 3-kinase, PKA: c-AMP dependent protein kinase A, PKC: c-AMP dependent protein kinase C, PKG I: cGMP dependent protein kinase I, PKG II: cGMP dependent protein kinase II, ROS: Reactive oxygen species, SERCA: Sarcoplasmic reticulum ATPase, sGC : Soluble guanylyl cyclase, SOD: Superoxide dismutase, TNF- a: Tumor necrosis factor a, TxA2: Thromboxane A2

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