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**Arginine and nitric oxide synthase: regulatory mechanisms and cardiovascular aspects.**

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**Abstract:**

L-Arginine (L-Arg) is a conditionally essential amino acid in the human diet. The most common dietary sources of L-Arg are meat, poultry and fish. L-Arg is the precursor for the synthesis of nitric oxide (NO); a key signaling molecule via NO synthase (NOS). Endogenous NO synthase inhibitors such as asymmetric-dimethyl-L-Arginine (ADMA) inhibit NO synthesis in vivo by competing with L-Arg at the active site of NOS. In addition, NOS possesses the ability to be “uncoupled” to produce superoxide anion instead of NO. Reduced NO bioavailability may play an essential role in cardiovascular pathologies and metabolic diseases. L-Arg deficiency syndromes in humans involve endothelial, inflammation and immune dysfunctions. Exogenous administration of L-Arg restores NO bioavailability, but it has not been possible to demonstrate, that L-Arg supplementation improved endothelial function in cardiovascular disease such as heart failure or hypertension. L-Arg supplementation may be a novel therapy for obesity and metabolic syndrome. The utility of L-Arg supplementation in the treatment of L-Arg deficiency syndromes remains to be established. Clinical trials need to continue to determine the optimal concentrations and combinations of L-Arg, with other protective compounds such as tetrahydrobiopterin (BH<sub>4</sub>), and antioxidants to combat oxidative stress that drives down NO production in humans.

**Key words:**

Arginine/ Nitric oxide synthase/ cardiovascular/therapeutic potential

# 1 Introduction

L-Arginine (L-Arg) deficiency syndromes in humans fall into at least two broad categories involving endothelial dysfunction and immune cell dysfunction. Low intake of dietary L-Arg has recently been associated with high levels of C-reactive protein, a biomarker of inflammation. The deleterious effects of low L-Arg availability may lead to a proatherosclerotic environment and subsequent cardiovascular disease [1-4]. Thus, dietary intake of L-Arg may be a critical factor in the way the human body responds to inflammation and oxidative stress associated with cardiovascular dysfunction. The hydrolysis of L-Arg by L-Arginase produces urea and ornithine, which in turn can serve as precursors for the synthesis of polyamines, proline or glutamate. Moreover, there are significant secondary consequences of reductions in the availability of L-Arg due to its catabolism by L-Arginase. These include reduced nitric oxide (NO) synthesis. L-Arg is oxidized to NO and Citrulline by the action of endothelial nitric oxide synthase (NOS). There are three main NOS isozymes, the neuronal NOS (nNOS or NOS I), inducible NOS (iNOS or NOS II), and endothelial NOS (eNOS or NOS III). NO is produced by iNOS in the immune system [5]. In contrast, protein-incorporated L-Arg residues can be methylated with subsequent proteolysis giving rise to methyl L-Arg compounds, such as asymmetric dimethyl-L-Arg (ADMA), which competes with L-Arg for binding to NOS [6]. In this context, the L-Arg/ADMA pathway is emerging as critical to endothelial function. Interest in L-Arg intake and supplementation is likely to grow. It has been reported that exogenous administration of L-Arg restores NO bioavailability, but it has not been possible to demonstrate, in all the studies, that L-Arg supplementation improved endothelial function in cardiovascular disease [7, 8]. The utility of L-Arg supplementation in the treatment of L-Arg deficiency syndromes remains to be established. Now, there is increasing evidence that upregulation of L-Arginases (I and II) functionally inhibits NOS activity and contributes to the pathophysiology of related vascular dysfunction. Furthermore, pharmacological inhibition of L-Arginase 1 (L-Arg1) restore endothelial NO production and endothelial function ex vivo [9, 10]. L-Arginase inhibitors are competitive inhibitors and do not inhibit NOS enzymes at concentrations that inhibit L-Arginases. In this context, L-Arginase inhibitors represent a potential alternative for treatment of L-Arg deficiency with potential therapeutic effects. The major objective of this article is to examine the metabolic basis of L-Arg nutrition and to review the roles of L-Arg as well as the prevention and treatment of cardiovascular diseases.

## 2 Arginine synthesis and catabolism

### 2-1 Arginine needs and usual diets

L-Arg is an essential amino acid (AA) in the human diet and a basic AA in physiological fluids. The most common dietary sources of L-Arg are meat, poultry and fish. Its content is relatively high in rice protein concentrate, and soy protein isolate [11], but low in the milk of most mammals. A study has been reported concerning the patterns of dietary intake of L- L-Arg in the general US population [12, 13]. It is an analysis of adults aged 18 years and older who participated in the Third National Health and Nutrition Examination Survey, a national public-use nutrition survey of non-institutionalized persons. Mean L-Arg intake for the adult population was 4.40 g/d, with 25% of people consuming less than 2.6 g/d. Obese individuals, and patients with diabetes consumed more L-Arg than people without those characteristics. Smokers had lower intake than non-smokers.

