Therapeutic potential of nitric oxide donors in the prevention and treatment of atherosclerosis

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Introduction

The discovery of the endothelium-derived relaxing factor, its identification as nitric oxide (NO) released by the vascular endothelium, and the elucidation of its biosynthetic pathway opened a whole new chapter in the quest for understanding cardiovascular disease and the way in which it may be prevented.¹-⁴ A reduction in the generation and bioavailability of NO has been shown to occur in several disorders, including atherosclerosis, which is the major cause of death and disability in the United States, Europe, and much of Asia.⁵ This review aims to consider the implications of NO in atherosclerosis and to discuss the potential therapeutic benefits of NO donors or enhancers of NO availability in the prevention and treatment of this pathology.

NO and atherosclerosis

Pathogenesis of atherosclerosis

Early atherosclerosis is characterized by the deposition of intracellular and extracellular lipids and by the appearance of macrophages and T-lymphocytes in the vessel intima. As macrophages and smooth-muscle cells (SMCs) below the endothelial cells (ECs) accumulate lipids, they acquire a foamy appearance. Clusters of lipid-laden cells become macroscopically visible as fatty streaks.⁶ These flat, fatty lesions may be transformed into raised fibrolipid plaques and ultimately into a fibroatheroma, which has a characteristic microanatomy of a core of extracellular lipid covered on the luminal side by a thick fibrous cap. Surrounding the core are lipid-laden foam cells, while ischaemia in the necrotic core initiates angiogenesis.⁷ This type of plaque may cause narrowing of the lumen, once compensatory vascular remodelling becomes inefficient. The ultimate stage, the complicated plaque, may arise either from a fissure in the fibrous cap or from intraplaque haemorrhage. If the thrombus is not occlusive, it becomes incorporated into the plaque and is organized by invading macrophages, ECs, and SMCs, thereby further compromising the lumen of the vessel. The sequence of fissure, thrombus formation, organization, and incorporation into the plaque may occur repeatedly.⁸

Thromboembolic events following plaque fissure are a major cause of clinically manifest acute ischaemic syndromes. Major mechanisms leading to coronary thrombosis include frank rupture of a plaque's fibrous cap, intraplaque haemorrhage, and superficial erosion of the endothelium. Plaque rupture occurs when the mechanical stresses in the fibrous cap exceed a critical level that the tissue can withstand.⁹ Biological factors weakening the fibrous cap include infiltration with inflammatory macrophages and T-cells and a reduction of the SMCs number at critical locations. The macrophages can promote local expression or activation of matrix metalloproteinases, which decrease the strength of the cap by degrading collagen and other matrix components. Furthermore, activated macrophages in atherosclerotic lesions kill SMCs in their vicinity either by lytic damage leading to necrosis or by inducing apoptosis.¹⁰-¹² As SMCs are central to the biosynthesis and maintenance of the fibrous cap, their number may become insufficient to repair the degradation. Well-known accelerating risk factors for atherosclerosis include hypercholesterolaemia, hypertension, diabetes, and smoking. These conditions are associated with endothelial dysfunction, which itself is associated with reduced endothelial generation of nitric oxide (NO). This is an overview of the implications of NO generation in atherosclerosis and of the potential therapeutic benefit of drugs which donate NO, such as organic nitrates, nicorandil, and sydnonimines, or those which increase the availability of endogenous NO, such as statins, angiotensin-converting enzyme inhibitors, L-arginine, and tetrahydrobiopterin.

Keywords
Nitric oxide; Atherosclerosis; Nitric oxide donor

References


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Pathogenesis of atherosclerosis in 28 steps.

