

## GENERAL CARDIOLOGY

Endothelial function and nitric oxide:  
clinical relevance

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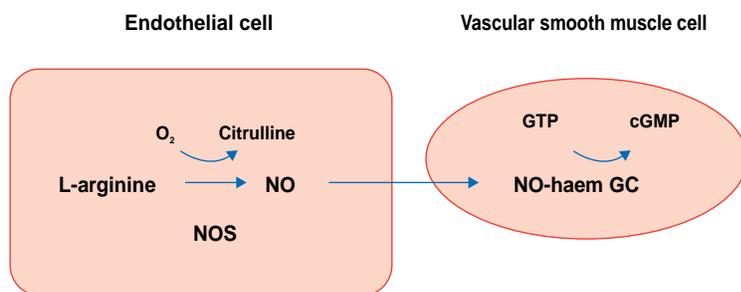
The vascular endothelium is a monolayer of cells between the vessel lumen and the vascular smooth muscle cells. Far from being inert, it is metabolically active and produces a variety of vasoactive mediators. Among these mediators, endothelial derived nitric oxide is essential in the maintenance of vascular homeostasis, and defects in the L-arginine: nitric oxide pathway have been implicated in a variety of cardiovascular diseases.

## Historic perspectives

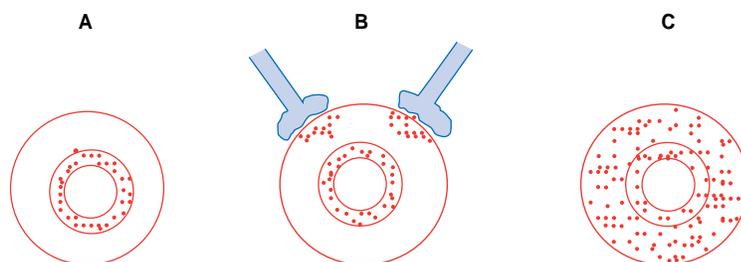
## From EDRF to nitric oxide

In 1980, Furchgott and Zawadzki showed that the presence of vascular endothelial cells is essential for acetylcholine to induce relaxation of isolated rabbit aorta.<sup>1</sup> If the vascular endothelium was removed, the blood vessel failed to relax in response to acetylcholine but still responded to glyceryl trinitrate. This endothelium dependent relaxation of vascular smooth muscle to acetylcholine is mediated by an endogenous mediator initially named endothelium derived relaxing factor (EDRF),<sup>1</sup>

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**Figure 1.** The L-arginine: nitric oxide pathway. NO, nitric oxide; NOS, nitric oxide synthase; GC, guanylate cyclase; cGMP, cyclic guanosine-3',5-monophosphate; GTP, guanosine triphosphate



**Figure 2.** (A) In most, if not all, vessels nitric oxide is synthesised within the endothelium. (B) In certain vessels (for example, cerebral vessels) nitric oxide is also synthesised by nerves in the adventitia (nitrogenic nerves). (C) After exposure to endotoxin or cytokines, iNOS is expressed throughout the vessel wall and produces large amounts of nitric oxide.

which was subsequently identified as nitric oxide.<sup>2,3</sup>

## L-arginine: nitric oxide pathway

Endothelium derived nitric oxide is synthesised from the amino acid L-arginine by the endothelial isoform of nitric oxide synthase, yielding L-citrulline as a byproduct.<sup>4</sup> Nitric oxide is labile with a short half life (< 4 seconds in biological solutions). It is rapidly oxidised to nitrite and then nitrate by oxygenated haemoglobin before being excreted into the urine.<sup>4</sup> Several co-factors are required for nitric oxide biosynthesis. These include nicotinamide adenine dinucleotide phosphate (NADPH), flavin mononucleotide, flavin adenine dinucleotide, tetrahydrobiopterin (BH<sub>4</sub>), and calmodulin. Once synthesised, the nitric oxide diffuses across the endothelial cell membrane and enters the vascular smooth muscle cells where it activates guanylate cyclase, leading to an increase in intracellular cyclic guanosine-3',5-monophosphate (cGMP) concentrations<sup>4</sup> (fig 1). As a second messenger, cGMP mediates many of the biological effects of nitric oxide including the control of vascular tone and platelet function. In addition, nitric oxide has other molecular targets which include haem or other iron centred proteins, DNA, and thiols. These additional reactions may mediate changes in functions of certain key enzymes or ion channels. Nitric oxide also interacts with enzymes of the respiratory chain including complex I and II, and aconitase, and through these effects alters tissue mitochondrial respiration. Interaction of nitric oxide with superoxide anion can attenuate physiological responses mediated by nitric oxide and produce irreversible inhibitory effects on mitochondrial function as a result of the formation of peroxynitrite (ONOO<sup>-</sup>), a powerful oxidant species.

## Nitric oxide synthase isoforms

Three isoforms of nitric oxide synthase (NOS) have been identified: the endothelial isoform (eNOS), neuronal isoform (nNOS), and macrophage or inducible isoform (iNOS). All three NOS isoforms play distinct roles in the regulation of vascular tone (fig 2). The genes encoding eNOS, nNOS, and iNOS are located on chromosome 7, 12, and 17, respectively.<sup>5</sup> Whereas eNOS and nNOS are normal constituents of healthy cells, iNOS is not usually expressed in vascular cells and its expression is seen mainly in conditions of infection or inflammation.

