The role of nitric oxide in cardiovascular diseases

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Abstract

Nitric oxide (NO) is a gaseous lipophilic free radical cellular messenger generated by three distinct isoforms of nitric oxide synthases (NOS), neuronal (nNOS), inducible (iNOS) and endothelial NOS (eNOS). NO plays an important role in the protection against the onset and progression of cardiovascular disease. Cardiovascular disease is associated with a number of different disorders including hypercholesterolaemia, hypertension and diabetes. The underlying pathology for most cardiovascular diseases is atherosclerosis, which is in turn associated with endothelial dysfunctional. The cardioprotective roles of NO include regulation of blood pressure and vascular tone, inhibition of platelet aggregation and leukocyte adhesion, and prevention smooth muscle cell proliferation.

Reduced bioavailability of NO is thought to be one of the central factors common to cardiovascular disease, although it is unclear whether this is a cause of, or result of, endothelial dysfunction. Disturbances in NO bioavailability leads to a loss of the cardio protective actions and in some case may even increase disease progression. In this chapter the cellular and biochemical mechanisms leading to reduced NO bioavailability are discussed and evidence for the prevalence of these mechanisms in cardiovascular disease evaluated.

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1. Introduction

Cardiovascular disease (CVD) is most common among populations of the industrialised countries, where standards of living are high. CVD is also one of the main causes of death globally. Within Europe, the UK has one of the worst records of cardiovascular disease, particularly in Scotland and Northern Ireland.
This may be associated with the distribution of social class groups in these regions. It is rising in countries of the Indian sub-continent. In Japan, the levels of CVD are low, but may rise with increased westernisation. In the USA, Western Europe, the deaths from CVD are falling, but the number of individuals with symptoms is not following this trend and many lives are being saved by the availability of new drugs such as the statins. The death rate from CVD is increasing rapidly in Eastern Europe.

Cardiovascular disease encompasses a number of different diseases including coronary heart disease, stroke and peripheral vascular disease. However, the underlying pathology for all of these disorders is atherosclerosis. The pathology of atherosclerosis is extremely complex and involves all the structural elements of the arterial wall, circulating cells such as platelets and leukocytes and a number of inflammatory cells particularly monocytes/macrophages (see Fig. 1). Central to the process is the vascular endothelium, which acts as a dynamic interface between the circulation and the arterial wall. Changes in the artery wall occur throughout life, but atherosclerosis is a pathological process that is different from ageing. It may even begin in childhood and is certainly present in many teenagers in the most industrialised countries. In itself, it is not usually life-threatening until the later stages, but many are struck down in middle age.

It is important to understand the pathogenesis of the atherosclerosis. A brief overview of the pathobiology of atherosclerosis is provided here: for more in depth material the reader is directed to an excellent review (Lusis, 2000).

Fig. 1. The structure of the artery and the main cell types involved in atherosclerosis and thrombosis.
2. The pathobiology of atherosclerosis

There are several clear phases in the development of atherosclerotic plaque which only in a minority of cases proceed to the final or thrombotic phase which normally is associated with myocardial infraction. The phases are as follows.

2.1. Endothelial activation or dysfunction

The endothelium is considered to be the main interface by which the deleterious effects of high lipid concentrations in the circulation, high blood pressure, toxins from smoking and high glucose levels in diabetes all lead to impairment of endothelium function. The response is the expression of docking proteins on the endothelial surface which permit the adhesion of blood monocytes to the affected areas, attracted by chemokines released from the damaged endothelium (Fig. 2A). Atherosclerosis is quite focal and influenced by local eddies, particular near branch points of blood vessels.

2.2. The inflammatory phase

The entry of docked monocytes into the artery wall and there transformation into macrophages is typical of the inflammatory response. The main purpose of the

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Fig. 2. The early stages in the development of the atherosclerotic plaque: (A) endothelial dysfunction; (B) the inflammatory phase.
macrophages is to get rid of damaged cells and to ward off infection by secretion of reactive oxygen and nitrogen species. Once complete the macrophages return to the circulation, but in the case of atherosclerosis the continued presence of high lipids etc. makes their presence permanent and this can lead to further damage. The macrophages also accumulate lipid (see below) and this phase is associated with the appearance of extracellular lipid deposits or the fatty streak (Fig. 2B). These can be seen even in quite young people.

2.3. The reparative phase

The fact that many atherosclerotic plaques take a lifetime to mature, indicates that there are some opposing defensive mechanisms to the persistent inflammatory condition that prevails within the plaque. This specifically involves the loss of contractile properties of the smooth muscle cells and their change to a more fibroblastic phenotype in which form they biosynthesise and secrete collagen and other matrix glycoproteins (Fig. 3C). The benefit of this change is that through remodelling the fatty tissue is covered with a fibrous cap with great mechanical strength; a kind of scar tissue. Many plaques do not progress beyond this point and there may be no clinical consequence.

There may be multiple phases of lipid deposition and the formation of new fibrous caps and sometimes the artery wall becomes thickened and the plaque protrudes into the arterial lumen and may partly restrict the flow of blood. Furthermore, it is difficult for the oxygen and nutrients to cross the thickened artery and it may become

![Fig. 3. Advanced atherosclerosis: (C) the fibrotic phase; (D) the thrombotic phase.](image-url)
hypoxic and, ultimately, necrotic. To counteract this, the process of angiogenesis, the formation of new blood vessels, may be activated and the nutrient supply improved. The downside of this is that it also permits the plaque to enlarge.

2.4. The thrombotic phase

All atherosclerotic plaques are not the same and may vary in their composition—especially in the number of macrophages (and lipid) and the thickness of the fibrous cap. Those with a thin cap may become vulnerable to mechanical fissure. In this event, the results may be catastrophic. Leakage of pro-thrombotic proteins such as tissue factor will activate the coagulation cascade and the exposure of collagen in the arterial intima will activate blood platelets to form a thrombus (Fig. 3D) which may totally block the coronary blood vessels or migrate to the cerebral blood vessels to precipitate a stroke. In these cases the repair mechanisms have failed and even the normally protective endothelium can become ruptured.

3. Lipoprotein oxidation

A central factor in the aetiology of atherosclerosis is the cholesterol-rich fraction of the blood, the low-density lipoproteins (LDL). LDL diffuse passively into the arterial wall and become trapped through an interaction between its protein moiety, apolipoprotein B$_{100}$ and matrix proteoglycans. In the subendothelium, LDL becomes oxidatively modified from prolonged exposure to reactive oxygen and nitrogen species (ROS and RNS). These are generated by vascular cells, particularly by monocyte/macrophages that have migrated there in an attempt to repair the damage. This oxidised LDL (oxLDL) exerts a pro-inflammatory response in the surrounding cells. It stimulates the endothelium to produce chemokines such as monocyte chemo-tactic protein-1 and growth factors such as macrophage colony stimulating factor, these factors attract monocytes to the area. oxLDL also stimulates the endothelium to express cell adhesion molecules P-selectin, vascular adhesion molecule-1 (VECAM-1), and intercellular adhesion molecule-1 (ICAM-1) on their surface. Expression of these receptors facilitates the entry of recruited monocytes into the arterial wall.

