Review Article

NITRIC OXIDE: AN ENDOGENOUS GAS WITH PLEIOTROPIC ACTIVITIES EMPHASIS ON THE CARDIOVASCULAR SYSTEM

Aiman Saad El-Khatib

شد أكسيد النيتريك انتباه العلماء في العقد الأخير حيث تمكنوا من معرفة أهميته في العمليات الفسيولوجية والمرضية المختلفة للعديد من الأنظمة الحيوية. ولأكسيد النيتريك وظائف محددة في النظام القلبي الوعائي ومن المعروف الآن أن النقص في تخليق و/أو فاعلية أكسيد النيتريك يساهم في بدء واستفحال العديد من الأمراض الوعائية كما أنه متورط في الاعتلالات المختلفة ذات الطابع الوعائي. إن التشوش في مسار L-Arg/NOS/NO قد يحدث في عدة مواقع منها: (أ) تلف مستقبلات الغشاء في جدار الوعاء الدموي الذي يتفاعل مع المشارك agonists أو المحرضات الفسيولوجية القادرة على توليد أكسيد النيتريك ، (ب) التراكيز المنخفضة أو خلل في استهلاك L-Arg ، (ج) انخفاض تركيز أو نشاط NOS ، (د) اختلال إطلاق أكسيد النيتريك من البطانة الوعائية المتحطمة ، (هـ) اختلال سريان أكسيد النيتريك من البطانة الوعائية إلى خلايا العضلة الوعائية الملساء متبوعاً بانخفاض في حساسيته لتأثيره الموسع للأوعية ، (و) تفكك أكسيد النيتريك الموضعي المعزز وذلك بالتوليد المتزايد للشقوق الحرة أو بالآليات الحساسة للأكسجين أو كلاهما ، (ز) التداخل المعطوب لأكسيد النيتريك مع إنزيمه المستهدف sGC وبالتالي الانحسار في انتاج cGMP. لذلك يجب الأخذ في الحسبان أن الآليات المتعددة غالباً ما تكون هي الأساس لأي من الاعتلالات الوعائية. وكما سنورد في المقالة، فإن التعرض لعوامل الخطورة مثل فرط كوليسترول الدم قد تقلل من الفعالية الأحيائية لإكسيد النيتريك نتيجةً لعدد من الآليات التي تتلخص في التالي: (1) يقوم البروتين الدهني منخفض الكثافة LDL بتبديل مسارات تحويل طاقة الإشارة التي تشمل بعض بروتينات G المنشطة و IP_3 وليس جميعها ، (2) يثبط نقل L-Arg بواسطة CAT-1 ، (3) ويتدخل البروتين الدهني منخفض الكثافة مع إنتاج BH_4 ، (4) ومن المحتمل أنه يعطل الاتحاد المزدوج لـ NOS واتحاد BH_4 مع الإنزيم ، (5) إن البروتين الدهني منخفض الكثافة يقلل ألفة NOS لـ L-Arg ، (6) بواسطة آليات توليد الأكسجين والإجهاد المؤكسد، يقوم البروتين الدهني منخفض الكثافة بتسريع تفكك أكسيد النيتريك ومن المحتمل أن يتدخل مع تفكك الـ ADMA المناهض الداخلي المنشأ الدوار وبالتالي يرفع من مستواه ، (7) يتدخل مع إصدار sGC mRNA وانتقاله. إن فهم الآليات المختلفة المسؤولة عن أداء أكسيد النيتريك لوظيفته أو اختلاله الوظيفي يتيح التوجيه العقلاني والتدخل العلاجي الذي يحفظ أو يسترد له وظائفه الفسيولوجيه ويفسر التأثير الضار لبعض العلاجات على الصحة الوعائية. فعلى سبيل المثال ، فإن التخليق المعطوب لأكسيد النيتريك و/أو مقاومة تأثيره الموسع للأوعية هما من بين الآليات المرشحة المسؤولة عن فرط ضغط الدم الذي يصاحب علاج الجلوكوكورتيكويد والإريثروبويتين. ومن ناحية أخرى ، فإن الأحماض الدهنية المشبعة طويلة السلسلة قد تؤثر على ضغط الدم عن طريق تثبيط نشاط eNOS وتعطل توسيع الأوعية المعتمد على البطانة الوعائية. ومن الأستراتيجيات العلاجية التي تعيد نشاط أكسيد النيتريك إعطاء مانحات أكسيد النيتريك ، و L-Arg ، ومضادات الأكسدة ، ومن المؤمل أن تساهم الدراسات الحالية في التعرف على طرق جديدة، وخصوصاً نقل المورث (الجين) الذي يقوم بتشفير إنزيم NOS إلى البشر.

During the last decade, NO has attracted the attention of scientists, who have revealed its significance in various physiological and pathological processes of many biological systems. NO has distinct functions in the cardiovascular system. It is now appreciated that reduction in NO synthesis and/or activity contributes to the initiation and progression of many vascular diseases and is involved in various disorders with vascular components. Derangement in L-Arg/NOS/NO pathway could occur at many sites including: (a) impairment of membrane receptors in the vessel wall that interact with agonists or physiological stimuli capable of generating NO; (b) reduced concentrations or impaired utilization of L-Arg; (c) reduction in concentration or activity of NOS; (d) impaired release of NO from the damaged endothelium; (e) impaired NO diffusion from endothelium to vascular smooth muscle cells followed by decreased sensitivity to its vasodilator action; (f) local enhanced degradation of NO by increased generation of free radicals and/or oxidation-sensitive mechanisms; and (g) impaired interaction of NO with its target enzyme sGC and consequent limitation of cGMP production. It should, however, bear in mind that multiple mechanisms often underlie any one of the vascular disorders. As mentioned in the text, exposure to risk factors such as hypercholesterolaemia decreases the bioactivity of NO as a result of a

Department of Pharmacology, College of Pharmacy, King Saud University, P. O. Box 2457, Riyadh 11451, Saudi Arabia.

variety of mechanisms summarized as follows: (i) LDL alters signal transduction pathways involving some, but not all, G stimulatory proteins and IP_3; (ii) it inhibits L-Arg transport via CAT-1; (iii) LDL interferes with BH_4 production; (iv) it possibly disrupts NOS dimerization and association of BH_4 to the enzyme; (v) LDL reduces the affinity of NOS for L-Arg; (vi) via O_2^- generation and oxidative stress mechanisms, LDL accelerates NO degradation and likely interferes with the degradation of the circulating endogenous antagonist ADMA and thus elevates its level; and (vii) it interferes with sGC mRNA transcription and translation. Understanding the various mechanisms responsible for NO function and dysfunction allows for rationale targeting and therapeutic interventions that preserve or restore its physiological functions and also provide the rationale for why certain therapies can detrimentally affect vascular health. For instance, impaired NO synthesis and/or resistance to its vasodilator action are among candidate mechanisms responsible for the hypertension that associates glucocorticoid (210) and erythropoietin (211,212) therapy. Long-chain saturated fatty acids, on the other hand, may affect blood pressure by inhibiting eNOS activity and impairing endothelium-dependent vasodilatation (213). Therapeutic strategies that restore NO activity include administration of NO-donors, L-Arg and anti-oxidants, among others and it is to be hoped that current studies will contribute towards the identification of new approaches, particularly the transfer of gene encoding the enzyme NOS to human subjects.

