Review Article

Nitric oxide synthesis in atherosclerosis

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Introduction

In 1980 Furchgott and Zawadzki[1] discovered that acetylcholine-induced relaxation of rabbit aortic strips required an intact endothelium. They found that stimulation of muscarinic receptors on the endothelium led to the release of a non-prostanoid relaxing factor which they named endothelium-derived relaxing factor. Further studies[2–10] have established the importance of endothelium-derived relaxing factor in human coronary arteries.

Endothelium derived relaxing factor

Endothelium-derived relaxing factor can be released in response to a variety of stimuli, including shear stress and pressure, aggregating platelets, thrombin, adenosine diphosphate (ADP), serotonin, bradykinin, histamine, norepinephrine, vasopressin, substance P, and acetylcholine[11–13]. Endothelium-derived relaxing factor has been shown to stimulate guanylate cyclase thus increasing cyclic GMP which mediates smooth muscle cell relaxation[14,15].

In certain circumstances, the endothelium may release factors which cause vasoconstriction. Endothelin is a potent vasoconstrictor peptide[16] which may cause prolonged contraction of vascular smooth muscle[17,18]. Whether the endothelium-derived contracting factor described in hypoxic canine arteries is endothelin is uncertain[19]. Moreover, endothelial cells can activate a cyclooxygenase-dependent pathway and produce endothelium-derived contracting factors[20] that are very likely to be endoperoxides such as thromboxane A₂ or prostacyclin H₂[21]. High local concentrations of angiotensin II may also be produced by the endothelium[22]. Thus, in pathological states these vasoconstrictor compounds could override the normal vasomotor tone associated with endothelium-dependent vasodilatation maintained by autoregulation, sympathetic innervation and endothelium-derived relaxing factor[23].

The nature of endothelium-derived relaxing factor

The first proof that nitric oxide was at least one of the relaxing factors resulted from studies by Palmer et al.[31] who, using a chemiluminescence assay, demonstrated that cultured endothelial cells release nitric oxide when exposed to bradykinin.

Nitric oxide is formed from the N-guanido terminal of the amino acid L-arginine and from molecular oxygen by nitric oxide synthase enzymes[24]. One of these enzymes is Ca²⁺-dependent and is constitutive in various types of cells, including endothelial cells[25]. Nitric oxide synthesized by constitutive nitric oxide synthase (iNOS), plays a primary role in the regulation of blood pressure[26]. A nower type of nitric oxide synthase (iNOS) is Ca²⁺-independent and inducible by immunological stimuli[27]. Endocardial endothelial cells express the constitutive form of nitric oxide synthase and this enzyme is known to modulate myocardial contraction and coronary tone[27]. The inducible form of nitric oxide synthase is located in myocytes and endothelial cells[28] and may be involved in the depression of myocardial contractility in septic shock[29].

In the intact blood vessel wall, most of the nitric oxide is presumed to arise from the activity of endothelial cNOS[30]. There is continuous basal release of nitric oxide (constitutive) which represents a sizeable portion of the total nitric oxide-releasing capacity of native endothelial cells. The rate of nitric oxide formation under basal conditions seems to be substantially smaller in cultured endothelial cells, implying that native endothelial cells in vivo may be continuously exposed to stimuli, for example shear stress, which affect nitric oxide synthase expression[31]. Once the endothelium has been damaged, exposure of smooth muscle cells to cytokines and other stimulators of NOS induction may have important physiological consequences for the blood vessel. Medial smooth muscle cells exhibit a rapid proliferative response immediately after endothelial denudation, but this is followed by endothelial regrowth.


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The mechanisms of action of nitric oxide production and activity have been examined in considerable detail\cite{32,13}. Receptors on the endothelial cell membrane, for example serotonin receptors, interact with signal transduction proteins (the G protein family)\cite{32} to modulate the synthesis and release of nitric oxide\cite{33}. After release from the endothelium, nitric oxide diffuses across the extracellular space into the smooth muscle cell to activate soluble guanylate cyclase, leading to an increase in the intracellular concentration of cyclic GMP\cite{14,15}.