## Biological effects of nitric oxide

## Nitric oxide and the vasculature

Endothelium derived nitric oxide is a potent vasodilator in the vasculature, and the balance between nitric oxide and various endothelium derived vasoconstrictors and the sympathetic nervous system maintains blood vessel tone. In addition, nitric oxide suppresses platelet aggregation, leucocyte migration, and cellular

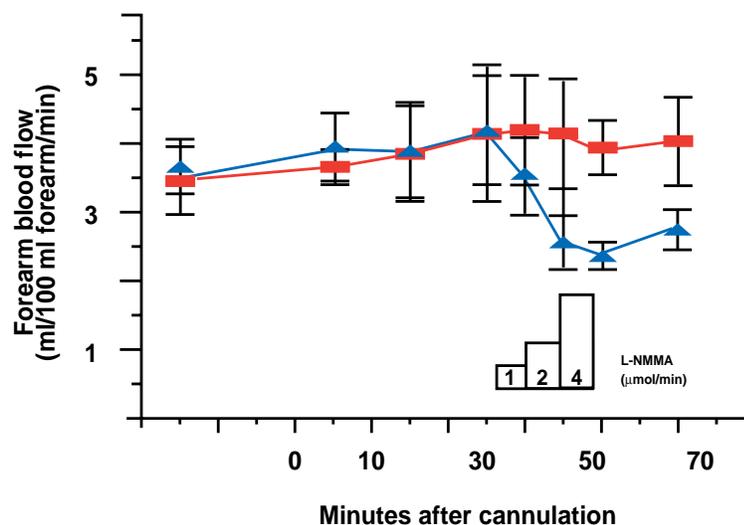
### The L-arginine: nitric oxide pathway

- Nitric oxide is synthesised from L-arginine to yield citrulline
- Co-factors required for nitric synthesis:
  - nicotinamide adenine dinucleotide phosphate (NADPH)
  - flavin mononucleotide
  - flavin adenine dinucleotide
  - tetrahydrobiopterin (BH<sub>4</sub>)
  - calmodulin
- Enzymes responsible for nitric oxide synthesis:
  - endothelial nitric oxide synthase (expressed in normal cells) (eNOS)
  - neuronal nitric oxide synthase (expressed in normal cells) (nNOS)
  - inducible nitric oxide synthase (expressed during infection/inflammation) (iNOS)
- Nitric oxide activates guanylate cyclase in vascular smooth muscle cells to synthesise cyclic guanosine-3',5-monophosphate (cGMP) which causes many of its biological effects

adhesion to the endothelium, and attenuates vascular smooth muscle cell proliferation and migration. Furthermore, nitric oxide can inhibit activation and expression of certain adhesion molecules, and influence production of superoxide anion. Loss of endothelium derived nitric oxide would be expected to promote a vascular phenotype more prone to atherogenesis, a concept supported by studies in experimental animals.<sup>6</sup>

#### Nitric oxide release from the vascular endothelium

There is a continuous basal synthesis of nitric oxide from the vascular endothelium to maintain resting vascular tone. A number of chemical and physical stimuli may activate eNOS and lead to increased nitric oxide production.



**Figure 3.** Inhibition of endogenous nitric oxide by L-NMMA produces a dose dependent reduction in forearm blood flow.

#### Basal nitric oxide release

The synthesis of nitric oxide in vascular endothelial cells in culture or intact vascular tissue can be inhibited by N<sup>G</sup> monomethyl-L-arginine (L-NMMA), an analogue of L-arginine in which one of the guanidino nitrogen atoms is methylated. This inhibitory effect of L-NMMA is readily reversed by L-arginine.<sup>7</sup> L-NMMA and similar substrate based inhibitors have been used to examine the role of nitric oxide in various vascular beds in vitro and in both human and animal models.

In rings of rabbit aorta, L-NMMA causes significant endothelium dependent contraction. Intravenous infusion of L-NMMA into experimental animals induces a dose related increase in blood pressure which is reversed by intravenous administration of L-arginine, and in the human forearm vasculature infusion of L-NMMA into the brachial artery causes substantial dose dependent vasoconstriction. Thus continuous generation of nitric oxide is crucial in maintaining peripheral vasodilatation in humans<sup>7</sup> (fig 3). This basal nitric oxide mediated dilatation has been seen in every other arterial bed studied including cerebral, pulmonary, renal, and coronary vasculature. In contrast, in the venous system inhibitors of NOS do not lead to an increase in basal tone in a variety of venous preparations from animals or humans,<sup>8</sup> suggesting that basal nitric oxide production does not have a major role in the maintenance of the resting tone in most veins. In conduit vessels, there is some basal nitric oxide mediated dilatation but it appears to be less than that seen in resistance vessels.