List of Abbreviations

L-Arg L-arginine
ADMA asymmetric dimethylarginine
CAT cationic amino acid transporter
cAMP cyclic adenosine monophosphate
cGMP cyclic guanosine monophosphate
eNOS endothelial nitric oxide synthase
DDAH dimethylarginine dimethylaminohydrolase
H_2O_2 hydrogen peroxide
iNOS inducible nitric oxide synthase
IkBa inhibitor kappa B alpha
IP_3 inositol 1,4,5-trisphosphate
IFN-γ interferon-gamma
IL interleukin
mRNA messenger ribonucleic acid
nNOS neuronal nitric oxide synthase
NADPH nicotinamide adenine dinucleotide phosphate, reduced form
NO nitric oxide
NFkB nuclear factor kappa B
OxLDL oxidized low-density lipoprotein
$ONOO^-$ peroxynitrite anion
sGC soluble guanylyl cyclase
O_2^- superoxide anion radical
SOD superoxide dismutase
BH_4 tetrahydobiopterin
tRNA transfer ribonucleic acid
$TGF-\beta_1$ transforming growth factor-beta 1
TNF-α tumor necrosis factor-alpha

Introduction

A 1:1 combination of two of the most abundant gaseous elements namely, oxygen and nitrogen constitutes the gaseous molecule, nitric oxide (NO). It is deceptively simple in structure but highly complex in activity. Initially, NO was recognized as a mere highly reactive noxious air pollutant. Its role as a biological signaling and effector molecule was virtually unknown until the late 1980s. Henceforth, NO has emerged as an endogenous chemical messenger in a multitude of biological systems having homeostatic activity in maintenance of cardiovascular tone, platelet regulation, and central nervous system signaling. It plays also a pivotal role in gastrointestinal, respiratory, and urogenital tracts, as well as in immune surveillance. This multi-functionality of NO led to its declaration by the American Association for the Advancement of Science as the molecule of the year in 1992. The seemingly ubiquitous involvement of NO has resulted in an explosion of interest in the field yielding more than twenty thousand publications in the last five years. This review is intended to provide an introduction to NO biology and to discuss some pathophysiological aspects of NO, focusing primarily on the impact of its impairment on the cardiovascular system.

1- Physical and chemical properties of nitric oxide

NO is an uncharged lipophilic molecule containing a single unpaired electron. By virtue of the latter, the molecule is unstable, chemically reactive, and short-lived. NO reacts as a free radical acting as oxidant or reducer and in some instances complexed with other biomolecules, depending on the surrounding microenvironment. In aqueous solution, where its estimated half-life is less than 4

minutes, NO undergoes rapid oxidation to relatively unreactive nitrite and nitrate. In biological systems, however, where its half-life is estimated to be few seconds, the most common chemical interactions of NO are characterized as stabilization of the unpaired electron.

NO does not activate typical receptor molecules and is capable of diffusing across membranes. It ultimately exerts its biological effects by reacting either directly or through other reactive nitrogen intermediates with a variety of cellular targets (1,2). NO has a high affinity for interaction with ferrous haemoproteins such as soluble guanylyl cyclase (sGC) enzyme and haemoglobin (3,4). As a result of binding to oxyhaemoglobin and to superoxide anion radical (O_2^-), the biological activity of NO is terminated (4). NO was originally thought to act as a protective free radical scavenger by binding O_2^- (5). The product, however, is peroxynitrite anion ($ONOO^-$), a relatively long-lived, very reactive oxidant, which subsequently decomposes into additional highly reactive intermediates (6,7). NO can also form nitrosotyrosine residues that inhibit the enzymatic activity of proteins such as caspases and can react with sulfhydryl groups to form S-nitrosothiols (8,9). This diversity of potential targets is reflected in the large number of different systems that utilize NO as a mediator and provides ample opportunity for abnormal regulation and development of pathological effects. Because NO is extremely labile in the biological milieu, its activities can be affected not only by NO itself but also by relatively stable physiological NO carriers or NO-donors. It has been proposed that NO circulates in mammalian plasma as an S-nitroso-adduct of albumin (10,11) and that dinitrosyl iron complexes are endogenous carrier molecules for NO (12,13). S-nitroso-compounds exhibit *in vivo* endothelium-derived relaxing factor (EDRF)-like properties mediated by a cyclic guanosine monophosphate (cGMP)-dependent process (11).

2- Biosynthesis of nitric oxide and its main biological effects

NO activity has been probed in almost every system in the human body where it exhibits diverse vital roles (9). In all cell types, NO is generated following a five-electron oxidation of the terminal guanidino nitrogen atom of the amino acid, L-arginine (L-Arg) in the presence of molecular oxygen and the electron donor NADPH to yield citrulline and NO (14). This reaction is catalyzed by a group of homodimeric haem protein enzymes, which are referred to as NO synthases (NOSs).Three well-characterized isoforms of NOS have been identified in various cells of mammalian systems. These isoforms are neuronal NOS (nNOS or NOS1), inducible NOS (iNOS or NOS2), and endothelial NOS (eNOS or NOS3). The names reflect characteristics of the activity or the original tissues in which the enzymes were first described, but it is now known that each of these isoforms is expressed in a variety of tissues and cell types (15). Human form of eNOS and iNOS genes have been cloned and localized to chromosomes 7 and 17, respectively (16). The gene for nNOS, isolated and identified in the cerebellum has been localized to chromosome 12 (9).

The three isoforms of NOS share structural similarities and have nearly identical catalytic mechanisms. They contain three distinct domains *viz.*, oxygenase domain, reductase domain, and an intervening calmodulin-binding domain, which are necessary for the catalytic activity. The oxygenase domain is located in the N-terminal region that contains the binding sites for haem, tetrahydrobiopterin (BH_4) and L-Arg. This domain actually catalyzes the conversion of L-Arg into citrulline and NO. The reductase domain is present in the C-terminal region that contains the flavin adenine dinucleotide and flavin mononucleotide moieties and transfers electrons from NADPH to the oxygenase domain. All NOS isoforms require bound calmodulin for activity but in case of nNOS and eNOS, such binding occurs only in response to transient increases in intracellular calcium concentration $[Ca^{2+}]_i$. iNOS, however, is unusual in that it carries a permanently bound molecule of calmodulin allowing for activity even at very low concentrations of Ca^{2+} (17). The activity of this particular isoform is thus not dependent on Ca^{2+} levels. The NOS isoforms display a number of differences related to their individual functions. Transcriptional and post-translational regulations of catalytic activity are distinct for each isoform. Some of the unique properties and functions of each isoform will be addressed below.

2-1- Neuronal nitric oxide synthase

nNOS is expressed in the central and peripheral nervous systems and in skeletal muscles (18). It generates physiological concentrations of NO in the

picomolar range for a short period of time. Although nNOS is usually reported to be a constitutive enzyme, its _expression is influenced by certain physiological and pathological stimuli such as shear stress and nerve injury (19). Due to a specific segment in its structure, nNOS is directed to sites of signal transduction (17,20). In neuronal tissues, Ca^{2+} influx triggered by the N-methyl-D-aspartate receptor activation is an important inducer of nNOS activity. In the brain, NO acts as a neuromodulator to influence functions such as behavior and memory formation. In the peripheral nervous system, NO acts as a neurotransmitter participating in functions such as smooth muscle control, gastrointestinal motility, and neuroendocrine function. In cavernous nerves, NO formed by nNOS is thought to diffuse into smooth muscle cells in the corpus cavernosum, relaxing it via cGMP formation and thus mediates sexual erectile function in males (21). In skeletal muscle, nNOS is anchored to the sarcolemma and is activated by membrane depolarization. NO functions in skeletal muscles as a signal transducer to regulate both metabolism and muscle contractility. Inappropriate regulation of nNOS has been implicated in a number of neurotoxicities which are known to be provoked by the release of excess glutamate such as those resulting from stroke and certain neurotoxins (18,22). This role of NO was clearly demonstrated when cultured neurons from nNOS knockout mice were shown to be resistant to glutamate toxicity. These mice also have much less tissue damage in response to focal ischaemia. Neurotoxicity appears to result from the action of O_2^- and $ONOO^-$ as evidenced by results with superoxide dismutase (SOD) knockout mice and transgenic mice that overexpress SOD. The source of O_2^- could be nNOS itself under conditions of L-Arg depletion. Some evidence suggests that nNOS may also be involved in neurodegenerative diseases such as Parkinson's disease (20).