### Studies using stimulators and inhibitors of nitric oxide synthase

Stimuli causing nitric oxide production and release include receptor-dependent agonists such as substance-P, acetylcholine, ATP and bradykinin\cite{34}. The identification of N\textsuperscript{G}-monomethyl-L-arginine (LNMMA) as a competitive inhibitor of the enzyme nitric oxide synthase has provided an important tool to investigate the relevance of nitric oxide in biological processes\cite{35,36}. LNMMA causes constriction of various vascular beds and produces a hypertensive response in animals and humans\cite{36}. It has no direct intrinsic constrictor activity on vascular smooth muscle and does not affect other systems in the vascular wall. Its actions are entirely endothelium-dependent, and result from the inhibition of nitric oxide synthase\cite{35}.

Endothelial vasodilator dysfunction has been demonstrated in a wide range of conditions, including hypercholesterolaemia, atherosclerosis, hypertension, diabetes mellitus, heart failure and post-ischaemic reperfusion\cite{2,5,36-38}. The mechanisms underlying this vasodilator dysfunction are unclear. Because the actions of endothelium-derived relaxing factor are complex, abnormalities could occur at many sites: (1) impairment of endothelial membrane receptors that interact with agonists or physiological stimuli to release nitric oxide; (2) diminished levels of or impaired utilization of L-arginine, the substrate of nitric oxide synthase; (3) reduction in concentration or activity of nitric oxide synthase, the enzyme responsible for the conversion of L-arginine to nitric oxide; (4) impaired release of nitric oxide from the endothelium; (5) enhanced degradation of nitric oxide by oxygen free radicals; (6) impaired diffusion from endothelium to smooth muscle cell; (7) impaired interaction of nitric oxide with guanylate cyclase and the subsequent limitation of the increase in intracellular cyclic GMP level; (8) generalized decrease in smooth muscle cell sensitivity to vasodilators.

### Forearm vascular bed

Cholinergic agonists infused into the brachial artery are known to increase flow. Vallance et al.\cite{39} demonstrated that this response could be attenuated by the infusion of LNMMA, which suggests that it is mediated by nitric oxide production. Several studies have shown that nitric oxide production makes an important contribution to the resting vascular tone in the human forearm and that its production is impaired in several cardiovascular disease states.

#### Hypercholesterolemia

In hypercholesterolaemic patients there is impaired receptor-dependent and flow-mediated endothelium-dependent vascular relaxation, but the relaxing effect of nitroglycerin is generally preserved\cite{40}. The role of nitric oxide in the regulation of vascular tone by the endothelium is therefore diminished\cite{41}. Impaired endothelium-dependent vascular relaxation was found in hypercholesterolaemic patients\cite{41}. Studies have suggested that depletion of L-arginine is responsible for this endothelial dysfunction. Accordingly, Creager et al.\cite{42,43} have demonstrated that an infusion of L-arginine improves the forearm blood flow responses to meta-choline in hypercholesterolaemic patients. The cause of the impaired endothelium-dependent vascular relaxation may be due to increased levels of intracellular superoxide inactivating the nitric oxide more rapidly\cite{44,45}.

#### Hypertension

Several abnormalities of endothelial regulation of vascular tone have been documented in genetic and experimental models of hypertension. In patients with essential hypertension endothelium-dependent relaxation is decreased, but endothelium-independent responses have been shown to remain intact\cite{51}. The response of forearm blood flow to N\textsuperscript{G}-monomethyl-L-arginine is also reduced in these patients suggesting that impaired generation of nitric oxide may contribute to their hypertension\cite{5,46,47}. This abnormality of endothelial function in patients with essential hypertension is not restricted to agonists of a single surface receptor type and it is not corrected by increasing the availability of substrate for nitric oxide synthase\cite{43,48}.

Vanhoutte et al.\cite{49} suggest that the generation of an endothelium-dependent contracting factor by endothelial cells diminishes the endothelium-dependent dilator responses. Indeed, several investigators have now reported elevation of plasma endothelin concentrations in human hypertension\cite{50,51}. This abnormal endothelial function, with an imbalance between endothelium-dependent relaxation and contraction, could contribute to the abnormal vascular responses with increased peripheral vascular resistance that is the central haemodynamic abnormality in hypertension.