#### Agonist stimulated nitric oxide release

Many chemical substances such as acetylcholine, bradykinin, serotonin, and substance P are able to induce endothelium dependent vasodilatation. In rings of rabbit aorta, endothelium dependent relaxation induced by acetylcholine, calcium ionophore (A23187) or substance P is inhibited by L-NMMA. This provides in vitro evidence that vasorelaxation induced by endothelium dependent agonists is nitric oxide mediated. L-NMMA also attenuates the hypotensive effect of acetylcholine in vivo in animals. However, the blockade is far from complete and there is now growing evidence for additional mechanisms underlying endothelium dependent responses to acetylcholine, particularly in resistance vessels. Similarly in humans, L-NMMA inhibits agonist stimulated relaxation in resistance,<sup>7</sup> conduit, and venous vessels in vivo. However, the degree of inhibition to agonist dependent dilatation varies between vascular beds, and mechanisms (for example, prostaglandins and endothelium derived hyperpolarising factors) other than that mediated by nitric oxide appear to be involved.

#### Physical forces and nitric oxide release

Haemodynamic shear stress exerted by the viscous drag of flowing blood is an important physiological stimulus in the regulation of nitric oxide release from the endothelium. The mechanisms of shear stress induced nitric oxide release is complex, involving

(1) extremely rapid initiation via ion channel activation, and (2) subsequent events related to signalling pathway activation such as phosphorylation of eNOS protein and increased expression of eNOS mRNA and protein. These complex events allow rapid and short lasting as well as slow onset and sustained vasodilatation in response to changes in shear stress.

#### *Ultra-quick: ion channels*

Numerous *in vitro* studies have provided strong evidence that ion channels—including certain calcium, potassium, and chloride ion channels—open seconds after exposure to haemodynamic shear. Application of shear stress to bovine aortic endothelial cells by fluid perfusion led to an immediate large increase in intracellular free calcium within one minute followed by a rapid decline. Notably, the increase in intracellular calcium occurs only in response to pulsatile flow and not to steady flow. The detection of potassium selective current with whole cell patch clamp recordings of arterial endothelial cells suggests activation of a distinct potassium channel in response to shear stress. Recently, a flow activated, chloride selective membrane current in vascular endothelial cells, distinct from the potassium current, was demonstrated. The balance between anionic and cationic current determines the net membrane potential and the subsequent change in calcium that alters eNOS activation and nitric oxide output.

#### *Quick: phosphorylation*

Mechanical activation of eNOS as induced by shear stress occurs also via phosphorylation and the effect is independent of intracellular calcium concentrations. It has been shown that in response to shear stress, the serine/threonine protein kinase B (Akt) directly phosphorylates and activates eNOS with a maximal increase up to sixfold after one hour of exposure to shear stress. The stimulation of Akt phosphorylation by shear stress appears to be mediated by phosphoinositide 3-OH kinase.

#### *Slow: increased transcription*

Shear stress also stimulates eNOS gene transcription to maintain long term nitric oxide production. Application of shear stress for three hours resulted in an induction of eNOS mRNA in a dose dependent manner in both bovine and human aortic endothelial cells.

These *in vitro* experimental data demonstrate the complexity of short medium and long term regulation of nitric oxide release in response to shear stress.

#### Loss of nitric oxide and predisposition to atherogenesis

A reduction in nitric oxide activity (manifested as impaired endothelial dependent vasodilatation) occurs very early in experimental and human hypercholesterolaemia, even before any structural changes in the vascular wall. In some cases, the impaired endothelial dependent vasodilatation appears to be reversed with L-arginine.<sup>9</sup> The action that nitric oxide normally has as an antiatherogenic factor is

supported by studies of long term NOS inhibition. Aortic rings from rabbits fed with cholesterol rich diet showed impaired endothelial dependent vasorelaxation to acetylcholine. Furthermore, blockade of nitric oxide with nitro-L-arginine methylester (L-NAME) causes structural changes with development of greater lesion surface area in the aorta of hypercholesterolaemic rabbits.

### Endothelial dysfunction in established cardiovascular disease

#### Atherosclerosis

Impaired endothelium dependent vasorelaxation to acetylcholine occurs in experimental models of atherosclerosis. In these studies, relaxation to endothelium independent nitric oxide donors such as glyceryl trinitrate and sodium nitroprusside was unaffected, indicating selective impairment of the L-arginine-nitric oxide pathway rather than a generalised reduced vascular smooth muscle cell response to nitric oxide. Similar findings were also confirmed in human coronary and peripheral circulation *in vivo*.<sup>10</sup> In patients with early coronary artery disease, abnormal responses to acetylcholine were found even in angiographically normal segments of coronary artery. Similarly, in patients with established coronary artery disease, endothelial dysfunction in the peripheral vessels as assessed by flow mediated dilatation is impaired. This impairment correlates with the extent of the coronary artery disease.