The major advantages of dietary L-Arg supplementation over drugs are that L-Arg is cheap and readily available from natural foods [14-16]. Oral administration of appropriate doses of L-Arg to humans is well tolerated and results in no side-effects [11]. However, higher oral doses of Arg-HCl (9 g/day) are occasionally associated with nausea, gastrointestinal discomfort, and diarrhea for some subjects, which may result from an excess production of NO by the gastrointestinal tract and from impaired intestinal absorption of other dietary basic AA such as lysine and histidine [17]. It would not be advisable to administer L-Arg to patients with severe inflammatory or autoimmune disorders, the risk being an excessive production of NO in cells [18].

Substantial amounts of orally administered L-Arg do not enter the systemic circulation in adults because 40% of dietary L-Arg is degraded by the small intestine in first pass metabolism and L-Arg, with a half-life of 1h in the circulation, is turned over rapidly in mammals [18]. L-Arg concentrations in hepatocytes are very low (0.03–0.1 mM), compared with 0.5–10 mM for other AA. In adults, endogenous synthesis of L-Arg involves the intestinal-renal axis via glutamine-glutamate - Citrulline metabolism. Inside cells, there are **multiple pathways** for L-Arg degradation to produce NO, ornithine, urea, polyamines, proline, glutamate and creatine. These pathways are initiated by L-Arginases, NO Synthases, L-Arg/glycine amidinotransferase, and L-Arg decarboxylase. In mammalian cells, 2% of metabolized L-Arg is utilized for constitutive NO production. Quantitatively speaking, the L-Arginase pathways are the most important for L-Arg catabolism. Two L-Arginase isozymes are expressed in humans and other mammals: L-Arginase I, which is cytosolic and expressed at high levels in the liver as a component of the urea cycle, and L-Arginase II, which is mitochondrial and expressed in moderate levels in the kidney [4, 19]. There is strong evidence that constitutive levels of L-Arginase activity in endothelial cells limit NO synthesis and NO-dependent vasodilatory function. L-Arginase inhibitors represent a potential alternative for treatment of L-Arg deficiency. Several potent L-Arginase inhibitors have been developed and have become commercially available within the past few years [20].

## **2-2 Arginine transport systems**

The regulation of L-Arg transport is essential in the regulation of NO synthesis. L-Arg in the plasma compartment contributes significantly (~60%) as a precursor pool for the synthesis of whole body NO. Intracellular concentrations of L-Arg range from 100 to 800  $\mu\text{M}$  in cultured endothelial cells while plasma L-Arg levels in humans reportedly range from 80 to 120  $\mu\text{M}$ , although levels as high as 210  $\mu\text{M}$  under normal physiological conditions have been reported [21]. Despite the substantial intracellular concentration of L-Arg, extracellular L-Arg has been demonstrated to be rate limiting for NO production.

Multiple transport systems mediate the influx of cationic and anionic amino acids across plasma membranes into mammalian cells. In addition, in most cells, arginine transport through the plasma membrane is not energized by coupling to the  $\text{Na}^+$  gradient. Thus, a single  $\text{Na}^+$ -independent transport system termed system Y+ is postulated to be the major entry route for cationic amino acids (CAAs) to most cells. (The letter  $\gamma$  is derived from lysine, the first substrate described for this system, and the + signifies the positive charge of the AA substrates.)

In mammalian cells, CAA transport via CAA transporters (CATs) is mediated by various transport systems that can be distinguished by their interaction with neutral amino acids and their dependence on  $\text{Na}^+$ . CATs are integral membrane glycoproteins with 14 putative transmembrane domains and an intracellular domain. Of these, the so-called system  $\gamma^+$  accounts for a significant part of CAA transport in most cell types. Carrier systems “ $\gamma^+$ ” or cationic amino acid transporter (CAT) and “ $\gamma^+\text{L}$ ” exist. L-Arg is transported into red blood cells (RBC) and is hydrolyzed to ornithine and urea by arginase (isoform I) or converted to NO and Citrulline by eNOS. L-Arg diffusion through RBC membranes (independently of CATs) requires supra-physiological concentrations of arginine, and is thus not clinically significant. System  $\gamma^+$  activity can be mediated by at least three different CAT proteins: CAT-1, -2B, and -3. All CAT proteins mediate influx as well as efflux of CAA [22]. Associations between CAT-1 and cytoskeletal proteins and caveolin are reported. These protein interactions, as well as the subcellular localization of CAT-1, seem to depend on the cell type. In human umbilical vein endothelium, L-Arg transport is mediated by the amino acid transport system  $\gamma^+$ /CATs and the cationic/neutral amino acid transport system  $\gamma^+\text{L}$  [23]. In addition, system  $\gamma^+\text{L}$  activity is downregulated by elevated concentrations of extracellular D-glucose [24]. It has been shown that insulin blocks D-glucose-increased L-Arg transport and cGMP accumulation in human umbilical vein endothelial cells (HUVEC). In these cells, insulin also modulates high D-glucose effects by activating transcriptional factors such as NF- $\kappa\text{B}$ . Evidence suggests that insulin blocks the stimulatory effect of D-glucose on L-Arg transport by reducing the transcriptional activity of CAT-1 activity via NF- $\kappa\text{B}$ - and reactive oxygen species (ROS)-dependent mechanisms [25].