2-2- Inducible nitric oxide synthase

iNOS was first reported in macrophages where its activity was found to be inducible in response to stimuli such as endotoxin or pro-inflammatory cytokines including interleukin-1 (IL-1), IL-6, tumor necrosis factor-alpha (TNF-α), and interferon-gamma (IFN-γ) (9,23). After induction, iNOS remains active yielding long-lasting release of NO in the nanomolar concentration range; 100-fold greater than those of the other two NOS isoforms. _Expression of iNOS has now been reported in a large number of cell types including vascular smooth muscle cells, endothelial cells and cardiac myocytes (15,23). The enzyme is inducible, located primarily in the cytoplasm and transcriptionally regulated, a process inhibited by glucocorticoids, transforming growth factor-beta 1 (TGF-β_1), IL-4, IL-8, and IL-10 (4,14,23). The activity of iNOS is also influenced by a variety of other control mechanisms that affect mRNA stability, translation and degradation of the protein, as well as availability of substrate and cofactors (17). By virtue of generating a number of highly reactive nitrogen and oxygen intermediates, NO synthesized via iNOS of activated macrophages, has been defined as an effector molecule that kills or inactivates invading pathogens (24,25) and cancer cells (26). The overproduction of NO by iNOS is implicated in a number of pathological situations such as septic shock (15) and inflammatory conditions including rheumatoid arthritis and asthma (23,27,28). Because of its regulatory role in the immune system signaling cascade and its effect as an effector molecule in macrophage-mediated immune response, NO can have detrimental or beneficial properties in inflammatory conditions depending on the setting (1).

2-3- Endothelial nitric oxide synthase

eNOS is expressed in vascular endothelial cells and cardiomyocytes, among many other cells. The unique N-terminal of the amino acid sequence of eNOS functions to localize the enzyme exclusively to membranes in close proximity to signal transduction sites (29). _Expression of eNOS is usually reported to be constitutive. Like nNOS, eNOS produces physiological concentrations of NO in the picomolar range for a short time period. In blood vessels, NO produced by the eNOS of endothelial cells controls vascular tone and reactivity and thereby regulates blood flow and pressure (9,27,30). Mutant eNOS knockout mice have blood pressure that is 30% higher than wild-type littermates (31). Within cardiomyocytes, eNOS affects Ca^{2+} currents and contractility (32). Within the cardiovascular system, eNOS generally has protective effects. It has been suggested, however, that eNOS-mediated overproduction of NO may be incriminated in oxidative endothelial cell injury (33). eNOS itself under conditions of BH_4 depletion can generate O_2^- from molecular oxygen resulting in endothelial dysfunction that associates several

disease states including, hypercholesterolaemia, atherosclerosis, and hypertension. Studies with nNOS and eNOS knockout mice clearly indicate that eNOS plays a protective role in cerebral ischaemia by preserving cerebral blood flow (31). During inflammation and atherosclerosis, low concentrations of NO prevent apoptotic death of endothelial cells and preserve the integrity of the endothelial cell monolayer (34,35). Likewise, NO also acts as an inhibitor of platelet aggregation and adhesion (4,9), leukocyte activation and migration (36), O_2^- generation (37), vascular smooth muscle cells proliferation (38), and adhesion molecules _expression (27,39,40).

3- Nitric oxide-cyclic guanosine monophosphate-calcium signaling cascade

As long ago as the late 1970s, pharmacological evidence indicated that NO, cGMP, and Ca^{2+} are interacted and played crucial roles in vascular cell physiology. The first discovery was that nitroglycerin and nitroprusside relax blood vessels by means of the generation of cGMP, and that NO is the active substance derived from these drugs (41). This was followed by the recognition of the existence of an endothelial-derived substance that was responsible for the cGMP-dependent relaxation of smooth muscle cells (42). cGMP was soon demonstrated to be able to reduce intracellular Ca^{2+} levels, a critical step in the inhibition of the enzymes involved in smooth muscle cell contraction such as myosin light chain kinase (43), as well as in the inhibition of platelet aggregation (44). After the discovery that the EDRF was actually NO (45,46), the role of NO in controlling Ca^{2+} homeostasis was investigated more thoroughly. This knowledge was subsequently extended beyond the vascular system, to many cell types of various organs.

Over the last few years, evidence gathered from a number of cell systems has indicated that NO is one of the key messengers governing the overall control of Ca^{2+} homeostasis, which is regarded as a fundamental regulator of cellular physiological functions. A negative modulation on Ca^{2+} release from its intracellular stores within various cell systems is a well-established function of the endogenous NO, which appears to work as a sensor for Ca^{2+}. Intracellular located NO may originate through its diffusion from the outside i.e. neighboring cells, and also through its intracellular generation by the constitutive Ca^{2+}-dependent NOS isoforms (47). The inhibitory effects of NO on Ca^{2+} release in various cells appears to be mediated through increased cGMP levels as a result of activation of the sGC, and the ensuing activation of cytosolic cGMP-dependent protein kinase I with the ultimate phosphorylation of its particular functional targets. The major effect of the latter enzyme is to reduce inositol 1,4,5-trisphosphate (IP_3) generation, an effect that accounts for the long-known ability of NO to inhibit the accumulation of this second messenger in many cell systems (47). cGMP-dependent protein kinase I-mediated phosphorylation of IP_3 receptors is another possible pathway by which NO exerts its inhibitory action on Ca^{2+} release (47). It seems, however, noteworthy that in endothelial cells, NO appears to have no effect at all on Ca^{2+} homeostasis *per se*, although its reaction with O_2^- may lead to the formation of $ONOO^-$, which is ultimately responsible for the depletion of intracellular Ca^{2+} stores (48).

4- Pharmacological regulators of nitric oxide biosynthesis

Many agents that regulate NO biosynthesis have been identified providing important pharmacological tools for investigating the pathophysiological relevance of NO in biological processes (49-51). Promoters of NO bioactivity include its precursor L-Arg, organic nitrates such as nitroglycerin, sodium nitroprusside, and S-nitrosothiols, while inhibitors include flavoprotein, calmodulin and haem binders, BH_4-depleting agents, and substrate analogues. Analogues of L-Arg such as N^G-nitro-L-arginine, N^G-methyl-L-arginine, and N^G-nitro-L-arginine methyl ester have become the most commonly used inhibitors both *in vitro* and *in vivo* (52). They act as both competitive substrate inhibitors and modulators of membrane transport of L-Arg (4). They exhibit variable affinities for various NOS isoforms, although none are truly specific. Inhibition of only the iNOS may prevent the consequences of massive overproduction while allowing basal NO release to continue. No physiological agents have yet been identified that specifically inhibit enzyme activity of any one of them. However, aminoguanidine, a nucleophilic hydrazine compound that shares structural similarity with L-Arg, is endowed with many activities and was found to inhibit in a selective manner iNOS, leading to decreased generation of NO (53-56).

5- Detection of nitric oxide in biological samples

In order to clarify the potential role of NO under various experimental and clinical settings, numerous methods have been developed for its estimation. The available techniques can detect NO either directly or indirectly in biological samples. Indirect assays include measurement of cGMP, which reflects the effect of NO on its target intracellular enzyme sGC and assessment of citrulline as a co-product of the action of NOS on L-Arg. Determination of nitrite/nitrate as stable end products of NO metabolism can also be performed (57). Such assays are simple and useful in evaluating the NO pathway at varying levels. Direct methods for the detection of NO include measurement by chemiluminescence reaction with or without acidification (58), detection by electron paramagnetic resonance with nitroso- or haemoglobin traps (59), and by spectrophotometric determination, which is based on the rapid oxidation of reduced haemoglobin to methaemoglobin by NO (3). The chemiluminescence method is one of the most widely utilized techniques for measurement of NO in exhaled air (28). Other available methods include detection of NO by chemical sensors and fluorescent probes. Using microsensors, it is possible to quantify NO release throughout the cardiovascular system in veins, arteries and the heart (60). Gas chromatography and mass spectroscopy are also described for the estimation of NO but less frequently used. By the aid of molecular biology tools, it is now possible to measure the NO activity as a function of NOS gene _expression (23).

II- Nitric Oxide and Cardiovascular System

As previously mentioned vascular NO is involved in many physiological and pathological processes throughout the body. It is worth to note that NO-related vascular diseases can be the result of either increased or decreased NO activity. Due to the diversity of these diseases as well as the multiple mechanisms responsible for their initiation and/or progression, herein emphasis will be given on those accompanied by a reduction in the activity of endothelium-derived NO as an important component involved in the pathogenesis of the disorder. Among these pathological conditions are atherosclerosis including coronary, peripheral and cerebral artery diseases, hypercholesterolaemia, hypertension, and heart failure (60-62). Some details of the known mechanisms of function and dysfunction of the NO pathway will be discussed in this part, which provide the rationale for why certain therapies can benefit vascular health while other therapies affect it deleteriously.