#### Chronic heart failure

Chronic heart failure (CHF) is characterised by a reduced vasodilator response to exercise and increased vasoconstriction; this is primarily a result of an imbalance between endothelium derived vasodilator and constrictor substances. There is general agreement that in CHF synthesis of endothelin-1, a potent vasoconstrictor, is greatly increased and this may contribute to the characteristic haemodynamic abnormalities. However, it remains unclear whether nitric oxide synthesis is decreased in CHF. The fact that endothelium dependent vasodilator response to acetylcholine, methacholine, and serotonin<sup>11</sup> is attenuated in peripheral resistance vessels suggests a reduction in agonist stimulated nitric oxide release. In contrast, response to L-NMMA appears to be either unchanged or even paradoxically exaggerated in CHF. This could be partially explained by an increased basal nitric oxide synthesis in the face of increased vasoconstrictor generation. Interestingly, endothelium independent vasodilator response to nitric oxide donors may also be attenuated,<sup>11</sup> suggesting that vascular smooth muscle sensitivity to nitric oxide might be reduced, with the degree of attenuation correlating with the severity of CHF. It is possible that the underlying mechanism relates to increased vascular generation of superoxide anion ( $O_2^-$ ) that inactivates nitric oxide.

Treatment of CHF with angiotensin converting enzyme (ACE) inhibitors has been shown to improve endothelium dependent vasodilatation induced by cholinergic stimuli. Furthermore, in a randomised, placebo controlled study of patients with moderate to severe CHF, spironolactone increased vasodilator response to acetylcholine with an associated increase in vasoconstriction to L-NMMA, suggesting enhanced basal nitric oxide mediated dilatation following spironolactone treatment. Whether these beneficial effects on endothelial nitric oxide contribute to the favourable outcome remains to be determined.

### Endothelial dysfunction and risk factors for coronary heart disease

#### Hyperlipidaemia

Experimentally induced hyperlipidaemia by either a fatty meal or intralipid infusion impairs flow mediated dilatation (FMD). In patients with hypercholesterolemia, endothelium dependent vasodilatation in both coronary and peripheral vessels is impaired before the development of clinical atherosclerosis.<sup>9-12</sup> Restoration of normal or near normal endothelium dependent vasodilatation in the forearm resistance vessels of hypercholesterolaemic subjects can be achieved after six months of lipid lowering treatment. Interestingly, reduced vasodilatation in response to acetylcholine has also been seen in patients with raised triglyceride but normal low density lipoprotein (LDL) cholesterol concentrations. In patients with familial combined hyperlipidaemia, lipid lowering treatment improved forearm blood flow in response to serotonin. Finally, raised Lp(a) lipoprotein appears to enhance acetylcholine and cold pressor coronary constrictor responses in patients with normal coronary arteries on angiography and impairs basal nitric oxide production in forearm resistance vessels. Thus, there is compelling evidence to indicate that endothelial function is impaired by hyperlipidaemia. However, recently the reversibility of this defect in coronary arteries has been called into question.

#### Hypertension

Substantial evidence from animal and human studies indicates that acetylcholine induced relaxation is impaired in patients with hypertension. However, no difference was found in endothelium dependent vasodilator response to acetylcholine or carbachol between patients with essential hypertension and matched normotensive controls in at least one study. This may be because of differences in methodology and in population subgroups in this heterogeneous condition. There is, however, evidence that basal nitric oxide synthesis is reduced in essential hypertension and that vasoconstrictor response to L-NMMA is reduced in untreated hypertension, although again this has not been a universal finding.<sup>13</sup> Current data would be most consistent with hypertension causing a decrease in nitric oxide mediated dilatation

rather than the loss of nitric oxide causing essential hypertension. This notion is supported by the observation that impaired endothelium dependent vasodilatation in essential hypertension can be restored with anti-hypertensive treatment,<sup>13</sup> and that endothelium dependent vasodilatation is impaired following acute elevation of blood pressure in normotensive subjects.

#### Diabetes mellitus

There is considerable controversy regarding the extent of endothelial dysfunction that occurs in type 1 diabetes mellitus, as endothelial function studies in both animal and human models of type 1 diabetes have produced conflicting results.<sup>14</sup> For example, agonist stimulated, endothelium dependent vasodilatation has been found to be either impaired or unchanged. Endothelial function may be modulated by several factors associated with diabetes such as the degree of acute hyperglycaemia, chronicity of hyperglycaemia (disease duration), accumulation of advanced glycosylated end products, insulin concentrations, and diabetic complications such as autonomic neuropathy and microalbuminuria. Variation in these factors between studies may in part explain the conflicting results. At present, there is no clear consensus about the level at which the disease might alter nitric oxide signalling. Most data would be consistent with a reduced responsiveness to nitric oxide in type 1 diabetes, and large scale definitive studies in the future would be required to show whether this is the case.

Vascular studies in type 2 diabetes have generally shown an impaired muscarinic, agonist stimulated, endothelium dependent response as well as an impaired endothelium independent response, although the impaired endothelium independent response was not confirmed by other investigators. Interestingly, it has recently been shown that even normoglycaemic subjects who are prone to develop type 2 diabetes and insulin resistance syndrome, such as those characterised by previous gestational diabetes and low birth weight, exhibit impaired FMD. This has led to the suggestion that endothelial dysfunction may precede the development of type 2 diabetes.