1. LDLs are oxidized to ox-LDL by ROS and by 15-lipoxygenase.
2. Ox-LDLs are cytotoxic for the ECs.
3. Ox-LDLs accumulate in the EC, intima, and SMCs.
4. The ECs and SMCs react by secreting monocyte MCP-1 and growth factors.
5. MCP-1 and growth factors stimulate monocytes and T-lymphocytes.
6. Ox-LDLs elicit an immune response and cytokines are produced.
7. Ox-LDL and cytokines induce the expression of VCAM-1 and ICAM-1 on ECs.
8. Leukocytes and monocytes bind to ICAM-1 and VCAM-1.
9. Macrophages migrate into the EC and phagocyte ox-LDL.
10. Macrophages also migrate in the intima and SMC layers.
11. They keep phagocytizing ox-LDL and become foam cells.
12. Macrophages are also cytotoxic for SMCs.
13. Ox-LDLs are chemotactic for monocytes and T-lymphocytes.
15. Macrophages and foam cells contribute to the production of ROS.
16. Ox-LDL upregulates Cav-1 and the complex between Cav-1 and eNOS inactivates e-NOS.
17. C-reactive protein is a biomarker of inflammation that is a common denominator in cell diseases related to atherosclerosis. It downregulates eNOS (step 18) and increases the production of ROS (step 19).
18. It stimulates macrophages and contributes to the activation of INOS.
19. INOS can also be activated by bacterial lipopolysaccharides (LPS) and cytokines such as IFN-γ, IL-1β, IL-12, and IL-18 and inhibited by IL-10.
20. Activation of INOS leads to a massive production of NO.
21. ROS can oxidize BH₄ into BH₂. BH₂ regulates eNOS and the resulting product is O₂⁻ instead of NO.
22. O₂⁻ can react with NO to form peroxynitrite (ONOO⁻).
23. ROS can also react directly with NO and amplify the production of reactive radicals ONOO⁻.
24. eNOS and uncoupled mitochondrial respiration products also contribute to ONOO⁻ formation.
25. By inhibiting NADPH and inducing production of O₂⁻, NADPH oxidase contributes to the production of ONOO⁻.
26. XO and uncoupled mitochondrial respiration products also contribute to ONOO⁻ formation.
27. By inhibiting NADPH and inducing production of O₂⁻, NADPH oxidase contributes to the production of ONOO⁻.
28. eNOS plays an important protecting role by reacting with O₂⁻ and producing hydrogen peroxide (H₂O₂).
The induction of vascular cell adhesion molecules (VCAM-1) and nuclear leukocyte adhesion to the endothelium through MCP-1 and growth factors involved in the stimulation of the ECs and SMCs to secrete monocyte chemotactic protein-1 (MCP-1) and intercellular cell adhesion molecules (ICAM-1). 

Monocyte-derived macrophages internalize ox-LDL through scavenger receptors. As these receptors are not downregulated by the intracellular cholesterol level, massive cholesterol accumulation occurs and the macrophages transform to foam cells, which are continuously recruited by the vessel wall to remove the lipoprotein particles that have invaded. Ox-LDL cholesterol increases the synthesis of caveolin-1 (Cav-1; the principal structural protein in caveolae that binds cholesterol), which inhibits production of NO by inactivating endothelial NO synthase (eNOS). Conversely, normal release of NO prevents oxidative modification of LDL cholesterol (Figure 1, steps 1–16).

Three NO synthase (NOS) isoforms have been identified and named after the site of their initial isolation. The neuronal synthase (nNOS or Type I) and eNOS (Type III) are constitutively expressed and synthesize NO in response to increased calcium. The high output of the third isoform NO synthase (iNOS or Type II) may be induced in selected tissues in response to a range of inflammatory mediators and its activity is functionally independent of calcium. It is not a normal constituent of healthy cells but is thought to be expressed in response to pro-inflammatory signals as part of an innate host defense mechanism.

Under normal conditions, low concentrations of NO are continuously involved in a variety of physiological functions, which include the regulation of blood pressure and flow and platelet aggregability. Under pathological conditions, however, high concentrations of NO, which are generated for potential anti-bacterial, anti-parasitic, tumoricidal, and anti-viral activities, may be detrimental to tissues, for example, in endotoxic shock and some immunological and degenerative diseases.

In 1986, it was reported that acetylcholine induced vasodilation in the coronary vessels of healthy volunteers, but not in patients with angiographic evidence of atherosclerosis. Since then, it has been clearly shown that in atherosclerosis there is impaired generation of NO in the vascular endothelium, leading to vascular dysfunction. This probably co-exists with the expression of iNOS in the plaque, generating excessive amounts of NO and leading to an interaction with oxygen-derived radicals, generation of ONOO−, and further impaired vascular function.

In mice lacking the LDL receptor, an animal model of familial hypercholesterolaemia, inhibition of endothelial NO production accelerates atherosclerotic lesion formation, whereas L-arginine (the amino-acid precursor of NO) treatment decreases lesion development. Moreover, it has been demonstrated that specific removal of eNOS from mice prone to develop atherosclerosis, i.e. the apolipoprotein E (apoE)-knockout mouse model, resulted in a marked acceleration of atherosclerotic lesion formation in the aorta and in the coronary arteries.