As the disease progresses the oxLDL stimulates the differentiation of monocytes into macrophages. These macrophages take up oxLDL through a number of scavenger receptors leading to the formation of lipid-laden foam cells. The activated macrophages secrete a number of growth factors and cytokines that act to promote the migration and proliferation of vascular smooth muscle cells (VSMC). Proliferating VSMC secrete excess extracellular matrix proteins which form the fibrous caps found on atherosclerotic plaques. In later stages of the disease, in some plaques, the foam cells die leaving a necrotic core of oxidised lipid which is highly thrombogenic in nature. Eventually the atherosclerotic plaque ruptures leading to the formation of platelet-rich thrombi (Fig. 3D).
4. The protective properties of nitric oxide

The cells of the vascular endothelium transduce circulatory stimuli to the arterial wall leading to the regulation of vessel tone, haemostasis, blood pressure and vascular remodelling. It is the ability of the endothelium to synthesise and release NO that accounts for the regulation of these physiological processes. Atherosclerosis, and diseases which predispose to atherosclerosis such as hypercholesterolaemia, diabetes, and hypertension, are characterised by endothelial dysfunction. Here endothelial cell function is compromised, which in turn facilitates altered activity of platelets, neutrophils and the underlying VSMC. Since the endothelium is a major source of NO in the vasculature, loss of normal cellular function would perturb regular NO production. The endothelium possesses a constitutive isoform of nitric oxide synthase (NOS), endothelial NOS (eNOS), and under certain conditions, for example inflammation, has the capacity to express iNOS. In contrast, VSMC have no constitutive isoforms, but can express high levels of iNOS (Kibbe et al., 1999). It is the regulation of these vascular enzymes and the bioavailability of their product that is critical to the development and progression of vascular disease. This review is aimed at providing the reader with an overview of the influence of NO on these important cellular processes such as angiogenesis and apoptosis, the biochemical and molecular mechanisms which regulate NO availability, and how these mechanisms are compromised in cardiovascular diseases. NO has a variety of properties that could be described as vasoprotective or anti-atherosclerotic (Table 1).

Luminal release of NO accounts for a number of the anti-thrombotic and anti-atherosclerotic properties of this molecule. Leukocyte adhesion and their subsequent migration into the subendothelial space are prevented by endothelial-derived NO. The anti-leukocyte actions of NO are achieved by inhibiting the expression of cell surface adhesion molecules P-selectin, vascular adhesion molecule-1 (VECAM-1), and intercellular adhesion molecule-1 (ICAM-1) (Kubes et al., 1991) and preventing the expression of monocyte chemoattractant protein-1 (Zeiher et al., 1995): NO is thought to down regulate gene expression of the adhesive proteins. It has also been proposed that NO can inhibit LDL oxidation by reacting with lipid peroxyl radicals (Rubbo et al., 1994). NO is a potent inhibitor of platelet adhesion to the

Table 1
The vasoprotective actions of nitric oxide and the possible mechanism by which these actions could reduce atherosclerosis

<table>
<thead>
<tr>
<th>Action</th>
<th>Effect</th>
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<tr>
<td>Inhibition of platelet activation and aggregation</td>
<td>Reduced thrombosis</td>
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<tr>
<td>Inhibition of platelet–leukocyte interaction</td>
<td>Possible reduced VSMC proliferation</td>
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<tr>
<td>Inhibition of cell adhesion molecule expression</td>
<td>Reduced leukocyte recruitment</td>
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<tr>
<td>Scavenging of lipid radicals</td>
<td>Reduced leukocyte adhesion and extravasation</td>
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<td>Inhibition of VSMC proliferation</td>
<td>Reduced LDL oxidation</td>
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<td>Inhibition of tissue factor expression</td>
<td>Reduced neo-intima formation</td>
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<td>Reduced production of extracellular matrix</td>
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<td>Reduced thrombosis</td>
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subendothelium and their subsequent aggregation (Radomski et al., 1987; de Graaf et al., 1992). Interestingly platelets also release NO upon stimulation, the more localised release of NO acts to limit the recruitment of platelets into a growing thrombus (Freedman et al., 1997). The activation of platelets at the sites of vascular injury leads to the release of a battery of growth factors, including platelet-derived growth factor and vascular endothelial cell growth factor (VEGF) (Ruggeri, 2002). By inhibiting the release of these agents NO prevents the underlying smooth muscle cells from being exposed to potent proliferating agents. NO also inhibits smooth muscle cell proliferation directly by a cGMP-dependent mechanism (Assender et al., 1992). These actions of NO are critical to disease prevention since the proliferation of VSMC and their over production of extracellular matrix is one of the key events in the progression of atherosclerosis (Lusis, 2000). NO also inhibits the expression of the prothrombotic protein tissue factor (TF), which initiates the intrinsic coagulation pathway, on the endothelial surface. Thus, NO has a number of important vasoprotective roles, and it is obvious that any loss in the availability of NO could significantly increase the progression of vascular diseases.

4.1. Nitric oxide and angiogenesis

Angiogenesis or collateral vessel formation is the name given to the formation of new capillaries from preexisting blood vessels. Angiogenesis is essential to provide oxygen and nutrients to growing tissues. It is a dynamic process that involves proliferation of endothelial cells and production of extracellular matrix by VSMC. NO is one of the key biochemical mediators of angiogenesis. Vascular endothelial cell growth factor (VEGF) is a strong angiogenic stimulus but also elicits the release of NO from endothelial cells (Hood et al., 1998). The ability of VEGF to stimulate the release of NO is crucial for its ability to stimulate angiogenesis. NO then reciprocally activates further biosynthesis of the VEGF receptor (Fig. 4). Human endo-

![Fig. 4. The interrelationship of nitric oxide with proteins linked to angiogenesis.](image-url)
thelial cells grown in a 3-D matrix of fibrin gel form capillary-like structures in response to VEGF, which is blocked by the inhibition of NOS (Babaei et al., 1998). The mechanisms by which NO stimulates angiogenesis is unclear, but is probably a combination of stimulating the proliferation and migration of endothelial cells (Ziche et al., 1994; Murohara et al., 1999), and inhibiting the release of angiostatin, an angiogenic antagonist (Matsunaga et al., 2002). Angiogenesis is stimulated by ischaemia such as that found in tissues supplied by atherosclerotic blood vessels, in order to re-establish normal blood supply. Thus it represents another possible NO-mediated vasoprotective mechanism.

Tissue factor (TF), which is normally associated with the initiation of the coagulation cascade is also essential to angiogenesis and also enhances the expression of VEGF receptor as well as the release of NO. TF, VEGF and NO therefore interact to promote angiogenesis (Fig. 4).

4.2. Nitric oxide and apoptosis

Apoptosis or programmed cell death is a highly regulated process that requires energy for protein synthesis (Hengartner, 2000) and results in a characteristic pattern of fragmented DNA. Often this is a signal-induced event leading to the activation of downstream signalling enzymes. It has been suggested by several studies that apoptosis is important to the pathogenesis of atherosclerosis (de Nigris et al., 2003). Animal models of atherosclerosis have demonstrated that apoptotic endothelial cells macrophages and VSMC are present in plaques (Lutgens et al., 1999). Indeed, it has been suggested that plaque development correlates strongly with the levels of apoptotic cells. Since NO availability is central to atherogenesis it is important to highlight how NO may affect this apoptotic process.