Treatment with inhibitors of nitric oxide synthesis induces a hypertensive response in animals accompanied by a decrease in the excretion of sodium, which may become irreversible because of glomerular damage\cite{52,53}. L-arginine treatment prevents the development of hypertension in animals prone to this disease\cite{54} and also causes a rapid reduction in systolic and diastolic pressures when infused into healthy humans and patients with essential hypertension\cite{55,56}.
Smoking
Cigarette smoking is an important risk factor for the development of atherosclerosis and has important effects on endothelial prostacyclin production and on vessel wall-platelet interactions. Studies have shown impaired flow-mediated forearm vasodilation in clinically healthy smokers. It is dose-related and consistent with endothelial dysfunction. Short- and long-term smoking did not affect the response to direct vascular muscle stimulation by nitroglycerin or sodium nitroprusside. A recent study has shown that long-term smoking is associated with a diminished nitric oxide dependent component of basal forearm vascular tone and an impaired endothelium-dependent vasodilator response to low-dose endothelin-1. Short-term smoking enhances endothelin-1 induced vasoconstriction.

Ageing
Advancing age is associated with a derangement of endothelial cells that leads to a decrease of endothelium-derived relaxing factor. A recent study showed a blunted response to acetylcholine with advancing age in normotensive control subjects. The mechanisms of this age-associated impairment of endothelial function in humans is unclear but a decrease in nitric oxide release has been proposed and production of endothelium derived contracting factors has also been implicated.

Coronary arteries
Normal coronary arteries
Previous studies have indirectly suggested that the endothelium contributes to a large extent to the exercise-induced dilation of epicardial coronary arteries through a flow-mediated release of endothelium-derived relaxing factor. This vasodilation of large coronary arteries during exercise is converted to constriction after administration of LNMMA. A angiographic studies demonstrated that normal coronary arteries dilate in response to acetylcholine. However, it has been shown that patients with normal coronary arteries and risk factors for coronary artery disease also show generalized or localized constriction in response to acetylcholine, indicating areas of dysfunctional endothelium in apparently normal coronary arteries. Studies using inhibitors of nitric oxide and nitric oxide synthase have confirmed that nitric oxide mediates acetylcholine-induced coronary artery dilation. Demonstrated that elevated serum cholesterol, male sex, family history of coronary artery disease, and age predicted the response to acetylcholine in normal coronary arteries. The more risk factors present, the more likely were the coronary arteries to constrict.

Diseased coronary arteries
In 1986 Ludmer et al. demonstrated that intracoronary infusion of acetylcholine caused coronary vasoconstriction in humans with atherosclerotic coronary artery disease. Since this seminal observation, growing evidence suggests that atherosclerosis impairs endothelium-dependent coronary vasodilation in humans and thereby may predispose to vasoconstriction. A paradoxical vasoactive response that represents a fundamental defect in endothelial regulation of vascular tone. Healthy young adults with a family history of premature coronary disease may also have impaired endothelium-dependent dilation even in the absence of other cardiovascular risk factors.

Recent studies from our laboratory in patients with coronary disease showed no significant effect of LNMMA (4 to 16 µmol.min⁻¹) on proximal segments but constriction of distal segments and stenoses. This indicated preservation of basal nitric oxide production not only in the distal segments but at the sites of stenoses at (16 µmol.min⁻¹) and confirmed loss of basal nitric oxide production in the proximal segments. As in patients with normal arteriograms there was considerable individual variation in vasomotor response, particularly at 4 µmol.min⁻¹. As the coronary stenoses in these patients were invariably located in the proximal segments it would be reasonable to assume that the endothelium at the site of the stenosis was also dysfunctional or absent. It is likely, therefore, that the site of the basal nitric oxide production was the...
endothelium of the new microvessels in the wall of the artery around the atheromatous plaque which is a pathological characteristic of atheromatous stenosis. Such neoangiogenesis of atheromatous plaques is well documented and a recent study[75] showed the presence of inducible nitric oxide synthase in the neomicrovasculature. The response of the distal segments to L-arginine may contribute to the dysfunction seen in hypercholesterolaemia.[77] 