## **2-3 Arginine and interactions with NO synthases**

### **2-3-1 Substrate and cofactors**

As we reported, the enzymatic activity of NOS is tightly controlled, depending on substrate and cofactor availability, as well as the rate of electron transfer. The substrate and cofactors that control NOS activity are also involved in other major metabolic pathways in the cell, thereby connecting NOS

activity with other metabolic pathways. In the presence of sufficient cofactors (nicotinamide dinucleotide phosphate (NADPH), flavin mononucleotide (FMN), tetrahydrobiopterin (BH<sub>4</sub>), flavin adenine dinucleotide (FAD)), NOS activity is dependent on the availability of L-Arg and oxygen [26]. Protein-protein interactions represent an important new element in the control of NOS activity [27].

The conversion of L-Arg into NO and L-Citrulline occurs in two mechanistic steps. First, L-Arg is hydroxylated to form N-hydroxy-L-Arg (NOHA), which is then converted to L-Citrulline with the concomitant release of NO. In each reaction, the NADPH in the reductase domain is utilized as the electron source. The three main NOS isozymes, catalyze the reaction. iNOS functions in an immunoprotective capacity, serving to fight off infection from sources ranging from bacteria to viruses. This isoform can be rapidly expressed in response to diseases such as myocardial ischemia [28]. In this context, iNOS can produce large amounts of NO in a short period of time. However, the increased production of NO has been implicated as a factor in myocardial dysfunction and the lack of response to vasoconstrictors during endotoxin shock induced by lipopolysaccharide (LPS). The spin-trapping technique allowed us to monitor organ-specific formation of NO after LPS administration and for the first time, we demonstrated direct NO production in the aorta and heart of LPS-treated animals [29, 30].

The reaction mechanism of NOS presents many similarities with that of cytochrome P450. The formation of a tight dimer through the heme domains is required for activity. In the periphery, many smooth muscle tissues are innervated by nitrergic nerves, i.e. nerves that contain nNOS and generate and release NO. NO produced by nNOS in nitrergic nerves can be viewed as an unusual neurotransmitter that stimulates NO-sensitive guanylyl cyclase in its effector cells, thereby decreasing the tone of blood vessels [31].

The structures of iNOS, eNOS and nNOS have been determined [32, 33]. The N-terminal catalytic domain binds the heme prosthetic group as well as the redox cofactor, tetrahydrobiopterin (BH<sub>4</sub>) associated with a regulatory protein, calmodulin (CaM). The C-terminal reductase domain has the binding sites for FMN, FAD, and NADPH. Both nNOS and eNOS are expressed constitutively in mammals and called constitutive NOS (cNOS). cNOS activity is controlled by CaM binding in a Ca<sup>2+</sup> concentration dependent manner. The enzyme is bound to caveolin-1, a protein that inhibits eNOS activity. Caveolin-1 interacts with the CaM binding sites and inhibits the electron transfer from NADPH at the reductase to the heme molecule in the oxygenase domain [32, 34]. NO synthesis is driven by electron transfer through FAD and FMN cofactors, and is controlled by CaM binding in the constitutive mammalian enzymes [35]. Electron transfer from FMN to heme has been shown to be the rate-determining step for overall NO synthesis and involves a conformational change in which the FMN domain moves from an electron input state to an electron output state [34]. Finally, the availability of substrate and cofactors as a limiting factor of NOS activity has been primarily attributed to pathophysiological situations. The use of selective NOS inhibitors has made it possible to investigate the roles of NOS in physiological and pathological functions. By using genetically modified mice, the cardiovascular roles of NOS have been studied. The findings provide important insights into the functions of NOS in cardiovascular diseases. All types of NOS gene-deficient animals have been developed. Furthermore, numerous types of NOS gene-transgenic (TG) animals, including conditional and non-conditional TG mice with endothelium-specific or cardiomyocyte-specific

overexpression of each NOS isoform, have been established [36]. Abnormal NOS signaling plays a key role in many cardiac disorders, while targeted modulation may potentially reverse this pathogenic source of oxidative stress. Improvements in the clinical translation of potent modulators of NOS function/dysfunction may ultimately provide a powerful new treatment for many heart diseases that are fueled by nitroso-redox imbalance [37].