In recent years, atherosclerosis has come to be recognized as active and inflammatory, rather than simply a passive process of lipid infiltration. The inflammatory immune system is strongly involved in the development of fatty streaks. Hypercholesterolaemic mice deficient either in monocyte-macrophages or in mature B- and T-lymphocytes (RAG-2 gene deficient), develop 10- and two-fold less fatty streaks than control mice, respectively. Mice deficient in various cytokines have demonstrated the aggravating role of pro-inflammatory cytokines, such as interferon-γ (IFN-γ) and interleukins (IL-1, IL-12, and IL-18), and the protective role of anti-inflammatory cytokines (mainly IL-10), in the development of the atherosclerotic process.

Macrophages and monocytes can produce excess NO through iNOS. In NOS is calcium-independent and is stimulated by cytokines such as IFN-γ and IL-1β. iNOS-derived NO plays an important role in numerous pathophysiological conditions (e.g. inflammation) It has also been reported to inhibit proliferation and to induce apoptotic cell death in SMCs and to activate matrix metalloproteinases.

The adhesion of leukocytes to the endothelium is a critical step in the initiation and the development of fatty streaks. Leukocytes first undergo an interaction between the selectin and the selectin ligand, which allows the cells to roll along the endothelial surface. Leukocyte chemokine receptors thereby come into contact with chemokines displayed by the endothelium, leading to activation of integrins. This is necessary for subsequent firm adhesion, through adhesion molecules of the immunoglobulin superfamily such as ICAM-1 and VCAM-1. This process is followed by the leukocyte migration from the vasculature to the subendothelial space.

Increased leukocyte–EC interactions have been observed in eNOS-deficient mice. At a molecular level, inhibition of eNOS results in increased expression of leukocyte-adhesion molecules and critical chemokines, such as MCP-1, which is thought to be responsible for the migration of monocytes into the intima at sites of atherosclerotic lesion formation. Conversely, NOS gene therapy rapidly reduces hypercholesterolaemia-induced leukocyte-adhesion molecule expression (VCAM-1) and ameliorates monocyte infiltration into the arterial wall of cholesterol-fed rabbits.

Endothelial dysfunction and nitric oxide

Over the last two decades, it has become evident that the endothelium is not merely an inert, single-cell lining covering the internal surface of blood vessels, but in fact plays a crucial role in regulating vascular function.

As the major regulator of vascular homeostasis, the endothelium maintains the balance between vasodilatation and vasoconstriction, inhibition and stimulation of SMCs proliferation and migration, and thrombogenesis and fibrinolysis. Endothelial dysfunction leads to the disruption of this balance and causes damage to the arterial wall. Endothelial dysfunction is considered as an early indication of atherosclerosis, preceding angiographic or ultrasonic evidence of atherosclerotic plaque. This has been demonstrated in acetylcholine- or serotonin-induced vasodilation tests or through the measurement of flow-mediated dilation. Endothelial dysfunction was detected at both
the conduit and microvascular levels in patients with coronary risk factors, but no angiographic or ultrasound evidence of structural coronary artery disease (CAD).\textsuperscript{41-43} These results confirm that endothelial dysfunction is present in the pre-clinical stage of atherosclerosis. Endothelium dysfunction of the microvasculature has also been associated with exercise-induced myocardial ischaemia in patients without haemodynamically significant CAD of the epicardial arteries, suggesting that endothelial dysfunction of the microcirculation may contribute to ischaemia when myocardial oxygen demand is increased.\textsuperscript{44}

Many of the traditional coronary risk factors that predispose a person to the development of atherosclerosis, such as hypercholesterolaemia, hypertension, smoking, diabetes, and a positive family history of premature CAD, are also associated with endothelial dysfunction.\textsuperscript{45,46} In a study in which vasodilatation was evaluated using plethysmography of forearm blood flow in response to acetylecholine (endothelium-dependent) and sodium nitroprusside (endothelium-independent), patients who experienced cardiovascular events over a mean follow-up of 4.5 years showed impaired vasodilator responses.\textsuperscript{47}

A defect in NO production or activity has been proposed to be a major mechanism of endothelial dysfunction and a contributor to atherosclerosis.\textsuperscript{48} Impaired production or activity of NO leads to events or to actions such as vasoconstriction, platelet aggregation, SMCs proliferation and migration, leukocyte adhesion, and oxidative stress.\textsuperscript{49} However, endothelial dysfunction is likely to be a multifactorial process. There is accumulating evidence that increased vascular production of reactive oxygen species (ROS) plays an important role (Figure 1, steps 18–28). Increased vascular production of superoxide anion has been demonstrated in all major conditions predisposing to atherosclerosis.\textsuperscript{50–53} For example, there is increased production of superoxide anion in coronary arteries in patients with CAD.\textsuperscript{54} In particular, superoxide anion reacts rapidly with NO, resulting in the formation of the highly reactive and cytotoxic ONOO\textsuperscript{-} and loss of the bioactivity of NO. Increased vascular production of ROS promotes the oxidative degradation of the critical eNOS cofactor tetrahydrobiopterin (BH\textsubscript{4}), leading to eNOS uncoupling and the consequent reduced production of NO and the increased production of superoxide anion from the enzyme.\textsuperscript{55–57} There is indirect evidence to suggest that eNOS uncoupling contributes to endothelial dysfunction and increased production of superoxide anion in the oxidant stress of ischaemia/reperfusion injury,\textsuperscript{58} hypercholesterolaemia,\textsuperscript{59} hypertension,\textsuperscript{60} diabetes,\textsuperscript{61} and heart failure.\textsuperscript{62}