One important family of enzymes involved in this apoptosis is the caspases, a set of cytosolic proteases. The enzymes are activated in response to pro-apoptotic signals, such as the release of cytochrome c from the mitochondria. Caspases cleave a series of important proteins resulting in cell death via protein and nucleic acid degradation, which may be regulated by NO. NO has been demonstrated to have both pro- and anti-apoptotic actions (for review see Hengartner, 2000). NO and peroxynitrite have been shown to induce DNA strand breakages which can promote apoptosis (Szabo and Ohshima, 1997). In macrophages, NO can increase the expression of the tumor suppressor protein p53, which leads to reduced levels of Bax and increased levels of cytochrome c, thereby stimulating apoptosis (Brockhaus and Brune, 1999). However, this must be viewed with caution since the effects of NO are dependent on concentration, cell type and the presence of other confounding factors. For example, in cultured endothelial cells exogenously generated NO inhibited TNF-α-induced apoptosis was partly dependent upon the cGMP pathway. However, when the experiments were repeated at higher concentrations of NO it led to a cGMP-independent increase in apoptosis (Shen et al., 1998).

The inhibition or induction of apoptosis by NO and NO-derived species is influenced by several other factors. These include the levels of Bcl2 family proteins, antioxidants, prostaglandins and glucose. The presence of, and interplay between, these
factors with varying concentrations of NO can determine if NO becomes pro- or any apoptopic. The levels of Bcl-2 and a number multiple related proteins have been shown to be involved both pro-apoptopic (Bax, Bld) and anti-apoptopic (Bcl2, Bcl-XL) events. NO has the capacity to inhibit caspase-3, and quite probably other caspases, leading to downstream changes in Bax and Bcl2 ratio and thus determining the apoptotic potential of the cell (Li et al., 1997; Kim et al., 1998). Again it is the levels of available NO which will determine its actions on the activity of caspases. Overall, NO has a dichotomous influence on apoptosis depending on the relative concentration of NO and the presence of other cellular factors. Apoptosis and necrosis are prominent features of advanced atherosclerotic lesions (de Nigris et al., 2003), although the importance of NO-induced and NO-inhibited apoptosis to the pathogenesis of vascular disease is yet to be fully resolved.

4.3. Nitric oxide and smooth muscle cell proliferation

VSMC proliferation is a fundamental component of vessel wall remodelling in response to both injury and disease. The development of an atherosclerotic plaque is associated with VSMC proliferation and neointima formation. The reduced NO bio-availability observed in cardiovascular diseases is thought to be intimately involved in this process. NO-donors, compounds that release NO, have been shown to inhibit VSMC proliferation in tissue culture studies using cells derived from rabbit, rat and human origin (Garg and Hassid, 1989; Mooradian et al., 1995; Tanner et al., 2000). Although there were differences in sensitivity to the effects of NO between the different cell types, the actions of NO were generally found to occur at physiologically relevant concentrations. Several studies have also demonstrated that the effects of NO donors can be replicated by using cell permeable analogues of cGMP, for example 8-bromo-cGMP, suggesting a cGMP/PKG dependent-mechanism (Garg and Hassid, 1989; Assender et al., 1992). It should be noted that NO can inhibit protein synthesis directly suggesting that inhibition of VSMC replication could occur at several levels (Garg and Hassid, 1993; Sarkar et al., 1997a,b).

Evidence also suggest that NO can influence VSMC proliferation in vivo. The administration of L-arginine or NO-donors to animal models of atherosclerosis inhibit VSMC proliferation in vivo as evidenced by reduced intimal thickening (Naruse et al., 1994; Groves et al., 1995; Cooke et al., 1992, Hamon et al., 1994). However, in these studies it is unclear whether NO directly influences the VSMC or exerts its effects through the inhibition of platelets and leukocytes. The activation of leukocytes and platelets lead to the release of factors known to promote VSMC proliferation. The increased NO availability in these animal studies would act to inhibit leukocyte and platelet activation and thus VSMC proliferation.

5. Factors that affect the bioavailability of nitric oxide in the cardiovascular system

The reduced bioavailability of NO is thought to be one of the central factors common to vascular disease, although it is unclear whether this is a cause of, or result of,
endothelial dysfunction. There are a number of factors which could potentially affect either the production of NO, or the ability of NO to diffuse to its cellular targets. Disturbances in NO bioavailability lead to altered regulation of key physiological and cellular processes such as vasodilatation, platelet function, angiogenesis, apoptosis and smooth muscle cell proliferation. In the following section, the factors affecting both the production and availability of NO will be discussed (Fig. 5). Furthermore these changes in NO bioavailability will be discussed in the context of the physiological processes they regulate. It is important to highlight these factors and explore their importance both individually and in combination.

5.1. Inhibition of nitric oxide synthase expression and activity

The normal function of eNOS and the tonic production of NO are associated with a fully functional and compliant endothelium. However enzyme activity can be affected by a number of factors. One of the more recent areas of eNOS function that has been explored are polymorphic variations of the enzyme and how these may be related to cardiovascular risk. The human eNOS gene contains 26 exons that span a region of 21 kb (Marsden et al., 1993). There are three classes of allelic variation that have been identified including variations in the intron regions, promoter regions and open reading frame (for review see Wang and Wang, 2000). Variations in the eNOS gene could have a plethora of effects on the enzyme including altered protein stability, altered post-translational processing such as acylation or phosphorylation, altered intracellular distribution or altered cofactor association: all of which could influence the enzymatic activity. Since the polymorphic variants were discovered a

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**Fig. 5.** Factors that may contribute to a reduction in nitric oxide bioavailability. One of the major contributing factors to the progression of cardiovascular diseases is a loss of NO-dependent actions. It is proposed that this is due to a reduction in the bioavailability of NO. There are a variety of factors which could reduce NO availability including (1) a reaction in the availability of the substrate L-arginine, (2) increased concentration of circulating inhibitors such as ADMA, (3) altered levels of eNOS expression, (4) perturbed signal transduction reducing agonist-induced eNOS activation, (5) reduced availability of tetrahydrobiopterin (BH4) an essential co-factor, or (6) the destruction of NO by other free radical species.
number of publications have performed linkage studies whereby a particular eNOS polymorphism, resulting in compromised enzyme function, is related to the alterations in the incidence of disease: the findings of these studies have proved inconclusive, and often contradictory (Wang and Wang, 2000). However, this is still a relatively new and plausible area of investigation, which may aid our understanding of how NO affects cardiovascular diseases.

Another important aspect of eNOS function is the availability of substrates and co-factors. It is highly unlikely that L-arginine becomes a rate-limiting factor since the $K_m$ of eNOS is approximately 2.9 $\mu$M (Stuehr et al., 1991), while the cytoplasmic levels of arginine are in the region of 600–900 $\mu$M (Baydoun et al., 1990). In addition the plasma concentration of arginine is 100 $\mu$M (Cynober, 2002), and therefore there should always be an adequate supply of arginine for optimal enzyme activity. The synthesis of NO from L-arginine can be blocked pharmacologically by a variety of arginine analogues, which have been used to elucidate the mechanisms of action of NO. In the cardiovascular system these structural analogues can induce vasoconstriction, increase thrombus formation and potentiate atherogenesis (Nava et al., 1995). One of these inhibitors, $N^G$-mono-methyl-L-arginine (L-NMMA), and another asymmetrical dimethylarginine (ADMA) are potentially very interesting since they are naturally occurring compounds that circulate in the plasma (Vallance and Leiper, 2004). Hence it was postulated that many of the changes in NO-production in disease states may be due, at least in part, to the generation of endogenously circulating enzyme inhibitors. ADMA is present in higher concentrations than L-NMMA and has therefore generated more interest. The levels of AMDA are regulated by a dynamic process, as it is synthesised by the methylation of arginine within proteins, is released as result of proteolysis, and metabolised to citrulline by the enzyme dimethylaminohydrolase (Leiper et al., 1999). Increased plasma AMDA levels have been described in a number on vascular disorders including hypercholesterolaemia, hypertension and has proposed as a strong predictor of the risk of acute coronary events (Vallance and Leiper, 2004).