Hypercholesterolaemia

Hypercholesterolaemia is associated with impaired endothelium-dependent vasodilation of the peripheral and coronary vasculature in humans. Cholesterol-lowering therapy has been shown to significantly improve endothelium-mediated responses in the coronary arteries of patients with atherosclerosis.[78] Such improvements in the local regulation of coronary arterial tone could potentially relieve ischaemic symptoms and stabilize the atherosclerotic plaque.[78] Egashira et al.[40] found an impairment of the coronary blood flow response to acetylcholine in patients with hypercholesterolaemia. Six months after treatment with lipid lowering therapy the coronary blood flow response was significantly increased indicating reversible endothelial dysfunction. One study has shown an improvement in the large vessel vasomotor response to acetylcholine after L-arginine infusion, indicating that impaired availability or utilization of L-arginine may contribute to the dysfunction seen in hypercholesterolaemia.[43].

L-arginine administration has been reported to restore endothelium-dependent vasorelaxation in hypercholesterolaemic humans and animals[43,79,80] and to dilate coronary stenoses in patients with advanced atherosclerosis.[81]. Superoxide anions rapidly inactivate nitric oxide but do not inhibit its synthesis.[79]. In states in which excessive free radicals are generated, oxidized low density lipoprotein is produced which has been shown to be a potent inhibitor of endothelium dependent relaxation[62], but there is no evidence that they inhibit nitric oxide synthesis. Superoxide dismutase can attenuate the vasoconstricting effect of acetylcholine in the coronary arteries of patients with atherosclerosis[45] suggesting a role for oxygen free radicals in the inactivation of nitric oxide in vivo. A large burst of free radicals, generated from endothelial cells shortly after reperfusion, could inhibit nitric oxide synthesis[83,84]. Furthermore, adherent activated neutrophils may release more free radicals[83,84]. Monocyte adherence to endothelial cells in culture is inhibited by introduction of nitric oxide into the culture medium[85]. In addition, nitric oxide-abrogates the ability of macrophages to oxidize low density lipoprotein[96].

Smoking

Smoking causes immediate constriction of proximal and distal epicardial coronary arteries and a decrease in coronary blood flow in patients with coronary artery disease, despite an increase in myocardial oxygen demand.[87,88]. Studies have described impaired acetylcholine-mediated coronary vasodilation in clinically healthy smokers.

Ageing

Ageing is associated with marked structural and functional changes in the blood vessel wall. In blood vessels of rats, endothelium-dependent relaxation appears to be impaired in older animals.[90]. Vita et al.[63] showed that the constrictor response to intracoronary infusion of acetylcholine was independently associated with age.

M yocardial ischaemia and reperfusion

Recent studies[91] of myocardial ischaemia and reperfusion have shown marked coronary endothelial dysfunction characterized by reduced endothelium-derived relaxing factor release in response to endothelium-dependent dilators. The mechanisms underlying the loss of basal nitric oxide release after myocardial ischaemia and reperfusion are not clear. Depletion of L-arginine might occur after ischaemia and reperfusion. It is known that the normal cells can recycle L-citrulline, a coproduct of nitric oxide synthesis from L-arginine, to produce additional L-arginine, thus maintaining a high concentration of L-arginine in endothelial cells.[92]. Ischaemia followed by reperfusion may block the recycling of L-citrulline to L-arginine, thereby decreasing the L-arginine concentration in endothelial cells.

Coronary arterioles and other resistance vessels

Endothelium-dependent relaxation contributes to the control of vasomotor tone in coronary microvessels, although it is unclear whether this is mediated by nitric oxide. Effective endothelial mediated dilatation of the resistance coronary arteries may contribute to abnormal blood flow regulation.[2]. The uncoupling of resistance vessel tone from metabolic factors may represent an important mechanism through which impaired endothelial function might contribute to the development of myocardial ischaemia even in the early stages of coronary atherosclerosis without obstructive lesions in the epicardial segments.[2]. Ryan et al.[38] demonstrated selective impairment of substance P induced endothelium dependent and preservation of adenosine induced endothelium-independent vasodilation of the coronary resistance vessels in patients with coronary disease. The close correlation between the extent of endothelial...
...dysfunction in resistance vessels and the failure of coronary blood flow to increase during cold exposure or with rapid atrial pacing in patients with atherosclerosis suggests that endothelial integrity in the microvasculature with relaxing factor production is crucial in regulating coronary blood flow during times of increased metabolic demands. Drexler et al. demonstrated an improvement in the flow response to acetylcholine after l-arginine infusion but did not demonstrate any effect on large vessel vasomotion. Other studies showed impaired endothelium-dependent coronary vasodilation in patients with angina pectoris and normal coronary angiograms which was associated with exercise-induced myocardial ischaemia.