#### Hyperhomocysteinaemia

Raised plasma homocysteine is thought to be an independent risk factor for coronary heart disease, and this may be mediated by endothelial dysfunction. Acute methionine induced hyperhomocysteinaemia impairs endothelium dependent FMD in healthy subjects which can be reversed by folic acid supplementation. Impaired FMD has also been found in chronic hyperhomocysteinaemia and homocysteinuric children. The concentration of plasma homocysteine associated with endothelial dysfunction in these in vivo human studies were several folds higher than the normal range. Recently, it has been shown that even mild physiological increments in plasma homocysteine concentrations were sufficient to impair endothelial function.

*Links between risk factors and atherogenesis*

In addition to various disease states, endothelium dependent vasodilatation is also impaired in old age,<sup>15</sup> and in young healthy subjects with a family history of premature coronary heart disease and cigarette smoking. The age related endothelial dysfunction may partially explain the increased cardiovascular risk in the elderly. In asymptomatic young smokers, impairment of endothelium dependent vasodilatation is reversible with smoking cessation. It may be that tobacco has a direct toxic effect on the vascular endothelium. Additionally, there is growing evidence of a link between infection/inflammation and risk of coronary heart disease. A recent study showed that acute systemic inflammation induced by vaccination causes impaired endothelium dependent vasodilatation in both resistance and conduit vessels. Thus various effects on the L-arginine: nitric oxide pathway exerted by hypertension, diabetes, hyperlipidaemia, hyperhomocysteinaemia, infection/inflammation, aging, cigarette smoking, and family history of coronary heart disease may form a link between risk factors and predisposition to atherogenesis or acute events.

**Alteration of the L-arginine: nitric oxide pathway in diseases: potential mechanisms**

The involvement of the L-arginine: nitric oxide pathway in disease states is complex and it can be altered in several ways.

**Reduced nitric oxide production***Deficiency of NOS co-factor*

Several conditions are associated with NOS co-factor deficiency which may contribute to endothelial dysfunction. For instance, insulin resistance is associated with deficiency of the essential co-factor BH<sub>4</sub>, resulting in impaired vascular relaxation. Additionally, chronic cigarette smoking contributes to depletion of BH<sub>4</sub>, resulting in decreased nitric oxide synthesis, and supplementation of this co-factor restores endothelial function in chronic smokers. In hypercholesterolaemia, impaired endothelium dependent vasodilatation can also be restored with BH<sub>4</sub> supplementation, suggesting that BH<sub>4</sub> deficiency plays an important role in impaired vascular function in these conditions.

*Role of endogenous inhibitors of NOS*

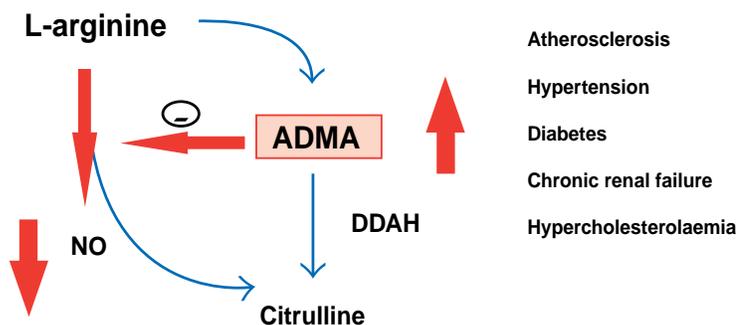
Overproduction of endogenous inhibitors of NOS in certain disease states may contribute to reduced nitric oxide synthesis (fig 4). Asymmetric and symmetric dimethylarginine (ADMA and SDMA) have been identified in human plasma. ADMA has properties similar to L-NMMA. It is synthesised by the human endothelial cells from arginine and is metabolised to citrulline before excretion into the urine (fig 4). The enzyme responsible for ADMA metabolism in the human vascular endothelial cells is dimethylarginine dimethylaminohydrolase of which two isoforms

(DDAH I and II) have been identified, sequenced, and cloned.<sup>16</sup> Circulating ADMA is increased in certain disease states. This includes animal models of hypertension, diabetes, hypercholesterolaemia, and atherosclerosis. In humans, raised ADMA concentrations were found in chronic renal failure,<sup>17</sup> childhood hypertension, pre-eclampsia, thrombotic microangiopathy, hypercholesterolaemia, and atherosclerosis. The mechanism whereby various disease states are associated with increased ADMA concentrations remains unclear but may involve alteration in DDAH activity, and this may be an important enzyme in atherogenesis. Accumulation of ADMA would be expected to enhance atherogenesis through loss of nitric oxide.

**Reduced nitric oxide bioavailability***Role of oxidative stress*

Even with adequate production, nitric oxide may not reach its biological targets (vascular smooth muscle and circulating cells) to exert its effect because of the lack of its bioavailability. For example, in hyperlipidaemia, excess LDL synthesis increases the formation of oxidised LDL. The resultant increase of oxidative stress enhances nitric oxide destruction, thereby reducing its biological effects. In atherosclerotic rabbit aorta, despite a threefold increase in total nitric oxide synthesis compared to normal rabbits, there is notably impaired endothelium dependent vasodilatation. This impaired vascular response can be partially restored following treatment with superoxide dismutase, suggesting that superoxide induced nitric oxide inactivation plays a major role. In humans, hypertriglyceridaemia with or without diabetes may have greater potential than cholesterol to increase superoxide production by leucocytes. Other atherogenic factors such as free fatty acids and low concentrations of high density lipoprotein also increase oxidative stress, contributing to reduced nitric oxide bioavailability. In addition to the associated atherogenic phenotype and oxidative stress, hyperglycaemia per se increases free radical production through increased arachidonic acid metabolism.