2-1- L-arginine-nitric oxide synthase-nitric oxide pathway

As alluded to in part I, NO is formed within endothelial cells, among other things, from L-Arg and eNOS. The bulk of L-Arg available to the endothelial cell originates from dietary sources, although it may pass through protein synthesis and metabolism before being available to endothelial cells (63). Another contribution of unknown proportion comes from synthesis of L-Arg from citrulline via this same pathway within the endothelial cell. Plasma L-Arg transport across membranes of endothelial cells is mediated by several independent transport systems. At physiological concentrations, about 70% of L-Arg is transported by the most important transport system namely, system y^+ which involves the high-affinity cationic amino acid transporters (CAT). This mechanism of transport appears to be rate limiting for intracellular L-Arg availability, but not necessarily NO synthesis, within endothelial cells under normal conditions. Two types of CAT have been identified; CAT-1 which is constitutively expressed, while CAT-2 is induced by various cytokines (28). At higher concentrations, however, 70% of the total L-Arg transported in human endothelium bypasses the y^+ system via the other non-saturable transport system (64,65).

eNOS gene _expression is considered constitutive and is influenced by the stability of its mRNA as well as its transcription rate. The promoter region of the gene encoding eNOS contains elements responsive to estrogen, shear stress, TGF-β_1, glucose, and agonists that activate protein kinase C (66). _Expression also responds to exercise training, chronic hypoxia and heart failure (19,62). Once expressed, the enzyme must be post-translationally modified, trafficked to its perimembrane location, assembled with haem and with its other cofactors (67), preferentially homodimerized, and phosphorylated to active form (68). Within the membrane, eNOS is targeted to the caveolae, small invaginations characterized by the presence of proteins called caveolins. These regions serve as sites for the sequestration of signaling molecules such as receptors, G proteins and protein kinases. The oxygenase domain of the eNOS contains a motif

that binds to caveolin-1, and calmodulin is believed to competitively displace caveolin resulting in enzyme activation (19). The homodimer form of eNOS is stabilized when its subunits are coupled with BH_4, a complex that is further stabilized by the presence of L-Arg (69). Phosphorylation of eNOS to its active form begins with the activation of the various cell surface receptors (sensing shear stress, bradykinin, acetylcholine, substance P, vascular endothelial growth factor, and β adrenoceptor agonists), followed by signal transduction via their respective signaling pathways. Activation of any of several agonist receptors as well as shear stress glycoproteins converge on the same intracellular pathway that involves G protein activation, IP_3 generation, Ca^{2+} influx and intracellular release, calmodulin activation, and finally the activation of a protein kinase B, named Akt (70). Shear stress also activates at least two other cascades; one involves endothelial cell membrane hyperpolarization, which leads to an increased Ca^{2+} influx (71), the other involves a Ca^{2+}-independent activation of Akt (72). β adrenoceptor agonists also activate eNOS although through an entirely different pathway; one that involves generation of cAMP and activation of protein kinase A (73). Other protein interactions have recently been shown to regulate eNOS activity. One such protein that interacts with and activates eNOS is heat-shock protein 90 (HSP90), which can be induced by certain stresses and by the presence of estradiol (74). HSP90 assists with the intracellular trafficking of eNOS and helps activate eNOS by disassociating it from caveolin-1, where it is thought to be kept inactive (75).

Once constituted optimally, eNOS in the presence of L-Arg generates NO, which exerts its biological activity by a number of mechanisms on proteins within the cytoplasm, at the level of the nucleus, or after diffusing to adjacent tissues such as the underlying smooth muscle and circulating elements such as platelets. Enzymes influenced by NO are often modified by direct nitrosylation such as that which occurs with sGC. Alternatively, following conversion to $ONOO^-$, it can interfere with enzymes that require metal cofactors or oxidized amino acids at the center of their active sites such as that which occurs with xanthine oxidase (76). NO inhibition of gene transcription can occur via the stabilization of the ubiquitous transcriptional protein, nuclear factor kappa B (NFkB), with its inhibitor kappa B alpha (IkBa), preventing its translocation to the nucleus and subsequent activation of many genes (77). NO activation of gene transcription can occur by its diffusion to the nucleus where it causes the disruption of zinc finger domains of inhibitory transcription factors, which sit on the promoter regions of NO-sensitive genes (78).

The NO molecule is eventually consumed via nitrosylation of proteins or through the conversion to $ONOO^-$ by combination with oxygen-derived free radicals; both processes often participate in the biological activity of NO. At the end, the products of NO undergo further oxidation to nitrite and nitrate, which can either be eliminated via the kidneys or, under conditions of hypoxia, might be reduced back to NO by xanthine oxidase (79,80).

2-2- Physiological actions of vascular nitric oxide

Before going to discuss how decreased NO impacts vascular disease, it is first necessary to understand its many roles in vascular homeostasis. The three major properties of NO in the vascular system are anti-ischaemia/anti-hypertension, anti-atherosclerosis, and anti-thrombosis.

2-2-1- Anti-ischaemia/anti-hypertension properties of nitric oxide

Under normal physiological conditions, a well-defined distribution of NO is maintained, which is dependent on flow rate of blood. The anti-ischaemia/anti-hypertension activities of NO follow from its actions to stimulate the production of vascular smooth muscle cGMP and from its action to promote angiogenesis (81,82). As mentioned earlier (section 1-3), elevations in cGMP within vascular smooth muscle cells through NO-mediated nitrosylation of sGC result in processes which inhibit the release of Ca^{2+} from its intracellular stores, prevent the entry and promote its extrusion (47) thereby leading to vasodilatation. This latter activity has many important clinical consequences including maintenance and enhancement of coronary and peripheral blood flow as well as maintenance of blood pressure and attenuation of hypertension in both systemic and pulmonary vascular beds (9,27,30). Inhibition of endogenous synthesis of NO, on the other hand, results in exaggerated vasoconstrictor responses in multiple human and animal models (9). Respecting anti-ischaemic and angiogenic properties, NO acts in concert with vascular endothelial growth factor to enhance endothelial cell proliferation as well as migration by stimulating podokinesis and by enhancing

_expression of urokinase-type plasminogen activator (83,84). NO prevents also apoptosis of newly formed vascular cells (34,35). As a vasodilator, NO decreases shear stress in the newly formed vessels, which can potentially disrupt endothelial cell interaction with the surrounding extracellular matrix (85,86).

2-2-2- Anti-atherosclerosis property of nitric oxide

One of the crucial functions of NO is that it inhibits circulating blood elements from interacting with the vessel wall (60). The anti-atherosclerosis activity of NO results most probably from its ability to reduce intracellular oxidative stress as well as from its capability to inhibit key early atherogenesis-signaling processes. Inhibition of these signaling processes leads to down-regulation of oxidative enzymes, the reduction in leukocyte accumulation, and the inhibition of vascular smooth muscle cell proliferation and migration. A reduction in intracellular oxidative stress by NO reduces the presence of damaging reactive oxygen species and is accomplished by several mechanisms (87,88). NO can scavenge O_2^- directly, although the product of this reaction, $ONOO^-$, is itself a highly reactive free radical (6,7). However, $ONOO^-$ may subsequently nitrosylate sulfhydryl groups to form *S*-nitrosothiols which can themselves participate in vasodilatation, and in the inhibition of platelet aggregation and monocyte adhesion (10). NO may also terminate the perpetuating chain reactions of lipid peroxidation that is initiated by oxidized low density lipoprotein (oxLDL) or by intracellular generation of oxygen-derived free radicals (89). The generation of these latter radicals can be directly suppressed by NO which nitrosylate oxidative enzymes such as NADPH oxidase keeping it inactive (90). Finally, NO can inhibit the gene _expression of oxidative enzymes through mechanisms described below.