Tetrahydrobiopterin (BH$_4$), an essential co-factor for NOS, is also thought to be of key importance to normal endothelial cell and eNOS function. BH$_4$ is an important reducing agent which is essential for the correct functioning of a wide variety of enzymes including aromatic amino acid hydroxylases. BH$_4$ also has profound effects on the structure and function of NOS including stabilisation of the dimeric structure, facilitating and enhancing the binding of L-arginine (Cosentino and Luscher, 1999). In vitro studies have demonstrated that reduced availability of BH$_4$ results in the ‘uncoupling’ of NOS, leading to the production of superoxide anion ($O_2^-$) and $H_2O_2$ (Stroes et al., 1998). Interestingly all three isoforms of NOS behave in a similar fashion when BH$_4$ availability is reduced. Hence it is conceivable that NOS itself could contribute to the oxidative stress associated with cardiovascular disease. In culture, endothelial cell production of NO is augmented if the culture medium is supplemented with BH$_4$ (Werner-Felmayer et al., 1993). This should be viewed with caution since it has also been demonstrated that BH$_4$ levels in endothelial cells fall with increased passage number (Rosenkranz-Weiss et al., 1994). Studies on isolated blood vessels have demonstrated that inhibition of BH$_4$ synthesis impairs endothelial func-
tion, again suggesting a role for reduced BH$_4$ and disease (Cosentino and Katusic, 1995; Kinoshita et al., 1997). However, the uncoupling of NOS has yet to be demonstrated in vivo. Since BH$_4$ availability may be a critical factor in optimal eNOS activity a number of studies have addressed how the supplementation of BH$_4$ affects vascular diseases. These studies have demonstrated that short-term administration of BH$_4$ led to improvements in endothelial cell function (Heitzer et al., 2000a,b; Stroes et al., 1998; Maier et al., 2000; Ueda et al., 2000). The mechanism is uncertain although the authors suggest an increase formation of NO is the most likely explanation.

5.2. The scavenging of nitric oxide by reactive oxygen species

In many vascular pathologies a combination of altered rates of NO production along with an increased removal of NO leads to an apparent reduction in the bioavailability of NO. This results in the loss of NO signalling and in some cases the generation of toxic NO-derived species. Currently the foremost mechanism for the loss of bioavailable NO is thought to be the reaction of NO with superoxide (O$_2^-$). In normal physiology O$_2^-$ is detoxified by the enzyme superoxide dismutase (SOD) to H$_2$O$_2$ and eventually water, therefore preventing its interaction with NO. However, if the levels of O$_2^-$ increase sufficiently, NO is able to outcompete SOD for O$_2^-$ (Fig. 6) The subsequent reaction is diffusion-limited for NO and is

![Fig. 6. The formation of peroxynitrite by the reaction of nitric oxide and superoxide anions. Endothelial cells have the capacity to produce NO and superoxide, the reactants required for the production of peroxynitrite, in both extra- and intracellular compartments. Under normal physiological conditions NO produced from L-arginine diffuses to its target soluble guanylyl cyclase (sGC) (1), while the levels of superoxide anions (O$_2^-$) are kept low by conversion to hydrogen peroxide (H$_2$O$_2$) and then water (2). However in the levels of O$_2^-$ increase such that the interaction with NO becomes the favoured reaction (3) leading to the production of peroxynitrite and the potent nitrogen dioxide (NO$_2^-$) and hydroxyl radicals (·OH). Under certain conditions hydroxyl radicals can been formed from hydrogen peroxide through the Fenton reaction (4).](image_url)
approximately six-times faster than the dismutation of $O_2^-\cdot$ by SOD (Beckman and Koppenol, 1996). This reaction has the dual effect scavenging NO and thereby reducing its availability, but also of producing the potent oxidant peroxynitrite (Fig. 6). Once formed, peroxynitrite can chemically modify amino acids, nucleic acids and thiol containing proteins and peptides (Koppenol et al., 1992). The apparent $pK_a$ of peroxynitrite is 6.8 (Beckman and Koppenol, 1996), so at pH 7.4, 20% of peroxynitrite will be protonated to form peroxynitrous acid, which is membrane permeable. Peroxynitrous acid, a strong oxidant itself, decomposes to form nitrogen dioxide radical ($NO_2^\cdot$) and a species similar in reactivity to hydroxyl radical ($OH^\cdot$) (Beckman and Koppenol, 1996). The $NO_2^\cdot$ attacks phenol groups to produce nitrophenols (Beckman et al., 1992). In biological systems this leads to modification of tyrosine residues to produce 3-nitrotyrosine. The formation of 3-nitrotyrosine can be thought of as a stable biological marker for the formation of peroxynitrite, and has been shown to be elevated in a number of cardiovascular diseases (see introductory chapter and Greenacre and Ischiropoulos, 2001). The formation of nitrotyrosine intracellularly can also have profound effects on cell function. Peroxynitrite-induced nitration of peptides has been shown to reduce their phosphorylation when exposed to specific protein kinases (Li et al., 1998), suggesting that protein nitration may interfere with protein phosphorylation signalling pathways (Mondoro et al., 1997; Low et al., 2002). Peroxynitrite can also induce physiological effects such as relaxation of aortic rings (Dowell and Martin, 1997) and activation of soluble guanylyl cyclase, probably through the formation of nitrosothiols (Mayer et al., 1995).

Under normal physiological situations there is insufficient $O_2^-\cdot$ available to drive the formation of peroxynitrite, but in disease states the production of $O_2^-\cdot$ is increased. $O_2^-\cdot$ is generated by the one electron reduction of oxygen by NADPH or NADH oxidases and particularly by respiratory chain enzymes in the mitochondria (Griendling and Ushio-Fukai, 1997). Endothelial cells and platelets constantly produce low levels of $O_2^-\cdot$, which are significantly increased when the cells become activated (Matsuba and Ziff, 1986; Maresca et al., 1992). Studies with experimental animals suggest that many vascular diseases are associated with increased formation of $O_2^-\cdot$. The enzymic origin of $O_2^-\cdot$ may vary in different types of disease and could potentially involve NAD(P)H oxidases, xanthine oxidase, lipoxygenase and NOS. However studies with both rabbit and human vessels suggest that the primary enzyme(s) responsible for $O_2^-\cdot$ production in the vasculature are the NAD(P)H oxidases. The best characterised NAD(P)H oxidase is that found in neutrophils and fulfils a role in the respiratory burst associated with destruction of phagocytosed microorganisms. It has been well established that neutrophils infiltrating the subendothelial space are a potent source of reactive oxygen species. Although significant evidence has recently emerged to suggest that NADH oxidases primarily present in endothelial cells, but also vascular smooth muscle, are a major source of $O_2^-\cdot$ (Souza et al., 2001). These enzymes have been shown to be upregulated in a variety of vascular pathologies (Guzik et al., 2000). Hence a dysfunctional endothelium can contribute to the reduced bioavailability of NO by releasing $O_2^-\cdot$, thereby increasing the propensity of peroxynitrite formation. A hypothesis supported by the observation
of peroxynitrite formation at the surface of cultured endothelial cells (Kooy and Royall, 1994).