The nicotinamide adenine dinucleotide phosphate NAD(P)H oxidase has been identified as an important vascular source of superoxide anion. This enzyme is stimulated by pro-atherosclerotic stimuli such as angiotensin II, mechanical stretch, and pro-inflammatory cytokines.\textsuperscript{63,64} Enhanced NAD(P)H oxidase protein subunit levels have been found in human vascular endothelium in atherosclerosis,\textsuperscript{65} hypertension,\textsuperscript{66} and diabetes\textsuperscript{67} in association with increased production of superoxide anion. Recent data have demonstrated that the coronary activity of the NAD(P)H oxidase is significantly increased in patients with coronary disease.\textsuperscript{68}

Another potential vascular source of superoxide anion is xanthine oxidase (XO).\textsuperscript{69} In patients with coronary disease, increased activity of coronary and endothelium-bound XO activity has recently been observed. This was inversely related to endothelium-dependent vasodilation, suggesting that increased activity of this enzyme contributes to endothelial dysfunction.\textsuperscript{67}

Vascular levels of superoxide anion and bioactivity of NO are determined not only by the rate of superoxide anion production but also by its rate of degradation. The major superoxide anion-degrading enzyme system is superoxide dismutase (SOD); the extracellular form of SOD (ecSOD) is of particular interest in the vessel wall because it is highly expressed and strategically located in the extracellular space around vascular SMCs.\textsuperscript{70,71} In coronary arteries from patients with coronary disease, ecSOD activity has been shown to be profoundly reduced. Furthermore, in patients with coronary disease, endothelium-bound ecSOD activity was shown to be reduced and closely related to impaired endothelium-dependent, NO-mediated vasodilation, suggesting that reduced ecSOD activity may contribute to endothelial dysfunction (Figure 1, steps 18–28).\textsuperscript{72}

In patients with CAD, endothelium-dependent vasomotion at the forearm is related to serum levels of a systemic marker of inflammation, the high-sensitive C-reactive protein (hs-C-reactive protein). Recent studies suggest that C-reactive protein, besides being a marker of inflammation, may also directly contribute to endothelial dysfunction. Exposure of ECs to C-reactive protein decreases endothelial NO production and downregulates eNOS expression due to decreased stability of eNOS mRNA.\textsuperscript{19,73,74} Numerous studies from various parts of the world have clearly established that C-reactive protein predicts future risk for cardiovascular disease in apparently healthy persons, independently of established risk factors in the majority of studies.\textsuperscript{75,76} These studies clearly support a role of C-reactive protein in atherogenesis.

Potential therapeutic effects of NO donors and enhancers of NO availability

Enhancers of NO availability

Statins

The statins are a group of compounds which lower LDL cholesterol by inhibiting the enzyme 3-hydroxy-3-methylglutaryl co-enzyme A. These drugs improve vascular relaxation, reduce vascular inflammation, reduce oxidative stress, decrease thrombosis and platelet aggregation, decrease adhesion of platelets and white cells to the vascular endothelium, stabilize vulnerable plaques, and promote new vessel formation.\textsuperscript{52,77} These beneficial effects of statins are, in part, mediated by an effect on eNOS because they can be blocked by L-NMMA, an inhibitor of eNOS,\textsuperscript{45,78} or are absent in eNOS deficient mice.\textsuperscript{79}

Simvastatin has been shown to prevent the decreased bioavailability of endothelium-derived NO and downregulation of endothelial eNOS that result from elevated levels of native LDL.\textsuperscript{80} Elevated LDL reduces NO production, in part, by increasing the interaction between Cav-1 and eNOS. Atorvastatin has been shown to reduce Cav-1 expression in ECs by inhibiting the interaction between Cav-1 and eNOS, resulting in increased NO production.\textsuperscript{81,82}