Inactivation of specific transcriptional factors such as NFkB by NO modulates various atherogenesis-signaling processes (77,91). This effect of NO appears to be partly due to direct stabilization and/or increased _expression of IkBa, which complexes with NFkB to inhibit its transcriptional activity (92). Stabilization of the inactive NFkB/IkBa complex prevents the gene transcription of oxidative enzymes, as well as of proteins involved in leukocyte accumulation. Specifically, inactivation of NFkB prevents the elaboration of glycoprotein adhesion molecules such as vascular cell adhesion molecule and chemokines such as monocyte chemotactic protein-1. _Expression of these proteins by endothelial cells, which occurs upon exposure to high levels of serum cholesterol, is inhibited dose-dependently upon exposure to NO (93,94). Inhibition of NO synthesis thus increases the _expression of these endothelial proteins (91).

The action of NO to regulate the growth and migration of vascular smooth muscle cells appears to be mediated through a cGMP-dependent mechanism. *In vitro,* NO-donors inhibit the proliferation of vascular smooth muscle cells; this effect is mimicked by exogenous administration of 8-bromo-cGMP, which is a stable analogue of cGMP (38). Activated platelets adhering to injured vascular endothelial cells may also participate in this process by releasing epidermal growth factor, platelet-derived growth factor, and other mitogens and cytokines. All of these factors induce smooth muscle cells in the vessel wall to proliferate and migrate into the area of the lesion where they undergo a change in phenotype from a “contractile” cell to a “secretory” cell. These secretory vascular smooth muscle cells elaborate extracellular matrix which transforms the lesion into a fibrous plaque.

2-2-3- Anti-thrombosis property of nitric oxide

The anti-thrombosis activity possessed by NO is due to its ability to inhibit platelet aggregation and adhesion (4,9). This occurs in part by the stimulation of intra-platelet cGMP activity and subsequent phosphorylation of proteins that regulate platelet activation and adherence (95). Platelets themselves contain small amounts of NOS and are capable of generating NO which may act as an autocrine mechanism preventing their activation (96).

2-3- Mechanisms of impaired nitric oxide activity

Mechanisms of decreased activity of NO, for which there is experimental evidence, can be divided into three major categories; decreased NO production, increased NO destruction, and decreased sensitivity to NO. While each one of these defects will be elaborated separately, it is likely that any given vascular disease has several points of dysfunction of the L-Arg-NOS-NO pathway. This is due to the fact that all the components of the pathway are interrelated and integrated together, and thus when one component stops working optimally, the resulting decrease in NO level or increase in O_2^-

production affects the other components of the pathway.

2-3-1- Decreased Production of Nitric Oxide

Aberrations at different levels of the L-Arg-NOS-NO pathway can result in decreased NO production. These aberrations include disruption of normal NOS signaling mechanisms, reduced availability of L-Arg, disruption of L-Arg/NOS metabolic channeling, altered activity and _expression of NOS.

2-3-1-1- Disruption of normal nitric oxide synthase signaling mechanisms

As stated previously, signal transduction pathways resulting from activation of cell surface receptors (agonist receptors or shear stress sensing glycoproteins) are linked with the activation of eNOS. There is evidence that one or more of these signal transduction pathways are dysfunctional in some vascular diseases as manifested by the differential alterations in responses created by various vasodilator agonists known to act via NO cascade without affecting the relaxing effect of nitroglycerin (60). This appears to be the case for a sub-population of patients with coronary artery disease (97), hypercholesterolaemia (98) and essential hypertension (99). It has been suggested that the defect is primarily confined to the IP_3/Ca^{2+}/calmodulin/Akt components (100). While a specific defect in the receptor-mediated signal cascade is an appealing theory in essential hypertension, flow-mediated vasodilatation, which utilizes a signal transduction cascade independent from those of the endothelial agonists, is also dysfunctional early in this disorder (101,102). This may reflect the heterogeneity of the disease or the fact that more than one pathway is often dysfunctional in any particular vascular disease.

2-3-1-2- Reduced availability of L-arginine

Availability of L-Arg to vascular endothelial cells is decreased in response to dietary deficiency, reduced transport, increased metabolism, and reduced recycling.

Dietary L-arginine deficiency: Experimentally, it has been reported that NO production as measured by urinary nitrate excretion is decreased in young rats following restriction of dietary protein or L-Arg intake as compared to normal diets (103). Although there is little evidence to support the view that dietary L-Arg deficiency elicits vascular dysfunction in humans, yet the concept of its deficiency in disease states is gaining popularity. This is because many of the described mechanisms of NO pathway dysfunction are responsive to L-Arg supplementation (for details see section 2-4).

Reduced L-arginine transport: Many pathological conditions alter L-Arg transport by endothelial cells leading to impaired production of NO. Co-localization of CAT-1 with membrane-bound eNOS within caveolae provides a mechanism for direct delivery of extracellular L-Arg to eNOS. It has been suggested that extracellular concentration of L-Arg and the activity of CAT-1 are potential determinants of the rate of NO synthesis (104). Factors that affect CAT-1 activity and in turn the rate of L-Arg transport in endothelial cells include membrane potential, the presence of endotoxin, the concentrations of potassium ion, L-Arg, other cationic amino acids, glucose and insulin (65). It has been demonstrated that hypoxia-induced membrane depolarization is responsible for reduced L-Arg transport in porcine pulmonary endothelial cells (105). Conversely, via its interaction with the A_2-purinoceptor, which causes a rapid and transient hyperpolarization, adenosine stimulates L-Arg transport into endothelial and smooth muscle cells (106). It has also been shown that endotoxin stimulates L-Arg transport systems through an autocrine pathway that involves cytokines such as TNF-α and IL-1 (107) and requires *de novo* RNA and protein synthesis (108). In addition, glucose, insulin and the diabetic state influence L-Arg transport. Acutely, glucose stimulates its transport and insulin does the same via induction of protein synthesis-dependent stimulation of L-Arg transport. Cultured umbilical veins from diabetic patients demonstrate an increased rate of L-Arg transport concomitant with elevation in basal NOS activity (64). On the other hand, in the face of long-standing hyperglycemia, insulin down-regulates elevated L-Arg transport (109). Likewise, oxidized lipoprotein inhibits L-Arg transport via CAT-1 (110). Concordant with this observation is the finding that oxLDL inhibits L-Arg transport into platelets (111). Finally, high circulating levels of cationic amino acids, notably L-lysine and asymmetric dimethylarginine (ADMA) competes with L-Arg and affects its rate of transport by CAT-1. Disruption of L-Arg transport seems to be responsive to exogenous administration of L-Arg, most probably via

bypassing the saturable y^+ system to the non-saturable transport components (64,65).

Increased metabolism of L-arginine: There is mounting evidence for the role of enhanced metabolism of L-Arg to ornithine by arginase in vascular disorders. Two isoforms of arginase are known to be present in the vasculature, arginase I (constitutive) and arginase II (inducible). In rat aortic smooth muscle cells and in macrophages, arginase II induction occurs in the presence of lipopolysaccharides which can be abolished by IFN-γ (112), while arginase I _expression can be increased by IL-4 and IL-13 and can be inhibited by the phytoestrogen and genistein (113). Induction of arginase activity has been proposed to explain NO dysfunction in the vascular components of heart failure. Patients suffering from this disorder showed increased serum arginase activity along with abnormally low plasma L-Arg levels as compared with healthy individuals (114). In this case, the increased arginase activity may not be from enhanced _expression but rather from spillage of enzyme from injured tissues such as a congested liver or from damaged myocytes such as that which has been observed in myocardial infarction (115). A novel mechanism for altered metabolism of L-Arg in smokers has recently been described. L-Arg solutions exposed to cigarette smoke extracts resulted in its depletion and the formation of cyanomethyl-derivative of L-Arg. The latter has been demonstrated to have an inhibitory effect on eNOS activity (116), which in turn might explain the decreased level of NO measured in nasal air from smokers (28).

Reduced recycling of L-arginine: It has been demonstrated that cultured endothelial cells can recycle L-Arg from citrulline (117). In addition to raising the possibility that this reaction could, in essence, provide a renewable source of intracellular L-Arg, it also raised the possibility that inhibition of this reaction may result in intracellular depletion of L-Arg during sustained NO production (117). At physiological levels, L-glutamine can actually inhibit this recycling process.