Clearly the reaction of NO and O$_{2}^{-}$ accounts for a significant part of the loss of bioavailable NO. However the use of O$_{2}^{-}$ scavengers does not completely restore NO bioactivity, suggesting that other reactions could also lead to the removal of NO. One such interaction is between NO and lipid oxidation products, in fact NO and NO-derived species can interact with lipids at several levels. In one sense this could be viewed as cardioprotective, since NO is a potent inhibitor of lipid oxidation in vitro (O'Donnell et al., 1997; Hogg et al., 1993). NO reacts with lipid peroxide radicals (LOO$^{\cdot}$) to terminate lipid peroxidation via the production of an organic peroxynitrite. A number of elegant studies have also suggested that NO can interact in a controlled fashion with the prostaglandin signalling cascade. NO can regulate the enzyme prostaglandin endoperoxide H synthase (PGHS) (Davidge et al., 1995; Salvemini et al., 1993), leading to stimulation of prostaglandin production in both endothelial cells and platelets. Hence changes in NO levels could have consequences for the regulation of prostaglandin production in the vasculature. PGHS and lipoxygenase also catalyse the consumption of NO via reactions with intermediates produced by these enzymes. Certainly in platelets the rate consumption of NO was high and suggests that this may also represent a mechanism by which NO could be removed by vascular cells under both physiological and pathological conditions (O'Donnell et al., 2000). Thus, the increased production of oxidants by vascular cells contributes to the removal of NO. This not only reduces NO bioavailability but also produces highly reactive secondary oxidants that can further compromise cell function.

6. Nitric oxide and cardiovascular disease

The dysregulation of NO metabolism as a contributing factor has been demonstrated in a number of vascular pathologies including atherosclerosis, hypercholesterolaemia, diabetes, hypertension and septic shock. The common factor is endothelial dysfunction in which an alteration in NO bioavailability is likely to play an important role. In the following section the role of NO in each of these diseases will be explored.

6.1. Atherosclerosis and hypercholesterolaemia

Atherosclerosis is a progressive disease that begins with fatty streaks and through a process of lipoprotein deposition and cellular dysfunction progresses to complicated plaques; NO in this setting can have both pro- and anti-atherosclerotic effects. The anti-atherosclerotic effects rely upon sufficient amounts of NO to regulate platelet function, leukocyte adhesion and extravasation, inhibit LDL oxidation and prevent SMC proliferation. However, endothelium-mediated vasorelaxation in vitro and in vivo, in both man and laboratory animals, is compromised in atherosclerosis, suggesting a loss of NO activity (Forstermann et al., 1988). The reduced
bioavailability of NO is thought to occur early in the development of atherosclerosis. Patients with hypercholesterolaemia show impaired acetylcholine-induced vasorelaxation prior to the development of atherosclerosis as visualised by angiography. The loss of NO has considerable effect on the development of the disease. In the early stages of the disease reduced NO would leave the endothelium vulnerable to increased leukocyte diapedesis and increase the possibility of LDL oxidation in the subendothelial space. Furthermore, the smooth muscle cell (SMC) proliferation associated with neointimal thickening would proceed unchecked. In the final stages, loss of NO could aggravate platelet activation, leading to thrombosis and myocardial infarction. Hence reduced bioavailability could contribute to the progression of atherosclerosis at all stages of the disease process.

A plethora of studies have attempted to address the reasons of the loss of NO. The expression of eNOS has been addressed in a range of studies often with contradictory results. In hypercholesterolaemia the production of NO actually increases, and may be due, at least in part, to an up regulation of NOS expression in the endothelium. Human atherosclerotic lesions showed a reduced expression and production of eNOS, while in hypercholesterolaemic animals the levels of NOS were the same as control animals (Shaull, 2003). Hence a clear consensus has not been reached with regard to altered expression of the enzyme in vivo. In cell culture systems the influence of lipoproteins on eNOS expression has proved just as controversial. Native LDL and HDL have no effect on NOS expression. Whereas the studies assessing oxLDL have shown both increased and decreased expression of eNOS (Hirata et al., 1995; Liao et al., 1995; Vidal et al., 1998). Some modified lipid components of oxLDL can also increase the expression of eNOS (Ramasamy et al., 1998). Although the influence of lipoproteins on eNOS expression is still unclear, there is evidence to suggest that a lipoprotein-induced reduction in enzyme activity may be the major site of action. Receptor-mediated activation of eNOS results in the translocation of eNOS from membrane caveolae and the disassociation of caveolin-1, a negative regulator of NOS. The incubation of endothelial cells with oxLDL results in the translocation of eNOS from the membrane. However, the pattern is distinct from that induced by agonists in that the enzyme remains in the unphosphorylated form and is inactive. oxLDL is thought to deplete caveolae of cholesterol resulting in the disruption of the receptor-eNOS activation complex (Blair et al., 1999). Other lipoproteins also have important effects on the bioavailability of NO either by decreases biosynthesis or enhanced formation of superoxide. LDL, VLDL and chylomicron remnants have all been shown to have negative effects (Goulter et al., 2003; Takahashi et al., 2002), Fig. 7. More recently HDL has been shown to have its own direct effects on enhancing NO biosynthesis via the SRB-1 receptor and the protein kinase B/Akt pathway, although possible mechanisms have also been suggested (Yuhanna et al., 2001; Mineo et al., 2003). This is supported by a report demonstrating HDL to improve vascular function in hypercholesterolaemic subjects (Spieker et al., 2002). In addition apolipoprotein apo E, present on HDL, enhances NO synthesis in some cell types (Sacre et al., 2003).

L-arginine supplementation has profound positive effects on a range of antiatherosclerotic actions of NO (Fig. 7). In hypercholesterolaemic rabbits L-arginine
has a restorative effect on vasodilator function (Cooke et al., 1991), inhibits platelet function and leukocyte adhesion. Similar effects have also been observed in studies on patients with atherosclerosis (Drexler et al., 1994). Initially it was proposed that the excess arginine facilitated an increased production of NO, although this was never confirmed in vivo. At a cellular level this mechanism seems unlikely, since NO production is largely independent of extracellular L-arginine concentrations. The cytoplasmic concentrations of L-arginine in cultured endothelial cells is ranges between 600 and 900 $\mu$M (Baydoun et al., 1990) depending on conditions, while the plasma concentrations of L-arginine are approximately 100 $\mu$M (Cynober, 2002); both of these are far higher than the $K_m$ for eNOS which is 2.9 $\mu$M (Stuehr et al., 1991). This would imply that it is highly unlikely that L-arginine would become rate limiting. Thus the mechanism by which L-arginine supplementation improves NO-mediated actions remains unclear. eNOS activity may also be affected by ADMA since increased circulating levels of this endogenous inhibitor have been demonstrated in patients with coronary atherosclerosis (Miyazaki et al., 1999), peripheral vascular disease (Boger et al., 1997) and hypercholesterolaemic rabbits (Bode-Boger et al., 1996). It is interesting to speculate that excess L-arginine could effectively compete with ADMA for NOS binding thereby abrogating the effects of ADMA.