In addition, statins block rac 1 isoprenylation,\textsuperscript{83} an important component of NAD(P)H oxidase complex, thus reducing...
the expression of NAD(P)H oxidase subunits.\textsuperscript{84} This results in an inhibition of endothelial production of superoxide anion, and a shift in the balance between NO and the free radical, thus improving endothelial function. A further proposed mechanism for the increase in eNOS expression with statin therapy is an increased stability of eNOS messenger RNA, which would permit preservation of eNOS expression in the presence of ox-LDL.\textsuperscript{85}

Augmented endothelium-dependent dilation has been noted in the forearm of healthy normocholesterolemic men after only 1 day of high-dose atorvastatin (80 mg), even before appreciable reduction in plasma LDL cholesterol or in C-reactive protein could be detected. This rapid increase in dilation is consistent with a cholesterol-independent effect of statins.\textsuperscript{86} In the RECIFE (Reduction of Cholesterol in Ischaemia and Function of the Endothelium) trial, 6 weeks of pravastatin therapy (40 mg/day) rapidly increased flow-mediated dilation compared with placebo in patients with acute coronary syndromes. Changes in flow-mediated dilation were not correlated with decreases in total and LDL cholesterol, suggesting that the improvement in endothelial function was not related to the lipid-lowering effects of the statin.\textsuperscript{87} Statin therapy also reduces circulating levels of the adhesion molecules P-selectin, E-selectin, and ICAM-1 in hypercholesterolemic and CAD patients\textsuperscript{88,89}. This reduction has been shown to be associated with an increase in levels of NO.\textsuperscript{90}

Furthermore, treatment with high doses of statin (80 mg atorvastatin) has been shown to decrease significantly hs-C-reactive protein and fibrinogen in parallel with an increase in NO in patients with CAD. Conventional treatment with 20 mg simvastatin, while significantly decreasing lipid concentrations, was unable to achieve this effect on inflammatory markers.\textsuperscript{91}

Angiotensin-converting enzyme inhibitors

Animal and human studies indicate that angiotensin-converting enzyme (ACE)-inhibition, leading to a decrease in the stimulation of angiotensin II type I receptors (AT-1), can improve endothelial dysfunction and oppose early atherosclerosis. These effects are attributable to the attenuation of the superoxide anion-generating effects of angiotensin II and to the enhanced endothelial release of NO, secondary to diminished breakdown of bradykinin and related kinins.\textsuperscript{8,52} Indeed, the AT-1 receptor mediates classical angiotensin II effects such as vascular contraction, SMC hypertrophy, extracellular matrix synthesis, increased platelet aggregation, monocyte adhesion and activation, and release of inflammatory cytokines. These events are crucial steps both in atherosclerosis and in the control of vascular homeostasis.\textsuperscript{52} Some effects of angiotensin II are mediated by ROS; for example, angiotensin II is a potent stimulus for the activation of NAD(P)H oxidase\textsuperscript{63} and ACE-inhibitors potently decrease NAD(P)H oxidase activity.\textsuperscript{52,92}

Several clinical studies have shown cardiac ischemic events to be prevented by ACE-inhibition in patients with a history of acute myocardial infarction (MI).\textsuperscript{93-96} However, a quantitative coronary angiography study in patients with normal left ventricular function could not demonstrate any effect of quinapril on stenosis progression or new stenosis development when compared with placebo treatment.\textsuperscript{97}

Though the improved EC function by ACE-inhibition may help to explain the beneficial effects of ACE-inhibitors in reducing the number of ischemic events in patients with left ventricular dysfunction,\textsuperscript{94} their clinical value as anti-atherosclerotic agents remains controversial, with both positive\textsuperscript{96,99} and negative\textsuperscript{100} results obtained in recent randomized placebo- or reference-controlled clinical trials.

**L-Arginine**

The beneficial effects of supplementation with L-arginine, the biochemical precursor of NO, have been documented both in animals and in humans in several conditions, including hypercholesterolaemia, hypertension, CAD, and diabetes.\textsuperscript{101-103} In a recent study investigating the extent of lymphocyte activation and anti-ox-LDL antibodies in patients with unstable angina pectoris undergoing percutaneous transluminal coronary angioplasty (PTCA) with stent placement, L-arginine has been shown to attenuate the systemic rise in peripheral lymphocyte activation, to limit oxidative stress markers induced by vessel wall injury, and to decrease anti-ox-LDL antibody levels.\textsuperscript{104}