2-3-1-3- Disruption of L-arginine-nitric oxide synthase metabolic channeling

The importance of metabolic channeling is becoming clear in the NO pathway with the elucidation of the mechanisms of trafficking, compartmentalization, and anchoring of the components of the pathway. Indeed, disruptions in metabolic channeling decrease the rate of NO synthesis without affecting the affinity of eNOS for the conversion of L-Arg to NO.

Indeed, disruption of nNOS compartmentalization and anchoring in skeletal muscle fibers demonstrates their importance to facilitate metabolic channeling. Within striated muscle fibers, nNOS is anchored to the membrane surface where it may participate in the regulation of synaptic actions as well as in contraction-induced increases in local blood flow (118). Anchoring is accomplished through a series of proteins one of which is dystrophin (119). Thus, in Duchenne's muscular dystrophy, a genetically inherited neuromuscular disease caused by the absence of the gene for dystrophin normally found on the X chromosome, patients lack dystrophin and, as a consequence, have no membrane association of nNOS (119). The loss of nNOS likely contributes to an alteration in skeletal muscle microcirculation and possibly to the selective destruction of the myofibril (120).

In endothelial cells, beyond its docking to the inhibitory protein caveolin-1 while inactive, the specific anchoring mechanisms of eNOS remain unknown but intracaveolar cholesterol may play a role. The effect of oxLDL cholesterol to act as a scrubber of intracaveolar cholesterol has been given in a recent study. In this study, while overall intracellular eNOS levels remained normal, in the absence of cholesterol, both eNOS and caveolin tended to leave the caveolar compartment resulting in reduced NO activity (121).

2-3-1-4- Alterations in the activity of endothelial nitric oxide synthase to form nitric oxide

The activity of eNOS to form NO can be altered through various mechanisms including decreased affinity towards L-Arg via presence of competitive inhibitors either exogenous or endogenous, reduced availability of cofactors, and covalent modification of NOS as well as reduced dimerization of NOS.

The affinity eNOS for L-Arg is blunted either by inhibition of the binding site for L-Arg or by a conformational change in the enzyme structure. Unfortunately, regardless of a lack of affinity for L-Arg, phosphorylated eNOS tends to produce O_2^- from molecular oxygen. Indeed, cultured endothelial cells exposed to native LDL cholesterol for several days begin to generate O_2^- (122), which can be decreased by the administration of L-Arg. One

explanation of this apparent paradox is that LDL may induce an alteration in the affinity of eNOS for L-Arg such that molecular oxygen becomes the preferred substrate for electron transfer and thus administration of L-Arg may effectively compete with molecular oxygen as a substrate. Administration of NOS antagonist, on the other hand, may sufficiently block electron transfer to both substrates. Confirmation of this mechanism of reduced NO activity has been provided in the reperfused rabbit hind limb (110). In this model, the levels of detectable NO in the hindlimb microvasculature declined in association with an increase in O_2^- production following reperfusion. The addition of SOD to the tissue samples fully restored NO production. However, stimulation of the tissue samples with agonists of eNOS only augmented O_2^- elaboration. Furthermore, antagonists of eNOS led to a reduction in O_2^- release.

Conformational change in the NOS structure may occur from unavailability of a cofactor or from covalent modification. BH_4 binding and eNOS dimerization appear as two critical determinants of the affinity of NOS for L-Arg (123,124). BH_4 likely acts to stabilize the eNOS dimer (125) and it may also have a role in scavenging O_2^- (126). BH_4 binding to eNOS is determined by its availability, which, in turn, is determined by its rate of synthesis. This mechanism of dysfunction seems to play a role in atherosclerosis and its risk factors. It has been shown that inhibition of BH_4 synthesis in coronary arteries results in decreased NO activity, increased hydrogen peroxide (H_2O_2) activity and vascular dysfunction (127). BH_4 availability appears to be rate limiting in the presence of high cholesterol and in tobacco use. Improvement in endothelial function by the infusion of BH_4 has been demonstrated in patients with hypercholesterolaemia (128), while pre-incubation of veins from smokers with BH_4 significantly increased nitrite and cGMP production in response to eNOS stimulation (129). It has recently been shown that ascorbic acid administration causes an increase in BH_4 levels *in vitro* with a potentiation of stimulated NO activity (130). This effect of ascorbic acid on BH_4 levels was probably via stabilization of the molecule. Another mechanism of conformational change altering the affinity of eNOS for its substrate is via covalent modification. One example of this is oxidation of eNOS by free radicals, which ultimately affects its affinity for L-Arg (131).

Analogues of L-Arg are capable of interacting with the active site of eNOS thereby reducing its availability to L-Arg. The cyanomethyl-derivative of L-Arg produced by cigarette smoke extracts is one example of this competitive inhibition (116). Perhaps a competitive inhibitor that is more pervasive in vascular diseases in one that is produced as part of normal metabolism. It has been observed that the endogenous compound, ADMA competitively inhibits NO synthesis thereby antagonizing endothelium-dependent vasodilatation (132). Elevation of plasma ADMA has been observed in patients with renal failure (132). Dialysis, which removes ADMA, also temporarily restores endothelial function. Increased ADMA levels are associated with reduced NO elaboration as judged by reduced urinary nitrate excretion and impaired endothelium-dependent, NO-mediated forearm vasodilatation in hypercholesterolaemic individuals. Likewise, exogenous ADMA affects the activity of the NOS in the mesenteric vasculature (132), and brain (133) of rats. It turns out that normal plasma levels of ADMA are elevated two-folds in hypercholesterolaemic patients (134). In elderly patients with peripheral arterial disease and generalized atherosclerosis, ADMA levels are also increased, corresponding to the severity of the vascular disease (134). ADMA has been shown to be elevated with tobacco use in diabetic individuals (135) and in Dahl (salt-sensitive) rats fed a high salt diet (136). Recently, plasma ADMA levels have been shown to be increased, independent of other risk factors in patients with multi-vessel coronary artery disease (137). All of these findings make ADMA a possible candidate as an independent risk factor for various vascular diseases.

The main source of ADMA within endothelial cells is the catabolism of proteins containing methylated L-Arg residues (138). The cause for ADMA elevation in most cases appears to be a dysfunction in its hydrolytic degradation to citrulline by the enzyme dimethylarginine dimethylamino-hydrolase (DDAH) (139). This enzyme is typically found in tissues containing eNOS. Inhibition of this enzyme with a specific inhibitor causes a gradual vasoconstriction of vascular segments, which is reversed by L-Arg (140). Hypercholesterolaemia and oxLDL, in particular, accelerate ADMA accumulation, which is associated temporally with a decline in DDAH activity (141). Such decrease in DDAH activity seems to be related to oxidative

stress as it can be prevented by ascorbic acid treatment (60). It has been suggested that ADMA may be a physiological break to NO synthesis. With this in mind, the dysfunction of the NO pathway as it relates to ADMA might be considered not at the level of eNOS inhibition but rather at the point where oxidative stress is increased.

2-3-1-5- Altered nitric oxide synthase gene _expression

Dysfunction of the NO pathway can occur by a decrease in the _expression of eNOS gene. This can occur via decreased gene transcription or by decreased half-life of eNOS mRNA. Attenuated transcription of the gene encoding eNOS is often due to decreased gene promoter activity either by an absence of factors to which the promoter region is sensitive or by the presence of an inhibitory factor blocking the promoter region. The promoter region of eNOS gene contains a number of binding domains, suggesting that it may be regulated by a variety of transcription factors (142). Signals that enhance transcription include estrogens, shear stress, TGF-β_1, high glucose and histamine while glucocorticoids and decreased estrogen availability inhibit transcription (66,143). Similarly, a sedentary lifestyle may result in decreased eNOS gene _expression as exercise is known to enhance its _expression (62). Decreased estrogen availability and sedentary lifestyle are known risk factors for accelerated atherosclerosis. TNF-α is known to interfere with the _expression of eNOS gene and appears to do so by destabilizing eNOS mRNA as do glucocorticoids (66,143).