Although the studies highlighting that eNOS expression and activity may be altered in atherosclerosis, the most common feature of the disease is oxidative stress, and there is strong evidence that NO plays a central role in this process. As highlighted earlier the increased production of $O_2^-$ is closely associated with the loss of NO. The dysfunctional arteries found in atherosclerotic and hypercholesterolaemic animals produce larger quantities of $O_2^-$ and probably other reactive oxygen species (ROS), than do healthy vessels (Ohara et al., 1993). Studies infusing polyethylene glycate SOD into atherosclerotic rabbits resulted in an improved endothelium-dependent relaxation, although one study in humans failed to confirm this (Mugge et al., 1991). Animal models of early atherosclerosis have shown that hypercholesterolaemia causes a twofold increase in NADPH-derived $O_2^-$ (Warnholtz et al., 1999), while in late atherosclerosis this increased to threefold (Miller et al., 1998). This was reversed by endothelial denudation, implicating the endothelium as the primary source of $O_2^-$. NOS may also contribute to the increased $O_2^-$ through
the uncoupling mechanism suggested in Chapter 1. The in vitro studies demonstrating that reduced BH₄ levels induce NOS to produce O²⁻ have yet to be confirmed in vivo. Although several studies, both human and animal, have confirmed that BH₄ supplementation has a beneficial effect on endothelial-dependent relaxation (Stroes et al., 1997; Higman et al., 1996).

6.2. Effects of anti-oxidants on endothelium-dependent vasodilatation

Since the oxidative and nitrosative stress found in atherosclerosis is associated with impaired endothelial-dependent relaxation many workers have examined the influence of anti-oxidant therapy on endothelial function. These studies have concentrated on α-tocopherol (vitamin E), and ascorbic acid (vitamin C), since these are the principal lipid and water-soluble anti-oxidants found in the body. In addition a smaller number of studies have addressed other anti-oxidants, including β-carotene and probucol.

The influence of ascorbic acid on NO bioavailability has generally been very positive in both human and animal studies. The extent to which ascorbic acid improves the disease is wide-ranging since the studies vary in nature in terms of both amounts of ascorbic acid used and type of outcome measured. Short-term infusion of ascorbic acid, maintaining the levels with physiological range, improved brachial artery flow-mediation dilation in patients with coronary heart disease (Levine et al., 1996), endothelial function of conduit arteries in patients with chronic heart failures (Hornig et al., 1998) and microvascular function in hypercholesterolaemia (Ting et al., 1997). In addition, long-term infusion of ascorbate into patients with coronary heart disease also obfuscates endothelial dysfunction (Gokce et al., 1999). The possible mechanisms by which ascorbate improves NO bioavailability is unclear. Certainly it is a powerful anti-oxidant that has the capacity to scavenge O²⁻ (Jackson et al., 1998), but the kinetics of the ascorbate-O²⁻ reaction probably preclude this mechanism. The reaction between NO and O²⁻ is approximately 10,000 times faster than the reaction of ascorbate and O²⁻. Thus, under these conditions peroxynitrite formation would be the favoured reaction. The scavenging of O²⁻ by supraphysiological concentrations of ascorbate acts to preserve NO and may explain the findings in some studies, but in studies where only physiological concentrations of ascorbate are used this mechanism is a less convincing explanation. Recent evidence suggests that the improvement of NO-mediated vascular function seen with ascorbate occurs at the cellular level. Firstly, it may occur by influencing the levels of glutathione, a tripeptide found in the plasma and cytosol. Glutathione improves endothelial dysfunction associated with the loss of NO, and may be important for the cellular actions of NO (Ghigo et al., 1993; Prasad et al., 1999). The anti-oxidant properties of ascorbate act to spare intracellular glutathione oxidation, thus helping to preserve NO. Secondly, ascorbate may directly enhance the production of NO by eNOS. Incubation of endothelial cells with physiological levels of ascorbate leads to enhanced synthesis of NO. It is proposed that ascorbate stabilises the dimeric structure of NOS by improving the binding of BH₄ (Heller et al., 1999, 2001). Alternatively NO may
prevent the cellular oxidation of BH$_4$ (Huang et al., 2000). Although the mechanisms are yet to fully deciphered, it appears that ascorbate does improve NO bioavailability.

In contrast to ascorbate, the beneficial effects of $\alpha$-tocopherol are far from convincing. In animal studies, $\alpha$-tocopherol prevents the endothelial dysfunction associated with both atherosclerosis and hypercholesterolaemia (Keaney Jr et al., 1993; Andersson et al., 1994), although this was dependent upon the dose given. In contrast, most patients based studies have generally failed to demonstrate beneficial effects (McDowell et al., 1994; Chowienczyk et al., 1998; Elliott et al., 1995), although there are some exceptions (Heitzer et al., 1999a). The mechanism by which $\alpha$-tocopherol exerts its effects in animals is unclear, but is thought that it may aid the endothelium to resist the effects of oxLDL (Keaney Jr et al., 1993; Cathcart et al., 1988) and thus preserving the function of eNOS. The lack of consensus in human studies, and the difference between animal and patient based work can partially be explained by experimental design. Studies in patients have concentrated on subjects who have either established disease or risk factors for atherosclerosis. In contrast many of the animal studies studied the influence of $\alpha$-tocopherol early in the disease process. Hence it could be speculated that $\alpha$-tocopherol may still be beneficial but in a more preventative role. An alternative strategy to combat the oxidant-mediated loss of NO is to target the enzymatic anti-oxidant defence mechanisms. SOD, catalase, glutathione peroxidase can all potentially affect the bioavailability of NO (Patel et al., 2000). Infusion of SOD has been shown to be beneficial in animal models of atherosclerosis (Mugge et al., 1991), but this has failed to be reproduced in human studies. In general anti-oxidant therapy does seem to prevent the development and progression of atherosclerosis, but the influence on NO axis is unclear. The main treatment for patients with cardiovascular disease is a class of drugs known as the statins, which lower plasma LDL cholesterol by competitively decreasing the activity of the rate-limiting enzyme in cholesterol biosynthesis HMG-CoA reductase and by increasing the number of LDL receptors on the liver and other tissues. Several studies have now shown that the statins also improve vascular function (vasodilatation) independently of their lipid lowering action (Wassmann et al., 2004). This is known as a pleiotropic effect, one unconnected with the primary action of the drugs. Recent evidence suggests that statins may lower plasma levels of ADMA (Lu et al., 2004). They therefore both lower lipids (which anti-oxidants do not) and improve vascular function and have save the lives of many patients.