Although the way in which L-arginine influences the synthesis of NO and alters the oxidant status of the atherosclerotic blood vessel is not well understood, it has been suggested that the beneficial effects of L-arginine administration are partially caused by a competition of this amino acid with the derivative asymmetrical dimethyl-L-arginine (ADMA), which is an endogenous inhibitor of eNOS activity.\textsuperscript{105,106} However, in men with stable angina, the increase in plasma L-arginine/ADMA ratio after 2 weeks oral supplementation with L-arginine was not associated with an improvement in endothelium-dependent vasodilation, oxidative stress, or exercise performance.\textsuperscript{107}

Since ECs contain L-arginine in concentrations thousand times greater (millimolar range) than those required for the activity of eNOS (micromolar range), it is unlikely that a lack of L-arginine accounts for reduced production of NO in cardiovascular disorders. However, it has been argued that native ECs in vivo are continuously exposed to hormonal and mechanical stimuli that might lead to relative intracellular deficiency in L-arginine, especially in the close proximity of eNOS.\textsuperscript{108}

Short-term effects are beneficial but long-term administration reveals that the effects are not sustained.\textsuperscript{109} Indeed, a recent study has even shown functional and biochemical evidences for an increased superoxide anion production in atherosclerotic aortas from hypercholesterolemic rabbits treated with L-arginine for 12 weeks.\textsuperscript{110}

**Tetrahydrobiopterin**

Tetrahydrobiopterin (BH\textsubscript{4}) is critical for eNOS activity. When ECs are in the presence of sub-optimal concentrations of BH\textsubscript{4}, eNOS generates superoxide anion instead of NO.\textsuperscript{111} Administration of BH\textsubscript{4} has been shown to improve endothelium-dependent relaxation in patients with hypercholesterolaemia\textsuperscript{112} and type II diabetes\textsuperscript{113,114} as well as in smokers.\textsuperscript{115} However, the benefit of such treatment remains uncertain in vascular disease states in which oxidative stress is much more pronounced\textsuperscript{56,116} such as in atherosclerosis. Indeed, in a recent placebo-controlled study testing the influence of BH\textsubscript{4} on insulin sensitivity index and flow-mediated dilation in type II diabetic patients suffering from CAD, it has been shown that BH\textsubscript{4} was able to increase insulin sensitivity without any discernable
improvement in endothelial function, i.e. no change in brachial artery diameter.117

NO donors

Because NO elicits protective and beneficial actions in various disease states, direct delivery of NO is expected to be effective in the prevention and/or treatment of essential hypertension, stroke, CAD, vascular complications of diabetes, and other disorders involving the vascular system. On the other hand, as previously mentioned, NO is known to have both beneficial and deleterious actions. For example, it reacts with the superoxide anion that is produced by activated macrophages and other cells, to form ONOO. For this reason, the tissue levels of NO need to be well controlled to obtain its therapeutic benefits.118

The pathways leading to NO formation differ significantly among individual NO-donor classes, as do their chemical reactivities. Some compounds require enzymatic catalysis, whereas others produce NO non-enzymatically. Some NO donors require interaction with thiols to release NO, some have to undergo reduction, and others oxidation. A further complication is the formation of different end products during decomposition or metabolism.119

Indirect NO donors

Nitrovasodilators, such as organic nitrates [nitroglycerin (NTG), isosorbide mononitrate (ISMN), and isosorbide dinitrate (ISDN)], have been used as therapeutic agents for over a century. These compounds have been administered successfully in the treatment of symptomatic CAD and hypertension, and evidence suggests that they offer benefit in the management of vascular disorders characterized by endothelial dysfunction and NO deficiency.120

Although studies on the effect of chronic administration of organic nitrates on the progression of atherosclerosis are very limited, long-term treatment of hypercholesterolaemic rabbits with a low dose of pentaerythritol tetranitrate (6 mg/kg/day for 16 weeks) has been shown to reduce the progression of lesion formation, endothelial dysfunction, and LDL-oxidation.121

Continuous transdermal administration of NTG has been found to be associated with increased vascular production of superoxide anion and endothelial dysfunction. In contrast, it was unclear whether vascular production of superoxide anion increased during eccentric administration of oral nitrates, which is a widely used therapeutic dosing regimen. However, recent data suggest that eccentric ISMN (200 mg/kg/day during 16 weeks) can decrease superoxide anion concentrations and partially prevent intimal lesion formation and endothelial dysfunction in hypercholesterolaemic rabbits.122,123

A major limitation of the use of nitrates is the rapid development of tolerance during sustained therapy. Several hypotheses have been proposed to explain this loss of haemodynamic and anti-anginal efficacy but the exact mechanism of tolerance remains unresolved.124 Two hypotheses are frequently evoked. The free radical hypothesis suggests that nitrate tolerance is caused by an increased production of superoxide anion by the endothelium during nitrate therapy. Inactivation of the NO released from organic nitrates by the superoxide anion would then result in the loss of responsiveness to nitrates. According to the second hypothesis, tissue sulphydryl groups (−SH) are required for the expression of vasodilator action of organic nitrates, possibly in order to react with the nitrates to liberate NO from the intermediate S-nitrosothiols that are formed. Repeated administration of relatively large doses of NTG would lead to the depletion or to the oxidation of tissue thiols, resulting in a gradual attenuation of the action of NTG.