Microvascular endothelium-dependent relaxation is attenuated in the presence of atherosclerosis in animal models. The blood flow response to acetylcholine and substance P is impaired in the coronary microvasculature of patients with non-obstructive coronary artery disease. Vallance et al. demonstrated a 50% decrease in forearm blood flow to locally infused L-NMMA, which suggests a role for nitric oxide in the maintenance of forearm resistance vessel tone. In contrast to the forearm vascular bed, Lefroy et al. demonstrated that L-NMMA did not abolish the coronary blood flow increase in response to acetylcholine. They concluded that nitric oxide may not be the mediator of acetylcholine-induced coronary microvascular dilation. Other mediators, such as adenosine and endothelium derived hyperpolarizing factor may be involved.

Nitric oxide-dependent vasodilator tone seems to be maintained through the physical activity of endothelial cells by stimuli such as pulsatile flow and shear stress. In a recent study in patients with coronary artery disease and patients with normal coronary arteriograms we demonstrated that L-NMMA infusion reduced the magnitude of dilation induced by atrial pacing of both normal and diseased proximal and distal epicardial coronary segments and partly inhibited the pacing induced increase in flow velocity. Quyyumi et al. also found that L-NMMA inhibits the pacing related dilation of angiographically normal or near normal epicardial coronary arteries and indicated that nitric oxide is the mediator of this dilation. These findings suggest that endothelial production of nitric oxide extends in part to the resistance vessels and is an essential component of flow-mediated coronary artery epicardial artery dilation.

Nitric oxide and thrombosis

When human platelet-rich plasma is incubated ex vivo with aspirin treated endothelial cells an inhibition of platelet aggregation is observed that can be reversed by haemoglobin (which scavenges nitric oxide) or methylene blue (which inhibits the action of nitric oxide on soluble granulate cyclase). The effect of nitric oxide in inhibiting platelet adhesion and aggregation is associated with increases in the level of platelet cyclic GMP. Because nitric oxide inhibits platelet adhesion to the vessel wall and aggregation within the lumen, it is likely that this endothelial factor plays an important role in preventing coronary thrombosis.

Nitric oxide subserves the antithrombotic process by mechanisms other than its anti-aggregatory and anti-adhesive effects on platelets. The products released by 'activated platelets' mediate changes in vasomotor tone in part through endothelium-dependent mechanisms. These factors, which in general result in vasoconstriction if the endothelium is intact, have been shown to produce constriction in patients with atherosclerosis. Thus, the released platelet products can amplify the ischaemic effect of platelet aggregation and thrombosis by causing constriction at sites of endothelial dysfunction.

Not only is nitric oxide a potent vasodilator but it also inhibits the interaction of circulating blood elements with the vessel wall. Platelet adhesion and aggregation induced by intraluminal injury of the canine coronary artery are attenuated by nitroglycerin as well as by intravenous infusion of l-arginine.

Conclusions

Endogenous nitric oxide production plays a dominant role in modulating the diameter of conduit vessels and stenoses. It extents partly into the resistance vessels causing flow-mediated coronary artery epicardial artery dilation. Nitric oxide is produced at the site of stenosis but its production is not enhanced by an increase in flow velocity in the lumen of the epicardial vessels. When nitric oxide production is reduced by disease, dilation in response to various stimuli is often replaced by constriction with an associated increase in vascular resistance. Alterations in its synthesis or activity may play an important role in the clinical manifestations of atherosclerosis, in hypertensive states and some vasospastic disorders. Because of its ability to inhibit interactions of circulating blood elements with the vessel wall a deficit of endogenous nitric oxide may promote vascular thrombosis, atherogenesis and reperfusion injury. Novel therapeutic strategies for these cardiovascular disorders are likely to derive from interactions to enhance the synthesis or effect of endogenous nitric oxide or to supplement its activity with exogenous nitric oxide donors.

References

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