In human aortic endothelial cells, although prolonged exposure to high glucose concentration causes increased eNOS expression, it also



**Figure 4.** Asymmetric dimethylarginine (ADMA) is synthesised from L-arginine by protein methylase I and subsequently metabolised by dimethylarginine dimethylaminohydrolase (DDAH) yielding citrulline. ADMA acts as an endogenous inhibitor of nitric oxide (NO) synthesis and its concentration is increased in certain disease states, possibly as a result of decreased DDAH actions.

leads to a concomitant increase in superoxide anion production (probably from NADH/NADPH oxidase) resulting in nitric oxide inactivation. Oxidative stress may also be a key mechanism for endothelial dysfunction in hyperhomocysteinaemia. Studies *in vitro* and in animals suggest that elevation of homocysteine enhances lipid peroxidation, which may contribute to impaired endothelium dependent vasodilatation. This potential mechanism is supported by the findings that the antioxidants folic acid and vitamin C rapidly reverse the impaired endothelium dependent vasodilatation without reducing the raised homocysteine concentrations. Similar improvement in endothelial function in type 2 diabetes with antioxidants such as vitamin E have also been observed, suggesting yet again the central role of oxidative stress in endothelial dysfunction under various pathophysiological conditions.

#### *Advanced glycation endproducts*

Accumulation of advanced glycation endproducts (AGEs)—the product of non-enzymatic glycation and crosslinking of collagen protein in sustained hyperglycaemia—may lead to quenching (or inactivation) of nitric oxide in diabetes. In experimentally induced diabetic rats, there is *in vitro* and *in vivo* evidence that reactive intermediates resulting from glycation quench nitric oxide rapidly. Furthermore, impairment of endothelium dependent vasodilatation in diabetic rats can be partially restored by aminoguanidine, an inhibitor of AGEs formation

#### Diminished vascular smooth muscle sensitivity

Vascular smooth muscle sensitivity may be decreased even with adequate nitric oxide supply. In human vascular studies, nitrovasodilators or nitric oxide donors (such as glyceryl trinitrate or sodium nitroprusside) have frequently been used as a control for agonist stimulated endothelium dependent vasodilatation. These agents act directly upon vascular smooth muscle and resultant vasodilatation is endothelium independent. There is evidence to suggest that vascular smooth muscle sensitivity to nitric oxide is reduced in diabetes and hyperglycaemia interferes with nitric oxide induced guanylate cyclase activation *in vitro*. Consistent with this finding, impaired vascular response to nitric oxide donors *in vivo* have been demonstrated in patients with type 1 diabetes.

#### Overproduction of nitric oxide in sepsis

Bacterial endotoxin and certain proinflammatory cytokines can lead to profound vasodilatation and decreased vasopressor responsiveness—the main clinical features of septic shock. These cardiovascular effects result from excessive nitric oxide production thought to be caused by induction of iNOS.

Under normal physiological conditions, iNOS is not expressed in the vasculature. Exposure to bacterial lipopolysaccharide or proinflammatory cytokines stimulate iNOS expression. In experimental animals, administration of tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) in doses similar to those produced endogenously during endotoxaemia rapidly results in a fall in arterial pressure, and longer exposure to TNF $\alpha$  and interleukin-1 $\beta$  (IL-1 $\beta$ ) *in vitro* leads to hyporesponsiveness to vasopressors.

Several animal studies provide evidence for iNOS involvement in the pathogenesis of sepsis. Administration of bacterial lipopolysaccharide to mice causes an increase in nitrite concentration, which is attenuated by a selective iNOS inhibitor. Moreover, selective inhibition of iNOS greatly increases vascular catecholamine reactivity in experimental mice with sepsis but not in control mice. Studies in mice genetically engineered to lack the gene for iNOS also confirm the role of this isoform in septic vasodilatation. In wild type mice, treatment of carotid arteries with lipopolysaccharide leads to impaired constrictor responses which is improved with selective iNOS inhibitors. In contrast, lipopolysaccharide treatment causes no impairment of vasoconstrictor responses in carotid arteries from iNOS deficient mice.