Nitroglycerin, when administered intra-arterially for 20 min at a dose that does not affect resting forearm blood flow (1 nm), specifically increased the vasodilator response to intra-arterial administration of acetylcholine in patients with congestive heart failure but not in normal subjects. The vasodilator response to acetylcholine was consistently enhanced by low-dose NTG throughout a 12 h period.125 Furthermore, in a recent study, the addition of a fixed dose of ISDN plus hydralazine to standard therapy for heart failure including neurohormonal blockers was found efficacious and increased survival (10.2 vs. 6.2% mortality in the placebo and ISDN + hydralazine groups, respectively) among 1050 black patients with advanced heart failure (NYHA Class III or IV).126

In contrast, in the ISIS-4 (Fourth International Study of Infarct Survival) placebo-controlled study, there was no significant reduction in 5 week mortality in patients with acute MI receiving oral controlled-release mononitrate (30–60 mg once daily) during 1 month when compared with patients receiving placebo. Further follow-up did not indicate any later survival advantage.95 Furthermore, chronic administration of long-acting nitrates in patients with a healed MI resulted in an increased number of patients with fatal and non-fatal cardiac events during a 18 month observation period.127 These results were in agreement with the increased risk of cardiac death in patients with CAD treated with long-acting nitrates during the chronic phase of the disease.128 However, due to shortcomings in both studies, the association of an increased risk of cardiac death and the use of long-acting nitrates is controversial and randomized controlled trials are necessary to resolve this important issue.129

Direct NO donors

Nicorandil. Nicorandil is a nicotinamide ester with a dual mechanism of action. Its distinctive pharmacological effect is to open ATP-sensitive potassium channels (K\textsubscript{ATP}), thereby dilating peripheral and coronary resistance arterioles; but it also possesses a nitrate moiety, which dilates systemic veins and epicardial coronary arteries.130 Thus nicorandil increases coronary blood flow, reduces pre-load and after-load,131–133 and has an anti-anginal efficacy and safety profile similar to that of organic nitrates.134

In 44 patients with angina who underwent PTCA, nicorandil pretreatment resulted in the induction of myocardial pre-conditioning, independently of the severity of ischaemia. The same effect was not observed with ISDN, proving that the opening of K\textsubscript{ATP} channels plays an important role in the protecting effect of nicorandil.135,136

In addition to its anti-ischaemic effects, nicorandil is thought to have cardioprotective properties. The IONA (Impact Of Nicorandil in Angina) placebo-controlled trial in patients with stable angina showed a significant improvement in outcome due to a reduction in major coronary events during a mean follow-up period of 1.6 years.137
Recently, nicorandil has been shown to affect fibrinolysis in 11 patients with CAD. There were no significant changes in the plasma concentrations of tissue-type plasminogen activator or type-1 plasminogen activator inhibitor (PAI-1) antigens after oral administration of nicorandil for 2 weeks. However, the plasma activity of PAI decreased significantly after the treatment. This finding suggests that nicorandil improves the fibrinolytic capacity and may reduce the risk of coronary thrombus formation in such patients.\textsuperscript{18}

**Sydnonimines.** The most studied representative of the heterocyclic direct NO donors is molsidomine, a sydnonimine that has been used since the 1970s in several European countries in the treatment of stable angina pectoris.\textsuperscript{139–143} Although molsidomine itself is only poorly vasoactive in vitro, SIN-1 (the active metabolite of molsidomine) is a potent vasorelaxant and anti-platelet aggregating agent. These activities are thought to be mediated largely by the release of NO. Activation of soluble guanylate cyclase by sydnonimines is independent of the presence of thiols.\textsuperscript{144} Being thiol-independent, molsidomine and SIN-1 do not cause tolerance and are not cross-tolerant to organic nitrates.\textsuperscript{145}