6.3. Potential pathological roles of nitric oxide in cardiovascular disease

As indicated above, the increased production of O$_2^-/\text{HO}_2$ could be critical to NO bioavailability and disease progression, due to the possible formation of peroxynitrite. Seminal work by Beckman et al. (1994) demonstrated a high degree of staining for nitrotyrosine in atherosclerotic plaques at post-mortem, suggesting that increased levels of peroxynitrite are formed in the areas of atherosclerosis (Beckman et al., 1994). The effects of peroxynitrite formation in areas of atherosclerosis is twofold, firstly, loss of bioavailable NO, and secondly, the formation of secondary and tertiary pro-atherosclerotic oxidants. It should be made clear that the main source of
the NO which promotes oxidation of lipoproteins in the form of peroxynitrite is likely to be the macrophages rather than the endothelium. Nevertheless, the loss of NO would abrogate its anti-platelet and anti-leukocyte actions. However, the pro-oxidant effects of peroxynitrite formation are for more damaging. Peroxynitrite, but not NO, has the capacity to induce the oxidation of LDL, a critical event in the development of atherosclerotic plaques (Graham et al., 1993). Unlike other reactive species, peroxynitrite has the capacity to modify both lipid and protein moieties of LDL. Peroxynitrite can deplete LDL of the anti-oxidants, α-tocopherol and β-carotene, and induce the oxidation of lipids to produce lipid hydroperoxides and isoprostanes (Patel et al., 2000). Protein moiety of LDL, apoB100, is modified by both direct and indirect mechanisms. Peroxynitrite directly oxidises cysteine and tryptophan, while modification of lysine, and arginine probably occurs via secondary reactions with lipid hydroperoxide radicals. The modification of LDL converts it to a highly atherogenic particle, which promotes SMC dysfunction and foam cell formation. There is strong evidence for this as the fingerprint of peroxynitrite, the presence of nitrated proteins is present in atherosclerotic plaque as originally shown by Beckman et al. (1994) and also the location of these modified proteins is close to that of the macrophages in the plaque. Interestingly peroxynitrite also causes the oxidation of BH4 rendering it inactive (Kuzkaya et al., 2003). It has been suggested that the beneficial effects of BH4 supplementation in atherosclerosis may be by replacing lost BH4.

The excess production of NO by macrophages may have another negative effect. As indicated earlier NO keeps smooth muscle cells in their contractile phenotype and prevents proliferation to the fibroblastic form. In the reparative phase excess NO may impair the formation of the fibrous cap or reduce the mechanical strength of the cap. Macrophage accumulation appears to reduce the thickness of this cap. However, surgically induced atherosclerosis in animal models, which is mainly due to smooth muscle cell proliferation after vein grafting, increased availability of NO by gene transfection of NOSII prevents this occurring and keeps the new vessels open (Schwentker and Billiar, 2002).

7. Diabetes and nitric oxide

Diabetes mellitus is associated with increased rates of morbidity and mortality caused primarily by the accelerated development of atherosclerotic disease (Beckman et al., 2002). Similar to other atherosclerotic pathologies diabetic vascular disease is characterised by endothelial dysfunction. Human IDDM and NIDDM, and animal models of IDDM are all associated with a reduced endothelium-dependent relaxation, although the response to exogenous NO-donors is often normal (Durante et al., 1988; Diederich et al., 1994; Johnstone et al., 1993; McVeigh et al., 1992; Williams et al., 1996), suggesting a reduced bioavailability of NO. This has been strengthened by data demonstrating reduced accumulation of cGMP in response to acetylcholine in vascular tissues (Abiru et al., 1991). Similar to other vascular pathologies a plethora of studies have attempted to address the mechanisms leading
to reduced NO bioavailability in diabetes. These experimental studies need to be viewed with caution since there are several variable factors which could affect the disease: in particular the two types of diabetes, IDDM and NIDDM, may have different cardiovascular pathologies.

As with atherosclerosis and hypercholesterolaemia the evidence pertaining to either altered expression or activity of NOS is mixed. In studies where human endothelial cells are exposed to hyperglycaemic conditions eNOS mRNA has been shown to both increase and remain unchanged depending on the incubation time (Mancusi et al., 1996; Cosentino et al., 1997). Diabetic animal models have demonstrated both increased mRNA and protein for eNOS (Pieper et al., 1997), yet despite this other studies have shown that there is reduced L-citrulline formation (Rosen et al., 1995). Thus the expression of NOS is normal or maybe slightly enhanced but its activity is compromised, suggesting a problem with regulation of the enzyme. There have been no studies measuring NO production directly in diabetic arteries, although a number of studies have attempted to address NO production by the measurement of the metabolic products of NO, nitrite and nitrate. In animal studies nitrite/nitrate levels are elevated (Maree et al., 1996), while in humans no differences have been found when compared to controls (Schmetterer et al., 1997). However, as with all studies measuring nitrite/nitrate the result should be viewed with caution, since in several studies the nitrate intake via water was not accounted for. In addition, streptozotocin, the agent most often used to induce diabetes in animal models can act as an NO-donor (Kwon et al., 1994).

The levels of arginine in diabetes have been addressed in numerous studies, again with very mixed results. In animal models the general trend suggests that L-arginine levels in both plasma and endothelial cells is reduced (Rosen et al., 1995; Pieper and Peltier, 1995), however the supplementation of animals with L-arginine did not always result in improved vasodilation (Pieper et al., 1997). In human subjects both reduced and normal levels of arginine have been reported (Beckman et al., 2002). L-arginine supplementation in diabetic patients has been shown to increase plasma cGMP and L-citrulline levels, and endothelium-dependent relaxation in IDDM (Beckman et al., 2002). In contrast, one study found that supplementation of a diabetic cohort with high concentrations of L-arginine had no effect at all on vasodilator function (MacAllister et al., 1995). The effects of increasing the L-arginine concentration in vivo should be viewed cautiously, since the amino acid can also increase insulin secretion. Insulin has been shown to induce NO-dependent relaxation of blood vessels (Steinberg and Baron, 1999) and activate eNOS in cultured endothelial cells (Montagnani et al., 2001). The physiological importance of insulin-induced NO release is yet to be fully evaluated, although loss of insulin in diabetic subjects could contribute to a reduction in the bioavailability of NO.

Evidence for the destruction of NO by reactive oxygen species in diabetes comes from several different sources. The levels of plasma nitrotyrosine in diabetics are elevated in comparison with age-matched controls, suggesting enhanced O$_2^-$ and therefore peroxynitrite formation (Ceriello et al., 2001). Certainly endothelial cells exposed to hyperglycaemic conditions in vitro produced significantly great levels of O$_2^-$ (Hink et al., 2001). Interestingly, diabetic subjects have been shown to have
reduced anti-oxidant capacity which could favour oxidative stress (Beckman et al., 2002). While in animal models the impaired endothelium-dependent relaxation has to be abrogated after acute incubation with SOD (Hattori et al., 1991; Ohishi and Carmines, 1995; Rosen et al., 1995). All this evidence is suggestive of a major role for the destruction of NO by O$_2^-$ in diabetes-induced vascular dysfunction.

8. Nitric oxide and hypertension

NO is crucial to the maintenance of normal blood pressure (Huang et al., 1995) and therefore its relationship to essential hypertension has been the subject of intense investigation. As with coronary artery disease and diabetes initial evidence suggesting a NO-dependent component of the disease came from studies assessing endothelium-dependent vasodilatation. A number of studies have demonstrated the impairment of NO-mediated vasodilatation in brachial (Panza et al., 1995), coronary (Treasure et al., 1993) and renal arteries (Higashi et al., 1995) in patients with essential hypertension compared to controls. The reason for reduced NO dependent vasorelaxation is unclear. Evidence suggests that the impaired NO responses are genetically determined, since basal NO production is impaired in offspring of patients with essential hypertension (Kelm, 2003). This work also suggests that impaired NO production does not occur simply as a consequence of the disease but may be integral to disease development. The possible genetic component of the disease has led to a plethora of studies assessing the role of NOS polymorphisms in essential hypertension. As with many other studies the results are inconclusive. The polymorphism (894G to T) in exon 7 causes the conversion of Glu to Asp at position 298 and is thought of as one of the most promising polymorphisms in relation to disease. This polymorphism has an increased frequency in hypertensives compared to controls and was associated with a resistance to antihypertensive therapy, although NOS activity was not addressed (Miyamoto et al., 1998). Similarly a multi-centred assessment of the polymorphism in Japan found an increased frequency in the hypertensives (Shoji et al., 2000). However, a study assessing as similar number of hypertensive subjects found no difference in polymorphisms frequency (Lacolley et al., 1998). Other studies have addressed the frequency of a number of different polymorphisms in hypertension with varying degrees of success (Wang and Wang, 2000). However it is important that these findings should be considered in the context of the disease. Essential hypertension is a multifactorial disease where the interactions between neuronal, hormonal and cellular signalling processes all contribute to the pathogenesis. eNOS gene polymorphisms probably led to only very subtle changes in NO production, and thus the contribution of these polymorphisms may only become important in presence of other contributing factors. Therefore the group of patients used for each study is a critical determinant of the results.