2-3-2- Increased Degradation of Nitric Oxide

Dysfunction of the NO pathway can occur via an increase in oxidative stress. Reactive oxygen species such as O_2^- and H_2O_2 can combine with NO directly which not only reduces the half-life of NO but also results in the production of the highly reactive $ONOO^-$. The latter can be protonated to form peroxynitrous acid, which in turn can yield the hydroxyl radical. All of these reactive species can oxidize lipids, damage cell membranes, and oxidize sulfhydryl groups (144). They can also covalently modify intracellular proteins including eNOS damaging them or rendering them less active (131). Enzymatic sources of these free radicals include xanthine oxidase, 15-lipoxygenase, NADPH oxidase, and from eNOS itself, which are up-regulated and/or activated in the presence of such things as oxLDL (122,145), and advanced glycosylation end-products (AGEs) associated with diabetes as well as hyperhomocysteinaemia.

Homocysteine reduces NO activity by a number of mechanisms, many involving an increase in oxidative stress. It has been found that the sulfhydryl group of homocysteine could be oxidized producing either H_2O_2 or O_2^- (146). Homocysteine can also scavenge NO directly forming *S*-nitrosohomocysteine (146). In addition, homocysteine increases ADMA levels thereby inhibiting NO generation. By virtue of reducing NO activity, homocysteinaemia prevents the _expression of the anti-oxidant protein, haem oxygenase-1, leading to further oxidative stress (147). Finally, oxidation products of homocysteine such as homocysteine mixed-disulfides and homocysteine thiolactone demonstrate a direct cytotoxic effect on endothelial cells (148).

Diabetes mellitus also appears to cause NO pathway dysfunction by multiple mechanisms including enhancement of oxidative stress. Hyperglycaemia and hyperinsulinaemia increase both O_2^- and H_2O_2 formation (149). In addition, free radical generation in the presence of high glucose can be inhibited by cyclooxygenase inhibitors, demonstrating that products of cyclooxygenase catalysis can also be a source of free radicals (150). High blood glucose also results in the covalent, non-enzymatic modification of proteins (151). These modified proteins, referred to as AGEs, have been shown to accumulate in diabetic and aging tissues. AGEs are directly toxic to endothelial cells (152) and elicit a wide range of cell-mediated responses leading to vascular dysfunction (153).

2-3-3- Decreased Sensitivity Toward Nitric Oxide

Even if a stimulus generates normal levels of NO, its biological activity may still not be sufficient if there is a derangement in NO-sensitive targets. For example, sGC, which is the best characterized effector enzyme in NO signaling, can be affected under certain conditions including the presence of some risk factors for heart disease. Specifically, oxLDL, hypertension, aging, and hyperglycaemia have all been shown to decrease the _expression of this enzyme (154). It has been demonstrated that in spontaneously hypertensive rats _expression of both the alpha-1 and beta-1 subunits of heterodimeric sGC and the basal levels of cGMP are reduced specifically in aortic rings. Moreover, mRNA

_expression of the cGMP receptor and effector protein cGMP-dependent protein kinase type I alpha were also reduced (155). Interestingly, aspirin appears to increase cGMP levels via enhancing sGC gene _expression at least in platelets and kidney epithelial cells and in doing so, increases sensitivity to NO (156). In contrast to these conditions which decrease gene _expression, chronic exposure to NO, such as that which can occur with the use of nitroglycerin or other NO-donors, can also desensitize sGC to NO without affecting protein levels (157).

2-4- Therapeutic approaches that preserve or restore the physiological functions of nitric oxide

Derangement in the L-Arg-NOS-NO pathway is now recognized as a common phenomenon in an array of cardiovascular disorders such as atherosclerosis, hypercholesterolaemia, hypertension and heart failure. Availability of drugs and/or nutritional supplements that restore such impairment virtually benefits these disorders and maintains cardiovascular health. In principle, therapeutic interventions that help preservation or restoration of the physiological activities of NO can target the triad components of the pathway *viz.*, substrate, enzyme, and reaction product. Supplementation with L-Arg as a sole substrate for NO seems reasonable for restoring its endogenous synthesis. Having provided a tool for introducing the enzyme itself or the gene that encodes it to the appropriate site, NO can be generated endogenously. NO itself can be administered supposing that it reaches the site of its impairment. In most instances, however, drugs that donate NO inside the body are more commonly utilized. Manipulation of the endogenous NO production and/or activity can also be achieved by some available therapies.

2-4-1- Nitric Oxide-Based Therapies

Nitric oxide: Because of its rapid inactivation in the blood by oxyhaemoglobin, NO cannot be given systemically as a therapeutic agent. Inhalation of low concentrations of NO is, however, among the most currently available vasodilator therapies used to attenuate the vasoconstrictive component of pulmonary hypertension and to improve arterial oxygenation of human subjects with severe pulmonary disease (9,28,158).

Nitric oxide-donors: More than a century ago, Alfred Nobel had synthesized the prototype NO-donor drug namely, nitroglycerin (GTN). NO-donors decompose inside the body to generate NO. Such drugs have been used for many years as cardiovascular therapeutics, in particular organic nitrates and nitrites such as GTN and amylnitrite, respectively for the management of angina pectoris. On the other hand, sodium nitroprusside (SNP), an inorganic nitroso-compound, is employed for the treatment of hypertensive emergencies. However, patients taking long-term nitrates often develop tolerance, and prolonged administration of SNP can give rise to cyanide accumulation in the body. Newer NO-donor drugs, in particular the S-nitrosothiols, offer advantages over the existing drugs, since they do not share these drawbacks, and initial clinical studies suggest that they may be of benefit in a variety of cardiovascular disorders (159). It has been shown that NO-donors including *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP) and 3-morpholino-sydnonimine (SIN-1) behave as pro-drugs and mimic the effects of NO *in vivo*. They reduce dose-dependently the oxidation of LDL (160), possess anti-platelet activity and effectively provoke vascular smooth muscle relaxation (161,162). S-Nitroso-glutathione (GSNO), a stable nitrosothiol, has been identified *in vivo* as a potential storage and transport vehicle for NO in the body (163,164). It has been used in clinical trials to inhibit platelet aggregation and adhesion at doses causing minimal vasodilatation (161). Inhaled NO-donors are among the most currently available vasodilator therapies used to attenuate the vasoconstrictive component of pulmonary hypertension (158). Several other compounds are now available in the literature and catalogs of commercial companies. It has to be noted that prolonged exposure to NO-donors reduces its endogenous generation in the vasculature that could account for the rebound vasoconstriction observed in some patients after sudden withdrawal of NO therapy.

L-Arginine: As mentioned previously, L-Arg is one of the components of the normal NO pathway. Its administration could thus aid NO bioactivity within the cardiovascular system. L-Arg surpasses the capacity of arginases, bypasses limited CAT-1 transport, enhances CAT-1 transport and the binding of BH_4 to eNOS, stabilizes the eNOS dimmer, and competes with ADMA for eNOS active site. L-Arg can also confer other beneficial NO-independent vascular effects including plasmin generation and

fibrinogenolysis, O_2^- scavenging and inhibition of leukocyte adhesion to non-endothelial matrix. Compelling evidence collected from experimental and clinical trials showed that chronic enteral or parenteral administration of L-Arg reverses endothelial dysfunction associated with major cardiovascular risk factors such as hypercholesterolaemia, smoking, hypertension, diabetes, and aging and it ameliorates many common cardiovascular disorders such as coronary and peripheral arterial disease, ischaemia/reperfusion injury, and heart failure. It has been suggested that dietary L-Arg supplementation may represent a potential nutritional strategy for preventing and treating cardiovascular disease (37,165,166). It has been shown that diets rich in L-Arg prevent the development of hypertension in animals at risk, and its infusion causes rapid reduction of blood pressure in human with essential hypertension (9). Furthermore, in hypercholesterolaemic patients an infusion of L-Arg improved forearm blood flow of resistance vessels in response to methacholine (167). Interestingly, L-Arg induced apoptosis of macrophages in intimal lesions by its conversion to NO, which acts through a cGMP-dependent pathway (168); an effect that may partly explain the observed regression of atheroma in cholesterol-fed rabbits by L-Arg supplementation (60). When concurrently administered, L-Arg and BH_4 increased NO synthesis and decreased O_2^- generation by the atherosclerotic dysfunctional endothelium (169,170). Long-term oral L-Arg supplementation in humans showed a marked decrease in plasma endothelin concentrations (171) and in cell adhesion molecule and pro-inflammatory cytokine levels (60).