In human sepsis the evidence for iNOS involvement has been less consistent. Some studies have suggested an increased iNOS activity—for example, in urinary leucocytes from patients with urinary tract infection, in alveolar macrophages from patients with acute respiratory distress syndrome following sepsis, and in peripheral blood mononuclear cells and macrophages isolated from putrescent muscle areas in patients with cellulitis. However, other studies have suggested the involvement of eNOS rather than iNOS in human sepsis. In an *in vitro* study using human umbilical vein endothelial cells cultured with IL-1 $\beta$  and TNF $\alpha$ , the resultant increase in nitric oxide production was shown to originate from eNOS as a result of activation of guanosine triphosphate cyclohydrolase (GTPCH-I), the rate limiting enzyme responsible for the synthesis of BH<sub>4</sub>. Similar findings have recently been shown in human veins *in vivo*. While it is clear that overproduction of nitric oxide contributes to vasodilatation in human sepsis,<sup>18</sup> the molecular mechanisms and isoform of NOS activated are unclear. It is also not known whether inhibition of nitric oxide generation is beneficial. Expression of iNOS in active atherosclerotic plaques has also been detected. It is possible that this iNOS contributes to tissue damage or other features of plaque development or stability.

#### Therapeutic possibilities

##### L-arginine

Supplementation of the nitric oxide substrate L-arginine has beneficial effects in certain conditions in laboratory animals and humans. Dietary L-arginine for 10 weeks has been shown to prevent intimal thickening in the

coronary arteries and attenuates platelet reactivity in hypercholesterolaemic rabbits. Furthermore, oral L-arginine administration reduces neointimal formation following balloon catheter induced injury in both hypercholesterolaemic and normocholesterolaemic rabbit models. In humans, dietary L-arginine supplementation reduces the increased platelet reactivity in hypercholesterolaemic subjects. Additionally, intravenous L-arginine infusion reduces peripheral vascular resistance and decreases both systolic and diastolic blood pressure, at least in some studies, improves endothelium dependent coronary vasodilatation in response to intracoronary acetylcholine in hypercholesterolaemic subjects,<sup>9</sup> and improves blood flow in critical lower limb ischaemia.

#### *The arginine paradox*

The beneficial effects of exogenous L-arginine to the vasculature in various disease states with increased plasma nitrate and cGMP concentrations during L-arginine administration suggest provision of excess L-arginine supply can stimulate NOS activity. However, it is perplexing that extracellular arginine administration should drive nitric oxide production since the intracellular arginine concentrations are always available in great excess of the needs of NOS, a phenomenon known as the “arginine paradox”. This was first demonstrated in hypercholesterolaemic rabbits<sup>19</sup> and has also been observed in patients with pulmonary hypertension. Several explanations have been proposed to account for this paradox. Firstly, it is possible that the endogenous inhibitor of NOS, ADMA, might antagonise the normal intracellular concentrations of L-arginine, and additional arginine supplementation is required to overcome a functional defect of NOS substrate. Secondly, since eNOS is preferentially localised to specific intracellular sites known as caveolae, local concentration of L-arginine in this microenvironment may differ considerably from that within the endothelial cell. It remains unclear how specific localisation of eNOS by caveolae might affect local substrate availability, but the mechanism may involve the co-localisation of eNOS with certain arginine transporters (for example, cationic amino acid transporter-1). The formation of such a caveolar complex seems to facilitate arginine delivery to eNOS.

It is also important to recognise that the vasodilatory effects of L-arginine are not all mediated directly by nitric oxide. L-arginine may inhibit peripheral sympathetic tone leading to vasodilatation via its metabolite, agmatine, which stimulates central  $\alpha_2$  adrenoceptors. Additionally, arginine also stimulates the release of several other hormones such as glucagon, prolactin, and growth hormone which may account for its vasodilatory action. Furthermore, many of the vascular and other actions of L-arginine are shared by its stereoisomer, D-arginine, which is not a substrate for NOS. The complex mechanisms whereby L-arginine appears to improve cardiovascular

function in some conditions merits further investigation.

#### Nitrovasodilators/nitric oxide donors

Nitrovasodilators such as amyl nitrite, glyceryl trinitrate, sodium nitroprusside, and molsidomine are all pro-drugs and exert their pharmacological effects after metabolism to nitric oxide. Hence they are termed “nitric oxide donor”. Based on their venodilatory properties, nitrovasodilators have conventionally been used for the treatment of cardiac failure and angina. Nitrosoglutathione, a compound in the class of nitrosothiols, has been studied extensively in humans. It has profound antiplatelet effects and more balanced arterial and venous vasodilatory effects than organic nitrates. Nitrosoglutathione inhibits platelet activation in the coronary artery following angioplasty and in coronary bypass grafts. Hence, nitrosothiols are potential pharmacological agents in the treatment of nitric oxide deficient conditions, and it is possible that novel nitric oxide donors could be developed that differ greatly from existing drugs.

#### Inhalation of nitric oxide

Administration of nitric oxide as inhalation treatment has been shown to improve several conditions affecting the pulmonary vasculature, including persistent pulmonary hypertension of the newborn, pulmonary hypertension secondary to chronic hypoxia, and adult respiratory distress syndrome (ARDS). In ARDS, selective delivery of nitric oxide to the pulmonary vasculature reduces the pulmonary arterial pressure and increases arterial oxygenation by improving the matching of ventilation with perfusion. As the nitric oxide is rapidly inactivated by haemoglobin, inhalation of nitric oxide gas does not cause systemic vasodilatation. However, whether this form of nitric oxide treatment will improve survival in patients with ARDS remains to be determined by randomised controlled clinical trials. It does have potential adverse effects such as pulmonary oedema and methaemoglobinaemia. There are as yet no clear guidelines regarding the effective dose of inhaled nitric oxide for the above pulmonary conditions.