Several studies have demonstrated impaired endothelial dysfunction in essential hypertension, which is associated with a blunted response to NO-mediated effects. Although it is unclear whether this represents reduced synthesis or increased consumption of NO. The vasodilator effects of the NO-donor sodium nitroprusside,
was identical in both hypertensive and normotensives (Calver et al., 1992), suggesting that the cGMP-signalling pathway in smooth muscle cells is normal. Interestingly, hypertensive subjects have defective vascular responses to acetylcholine, but normal responses to β-adrenergic stimulation by bradykinin. These findings suggested that endothelial dysfunction associated with essential hypertension is due to a selective abnormality of the signalling pathways leading to NOS activation. Indeed, the hypotensive effects of angiotensin-converting enzyme (ACE) inhibitors in hypertensive subjects, has been shown to occur through increased formation of bradykinin and leading to enhanced NO formation (Ignjatovic et al., in press). Another study has suggested that the depressed levels of basal NO in hypertensive subjects could be due to increased circulating levels of ADMA (Achan et al., 2003), although this has not been confirmed.

The spontaneously hypertensive rat model was also used to show that NO production was normal, but O$_2^-$ production is elevated and led to increased oxidation of NO and resulting in decreased vasodilatation (Heitzer et al., 1999b). The O$_2^-$ could arise from a variety of different sources including NAD(P)H oxidases and cyclo-oxygenase activity, since the activity of both enzymes is seen to be unregulated in hypertension (Heitzer et al., 1999b; Taddei et al., 1997). This evidence is corroborated in human subjects where infusion of ascorbic acid reversed the impaired endothelium-dependent relaxation in hypertensives, again suggestive of an important role for oxygen free radicals (Taddei et al., 1998). Thus, similar to other vascular pathologies essential hypertension is associated with reduced endothelium-dependent relaxation, where the reduced effect of NO is probably due to a variety of contributing factors.

9. The importance of platelet nitric oxide production

The previous sections have concentrated on endothelial-derived NO, however the importance of platelet-derived NO in vascular disease is now recognised. In contrast, to the basal release of endothelial NO, platelet-derived NO is released in response to cell activation: it is very localised and is released specifically to attenuate thrombosis. NO released by activated platelets in vitro markedly inhibits the secondary recruitment of platelets into aggregates, consistent with a role limiting thrombus formation (Freedman et al., 1997). This was confirmed by elegant studies using a mouse model lacking the NOSIII gene. The mice had decreased bleeding times and platelets with reduced ability to inhibit activation of a second population of platelets, even after endothelial NO production was accounted for (Freedman et al., 1999). It can be speculated that the localised NO sets a level for which the activatory stimulus must surmount in order to form a thrombus. The clinical relevance of the importance of platelet-derived NO can be ascertained from several key patient studies. There is an inverse correlation between platelet NOS activity and other coronary risk factors such as total cholesterol, LDL cholesterol and arterial blood pressure (Ikeda et al., 2000). Patients with acute coronary syndromes (Freedman et al., 1998), diabetes (Rabini et al., 1998) and essential hypertension (Camilletti et al., 2001) all
produce significantly less platelet-derived NO than controls. Impairment of platelet-derived NO probably accounts, at least in part, for the increased athero-thrombotic risk associated with these disease states. Hence in pathologies such as atherosclerosis, diabetes and hypertension, which are associated with reduced bioavailability of endothelial-derived NO (Loscalzo, 2001), the availability of platelet-derived NO is critically important in determining the extent of thrombosis. The mechanisms leading to impaired platelet NO synthesis are unknown and are currently under investigation.

10. Nitric oxide and septic shock

This review has concentrated on vascular diseases, which are associated with reduced bioavailability of NO, however septic shock represents a disorder in which overproduction of NO is critical. Septic shock has a mortality rate of up to 60% and is characterised by hypotension, compromised vascular function and multi-organ failure. The hypotension associated with septic shock is brought about by a massive increase in NO production, emanating primarily from NOSII present in VSMC. Lipopolysaccharide (LPS) found in the cell wall of gram-positive bacteria is the endotoxin responsible for the induction of septic shock, although the mechanism of action is poorly defined. It has been postulated that LPS leads to a widespread increased expression of iNOS, resulting in a large systemic increase in NO production. LPS induces the production of several cytokines such as tissue necrosis factor (TNF-\(\alpha\)), interferon-\(\gamma\) (INF-\(\gamma\)), interleukins (IL)-1 and 6: these cytokines have the capacity to increase NOSII expression (Titheradge, 1999). Treatment of vascular smooth muscle cells with endotoxin, TNF-\(\alpha\) and IL-1 in vitro results in iNOS induction in endothelial cells and VSMC (Busse and Mulsch, 1990). While a combination of IL-6, INF-\(\gamma\) and TNF-\(\alpha\) can induce NOSII in the vascular wall (Kilbourn et al., 1997). The activation of NOSII by endotoxin results in far larger quantities of NO produced than under normal conditions. This leads to an increase in vasodilation and an increased resistance to the actions of vasoconstrictors.

The primary evidence for the role of NO is provided by the clinical use of NOS inhibitors and knock-out animals. NOSII mice knockouts are resistant to the endotoxin-induced hypotension, suggesting that the NOSII-derived NO is responsible for reduced blood pressure (MacMicking et al., 1995). While both \(N^G\)-methyl-L-arginine and \(N^G\)-nitro-L-arginine methyl ester have been used in patients and have shown to stabilise the endotoxin associated hypotension (Petros et al., 1991). However, the use of NOS inhibitors clinically must be viewed with caution, since there may also be detrimental effects. In animal models the use of NOS inhibitors, prevented the iNOS associated hypotension, but also led to the inhibition of eNOS in the microcirculation and possible tissue damage. Thus in septic shock excess iNOS-derived NO leads to circulatory failure and contributes to mortality from multi-organ failure, while eNOS-derived NO may be important in protecting against tissue damage in the microcirculation.
11. Concluding remarks

The aim of the review was to provide the reader with an overview of what is an enormous and extremely vigorous area of biomedical research. NO has emerged as a prominent protector against cardiovascular disease. The consensus suggests that the level of NO synthesis does not change dramatically with disease. However, the increased oxidative stress associated with cardiovascular pathologies has the potential to shift the balance from NO being a protective to an injurious agent. Therapeutic intervention aimed at preserving bio-available NO by reducing oxidative stress and regulating other risk factors such as hypercholesterolemia and diabetes will help to combat cardiovascular mortality.

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