2-4-2- Nitric Oxide Synthase-Based Genetherapy

Generally speaking, gene therapy aims to intervene in a disease process by transfer and _expression of specific functional genes in a target tissue or organ. Depending on the basis that in many cardiovascular disorders, the pleiotropic beneficial activity of NO is lacking, NOSs have received considerable attention as potential candidates for gene therapy (172-174). Blood vessels are among the easiest targets for gene therapy and adenoviral vectors are the most efficient means of transgene _expression. According to treatment requirements, gene transfer to the artery wall can be accomplished both from lumen and from adventitia. Transfer and functional _expression of recombinant NOS genes to cerebral and cardiovascular beds have demonstrated promising results both *ex vivo* and *in vivo*. It was found that *ex vivo* gene transfer of eNOS to atherosclerotic rabbit aortic rings improves relaxations to acetylcholine (175) suggesting that the reduced NO bioavailability observed in cholesterol-induced vascular dysfunction can be partially overcome by eNOS gene transfer. Increasing NOS _expression via transfection with eNOS/DNA of human endothelial cells augmented L-Arg and shear stress-induced NO synthesis leading to a reduction in endothelial adhesiveness for monocytes and ameliorated atherogenesis (176). Although the feasibility of the NOS gene transfer approach has been demonstrated in animal models, further studies regarding gene transfer techniques, vectors and safety of the procedures still have to be performed before human NOS gene therapy for cardiovascular disease can be attempted (173,174).

2-4-3- Common Drugs and Nutritional Supplements Augenting Endogenous Nitric Oxide

Many experimental and clinical studies showed beneficial modulation on NO bioavailability via many commonly utilized dietary supplements and drugs including anti-oxidants, lipid lowering agents particularly 3-hydroxy-3-methylglutaryl (HMG) CoA reductase inhibitors, angiotensin converting enzyme (ACE) inhibitors, certain calcium channel blockers and β adrenoceptor blockers as well as estrogens.

Anti-oxidants: The corollary of the oxidation hypothesis of atherogenesis is that anti-oxidants may reduce plaque formation and progression of the disease (177,178). Anti-oxidant intervention and restoration of the NO activity have been shown to mitigate functional and structural arterial alterations and improve cardiovascular outcomes (179). Vitamin E antagonizes mainly oxLDL-related events in atherogenesis with subsequent benefits on NO activity while vitamin C exerts its benefits on NO, most probably, as a result of the increase in BH_4 production (130) and the enhancement of DDAH activity via relief of oxidative stress (60). Chronic administration of vitamin E improved endothelium-dependent vasodilator function and systemic NO production, reduced vascular oxidative stress, and reduced the progression of atherosclerosis in cholesterol-fed rabbits with preexisting hypercholesterolaemia (180,181). In spite of encouraging results obtained with anti-oxidants in

animals, clinical trials could only show a clear positive effect of anti-oxidants treatment on the outcome of cardiovascular disease.

Angiotensin converting enzyme inhibitors: Animal studies have demonstrated the potential beneficial effects of ACE inhibition at a variety of sites, including improvement of endothelial function, inhibition of platelet aggregation and prevention of atherosclerotic lesions. In addition, ACE inhibitors inhibit the breakdown of quinines by metaloproteinases, thereby enhancing the stimulus for NO production from the intact endothelial cell. Thus, at the cellular level ACE inhibition shifts the balance of ongoing mechanisms in favour of those promoting vasodilatory, anti-aggregatory, anti-thrombotic and anti-proliferative effects (182-184).

Lipid lowering drugs: Clinical and experimental evidence has amassed suggesting that apart from cholesterol reduction, therapy with HMG CoA reductase inhibitors can improve endothelial function and thus benefit cardiovascular health. They increase the production of NO *in vivo* possibly via enhancement of the dissociation of 63 kDa protein from eNOS mRNA thereby preserving it from degradation (185-189).

Calcium channel blockers: It has been shown that calcium antagonists enhance the release of NO most probably via enhancing eNOS gene transcription and via stabilizing eNOS mRNA (184,190,191).

Beta adrenoceptor blockers: Recently, some of the third generation β blockers as nebivolol have showed novel properties including augmented release of vascular NO (192,193).

Folates: It has recently been reported that supplementation with folates benefits endothelium functions via direct interactions with the enzyme eNOS in addition to the anti-oxidant properties and the lowering effect on homocysteine level (194).

Long-chain n-3 polyunsaturated fatty acids: The cardiovascular health benefits of long-chain n-3 polyunsaturated fatty acids have been reported to be related to NO pathway (187,195).

Estrogens: There is evidence that estrogen therapy can stimulate eNOS activity and thus increase the bioavailability of NO. Enhancement of eNOS gene _expression can be brought about by activating the SP1 promoter region of the gene and by decreasing the _expression of the eNOS inhibitor protein, caveolin-1 (196,197).

Ibudilast: Ibudilast, a non-selective inhibitor of cyclic nucleotide phosphodiesterase, is widely used in Japan for improving prognosis and relieving symptoms in patients suffering from ischaemic stroke. This clinical application is based on the properties of ibudilast that inhibit platelet aggregation and improve cerebral blood flow which may be due to synergistic elevation of intracellular cyclic nucleotides and release of NO from endothelium (198).

2-4-4- Newly Developed Nitric Oxide Enhancing Therapeutics

Nitric oxide/aspirin combination: NO-releasing aspirin (NCX 4016, 2-(acetyloxy) benzoic acid 3-(nitro-oxymethyl) phenyl ester, Nicox company, Sophia Antipolis, France) is a recently developed drug that still in Phase I-II clinical trials in many European countries as anti-platelet agent (199). It combines the *in vivo* beneficial cardiovascular effects of NO and the anti-thrombotic activity of aspirin. It has been reported that this drug demonstrates the *in vitro* dose-dependent inhibition of arachidonic acid-induced platelet aggregation and thromboxane B_2 production (200), and platelet adhesion (201). In addition, both *in vitro* and *in vivo* cardioprotective effects in experimental models of ischaemia and infarction (202) as well as atherosclerosis (60,203) were also observed.

Nitric oxide/non-steroidal anti-inflammatory drugs combination: The protective effect of NO against non-steroidal anti-inflammatory drugs (NSAIDs)-induced gastroenteropathy has latterly been demonstrated (204). To lower the gastric injury commonly associating the use of NSAIDs, different compounds belonging to this class of drugs have been formulated by attaching NO releasing-moieties and showed promising results (205,206). The beneficial effects of NO are due to its involvement in the maintenance of gastric mucosal blood flow allowing adequate perfusion and also due to its mucosal protector effect via regulation of acid and mucus secretion as well as alkaline production (207).

L-Arginine/yohimbine: The combination of yohimbine and L-Arg is currently under clinical investigation in early phase III development for male erectile dysfunction therapy (208).

S-Nitrosocaptopril: S-nitrosocaptopril (CapNO) is a novel vasodilator possessing the capacities of both a NO-donor and an ACE inhibitor. Considering the absence of adverse effects of CapNO in the subchronic toxicity study, CapNO appears to be a

safe and effective drug for further clinical trials (209).

BiDil: BiDil is a novel NO-donor containing a combination of isosorbide dinitrate and hydralazine introduced by NitroMed company for NO-enhanced medicines (www.nitromed.com). It is a drug to potentially treat heart failure. The United States Food and Drug Administration has deemed BiDil ultimately approvable, pending positive results of the African American Heart Failure Trial (A-HeFT), a clinical study to confirm the product's efficacy specifically in patients of African decent. If approved, BiDil would be the first heart failure medication specifically indicated for these patients.

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