#### Antioxidants

Since oxidative stress has been strongly implicated in endothelial dysfunction, numerous studies have examined the role of antioxidants in vascular function and in the prevention of cardiovascular disease. Intrabrachial administration of ascorbic acid (vitamin C) improves endothelium dependent vasodilatation in patients with type 2 diabetes, smokers, patients with hypercholesterolaemia, and patients with heart failure. Similarly, oral administration of ascorbic acid in patients with coronary heart disease also improves flow mediated vasodilatation. This beneficial effect has been shown to occur rapidly after two hours and can be sustained for 30 days. Furthermore, intravenous infusion of ascorbic acid improved endothelium dependent vasodilatation in epicardial arteries in hypertensive patients without

### Summary

- Endothelium derived nitric oxide has important antiatherogenic properties:
  - enhancement of vasodilatation
  - inhibition of platelet aggregation
  - inhibition of leucocyte migration
  - inhibition of smooth muscle proliferation and migration
  - inhibition of adhesion molecule activation and expression
  - anti-oxidant
- Three distinct nitric oxide synthase isoforms exist which all have a role in vascular control under different conditions
- Reduced nitric oxide synthesis or defective nitric oxide activities predispose to atherosclerosis
- Different disease processes and risk factors affects the L-arginine–nitric oxide pathway via different mechanisms:
  - decreased synthesis
  - decreased co-factor availability
  - decreased enzyme expression/activity
  - increased endogenous nitric oxide synthase inhibitors
  - decreased nitric oxide bioavailability
  - decreased vascular smooth muscle sensitivity to nitric oxide
- Understanding these mechanisms in diseases may help therapeutic strategies in prevention of atherosclerosis

coronary heart disease. In patients with coronary heart disease, intracoronary co-infusion of vitamin C with L-arginine causes greatly increased vasodilatation. Despite these observations, not all experiments have shown beneficial effects and further studies are required. In addition, agents such as vitamin C can produce pro-oxidant effects in some circumstances.

#### Gene transfer

The direct transfer of NOS isoform genes to the vessel wall alters vasomotor function and may have a role in the treatment of cardiovascular diseases.<sup>20</sup> This approach has been shown to be effective in a variety of animal models of vascular disease. Vascular gene therapy of eNOS delivered to specific tissues enhances nitric oxide production at the site of interest. In 1995, NOS gene transfer was first reported when eNOS cDNA, in a complex with the haemagglutinating virus of Japan, was delivered to rat carotid arteries intraluminally following balloon injury. This resulted in a pronounced reduction in neointima formation at 14 days. Shortly after, similar results were confirmed by other research groups in iliac and coronary arteries and vein grafts. Gene therapy has also been extended from local to systemic delivery. Delivery of a single injection of naked eNOS cDNA into the systemic circulation through the tail vein of spontaneously hypertensive rats resulted in increased production and excretion of cGMP and nitrite/nitrate, and was associated with a significant reduction in systolic blood pressure lasting up to 12 weeks.

#### NOS inhibitors

Inhibition of iNOS has been shown to improve nitric oxide mediated haemodynamic changes in experimental models of septic shock.<sup>18</sup>

Intravenous administration of the arginine analogue L-NMMA reverses the decrease in peripheral vascular resistance and fall in arterial blood pressure in endotoxaemic dogs. The same effects with L-NMMA have also been confirmed in humans in a randomised, double blind, placebo controlled trial.<sup>18</sup> A potential pitfall in this therapeutic strategy is that none of the currently available NOS inhibitors are specific for the excess nitric oxide causing pathophysiology, and may therefore lead to potential hazardous effects, by also inhibiting “physiological” nitric oxide. Indeed, both pulmonary hypertension and reduced cardiac output have been reported following NOS inhibition. Future drug development in this area should focus on NOS isoform specificity as well as the degree of inhibition required in order to produce an optimal pharmacological effect.

### Conclusions

Nitric oxide is a major player in cardiovascular physiology and pharmacology. Its release from endothelium exerts a tonic hypotensive, anti-platelet, antiatherogenic influence in the arterial tree. Several common cardiovascular disease states or risk factors impair nitric oxide synthesis, increase nitric oxide destruction or affect the ability of cells to respond to this mediator. The net effect is to enhance vascular tone and reactivity and predispose to atherosclerosis. Clinicians already use drugs that either mimic nitric oxide (nitric oxide donors) or enhance its endogenous production (for example, by lowering blood pressure or serum cholesterol). In contrast, overproduction of nitric oxide is an important mechanism of inflammatory vasodilatation. Over the next few years a new generation of drugs will emerge that specifically modify nitric oxide production or mimic its action. Indeed, the first such drug is sildenafil which blocks the phosphodiesterase that degrades cGMP and thereby acts as an amplifier of nitric oxide signalling (fig 1). Diagnostic tests based on early assessment of endothelial function may also become part of cardiovascular risk stratification or assessment of novel therapeutics.

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