A few studies have looked directly at the effect of NO or NO donors on the prevention of intimal hyperplasia in humans, including ACCORD (Angioplastie Coronaire Corvasal Diltiazem), in which 700 patients undergoing elective PTCA were randomized to receive either an infusion of SIN-1 followed by oral molsidomine or calcium channel blockers, for 6 months. Although no improvement was documented in clinical outcomes such as death, non-fatal MI, or need for repeat procedures, there was a reduction in the rate of restenosis (>50% stenosis) from 47 to 38%, in patients who received the NO donors.\textsuperscript{146}

Flow-mediated dilation of human blood vessels is essential to adaptation and regulation of peripheral blood flow and is mediated by endogenous NO. Computerized ultrasonography was used in a randomized double-blind placebo-controlled study to measure diastolic diameters of the brachial artery before and after hyperaemia in two groups of 10 patients with CAD. Each group received orally either placebo or 12 mg molsidomine a day for 48 h. In the molsidomine group, flow-mediated dilation was improved with a 60% increase after the first intake and a less pronounced increase was observed after the last intake. A significant increase in diastolic diameter was observed after the last molsidomine intake, but not after the first one. Thus, it appears that molsidomine has an early positive effect on flow-mediated dilation in addition to a delayed vasodilator effect. Improvement of endothelial dysfunction by molsidomine in patients with CAD might uncover new therapeutic perspectives in the use of NO donors.\textsuperscript{147}

Although the haemodynamic effects of molsidomine and SIN-1 are well recognized, there are only a few studies having explored their potential therapeutic benefits in the prevention and/or treatment of atherosclerosis. Treatment of cholesterol-fed rabbits with molsidomine (1 mg/kg/day) during cholesterol withdrawal did not affect plaque size but increased the thickness of the subendothelial macrophage-free layer consisting of SMCs and normalized superoxide formation and SOD mRNA expression. This demonstrates that molsidomine can decrease signs of oxidative stress and can increase the features of stable atherosclerotic plaques.\textsuperscript{148}

Monocyte adhesion to vascular endothelium is a crucial step in the early stages of atherosclerosis, which may be mediated by the interaction with adhesion molecules expressed on the surface of ECs. In vitro, IL-1β markedly increases the expression of ICAM-1 and VCAM-1. This effect is antagonized by SIN-1 in a dose-dependent manner. This action of SIN-1 was abolished in the presence of a scavenger of NO, such as haemoglobin.\textsuperscript{149} In apoE-knockout mice\textsuperscript{150} and in clinical studies,\textsuperscript{151} the level of soluble ICAM-1 correlates with the degree of atherosclerosis and is suggested to be an appropriate biomarker reflecting the development of atherosclerosis.\textsuperscript{152} Therefore, lowering of soluble ICAM-1 levels might be beneficial, since in atherosclerotic mice models, a deficiency in ICAM-1 was shown to protect substantially against the progression of atherosclerosis.\textsuperscript{153,154} A recent review focusing on the relationship between adhesion molecules and atherosclerosis concluded that the levels of adhesion molecules might correlate with clinical risk and serve as therapeutic targets.\textsuperscript{155}

Patients with stable angina pectoris (n = 172) were treated daily for 1 year with molsidomine (16 mg once-a-day Geomatrix formulation). After 4 weeks of treatment, angina attacks and frequency of consumption of sublingual ISDN were significantly reduced without altering soluble ICAM-1 levels when compared with baseline values. The anti-anginal effect of molsidomine was sustained and even improved during the following year of treatment and a significant decrease of 10% was measured in soluble ICAM-1 levels. When the soluble ICAM-1 changes during the 1 year treatment period were distributed in four quartiles, it was demonstrated that the decrease in sublingual ISDN consumption between the start and the end of the study was most pronounced in the group with the largest decrease in soluble ICAM-1. The results of this open study may indicate that molsidomine, in addition to its anti-anginal function, promotes a less activated state of the endothelium and thereby may modulate the progression of atherosclerosis in patients with stable angina.\textsuperscript{156} However, further randomized and controlled studies are needed to confirm these preliminary results.

**Conclusion**

The vascular endothelium and its product NO are key regulators of vascular health. Reduced bioavailability of NO is involved in the initiation, progression, and complications of atherosclerosis. This pathology is the major cause of death and disability in the United States, Europe, and much of Asia. Elucidating the precise mechanisms by which NO elicits protective effects in atherogenesis will directly impact on the successful development of NO-based therapies. Appropriate treatment of vascular inflammation by direct or indirect NO donors, enhancement of the action of NO, and/or scavenging of ROS should be further explored for prevention of atherosclerosis. In this respect, new technologies such as controlled, slow, and regular release of NO from prolonged-release NO-donor tablets appear to be promising strategies.

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