Despite the name, nNOS can be found in nerve endings as well as muscles. Outside the brain, nNOS modulates various processes. A multitude of physiological functions, such as neurogenesis, are performed by the neuronal isoform under normal conditions, whereas pathologies such as ischemic brain damage and Parkinson's disease can also arise when the enzyme malfunctions [38].

### **2-3-2 Importance of BH<sub>4</sub>**

The major role of BH<sub>4</sub> (2-amino-6-(1,2-dihydroxypropyl)-5,6,7,8-tetrahydro-1H-pteridin-4-one) in vascular disease is frequently considered to be the regulation of eNOS function in the endothelial cell layer. This focus on endothelial cell biology overlooks important roles for BH<sub>4</sub> in the control of other NOS isoforms, in particular iNOS, which are expressed by other cell types. In contrast to the vasoprotective role of eNOS, iNOS expression is regarded as detrimental, in particular due to its role in septic shock [30, 39]. BH<sub>4</sub> distribution is highly tissue-specific in mammals, suggesting the presence of a tissue-specific regulatory mechanism to maintain cellular BH<sub>4</sub> homeostasis. BH<sub>4</sub> is synthesized through a multistep pathway initiated by guanosine triphosphate (GTP) cyclohydrolase I (GTP-CH). GTP-CH is the rate-limiting enzyme in BH<sub>4</sub> biosynthesis. BH<sub>4</sub> is formed de novo from GTP, via a sequence of enzymatic steps carried out by GTP-CH, 6-pyruvoyl tetrahydropterin synthase (PTPS) and sepiapterin reductase (SR) [40]. The iron is present as a heme ion. Heme and BH<sub>4</sub> are found in the oxygenase domain of the enzyme, the additional cofactors FAD and FMN bind to the reductase domain, which donates electrons from NADPH to the oxygenase domain. NOS are active as dimers, and the reductase domain of one dimer interacts with the oxygenase domain of the other dimer [41]. BH<sub>4</sub> is one of the most potent naturally occurring reducing agents. It is therefore reasonable to hypothesize that oxidative stress may lead to excessive oxidation and depletion of BH<sub>4</sub>. An important step in the elucidation of the reaction mechanism was the detection of a trihydrobiopterin radical (BH<sub>3</sub>). BH<sub>4</sub> was found to stabilize the dimeric form of NOS. BH<sub>4</sub> provides the two electrons required for the reduction of the second atom to water and therefore acts as a substrate rather than a tightly bound cofactor. A redox-active contribution to catalysis by reductive activation of the heme–oxygen complex has been demonstrated.

### **2-3-3 Uncoupling of NOS**

Under certain conditions, NOS loses its ability to convert L-Arg to L-Citrulline. Removing an electron from NADPH and donating it to molecular oxygen to yield superoxide instead of NO. Superoxide produced from the uncoupled NOS leads to the state of oxidative stress which can dramatically affect cardiovascular functions [42].

The balance between NO and superoxide production by eNOS appears to be determined by the availability of BH<sub>4</sub>. When BH<sub>4</sub> levels are sufficient, oxidation of L-Arg is coupled with the reduction of



molecular oxygen to form NO and L-Citrulline. When BH<sub>4</sub> levels become limiting, due to either reduced biosynthesis or to oxidative loss, NOS enzymes become uncoupled and superoxide is produced as an alternative product of the enzyme. This production of superoxide can cause further oxidative loss of BH<sub>4</sub> thus potentiating a state of oxidative stress in the cells of the vasculature. The half-life of NO and therefore its biological activity are decisively determined by ROS such as superoxide: this free radical rapidly reacts with NO to form the highly reactive intermediate peroxynitrite (ONOO<sup>-</sup>) in a reaction that is about 10 times faster than the dismutation of superoxide by superoxide dismutase. The reaction with superoxide and ONOO<sup>-</sup> formation may substantially limit NO bioavailability and its protective effects. ONOO<sup>-</sup> may impair the activity of endothelial NOS. Peroxynitrite in turn will oxidize BH<sub>4</sub> to the BH<sub>3</sub><sup>•</sup> radical, and the resulting decrease in endothelial BH<sub>4</sub> triggers eNOS uncoupling [5].

In most situations where endothelial dysfunction due to increased oxidative stress is encountered, the expression of eNOS has been shown to be paradoxically increased rather than decreased. It appears that eNOS may become “uncoupled”. In this uncoupled state, electrons normally flowing from the reductase domain of one subunit to the oxygenase domain of the other subunit are diverted to molecular oxygen rather than to L-Arg; in these conditions, superoxide rather than NO is produced. Superoxide produced from the uncoupled NOS leads to a state of oxidative stress [42]. The most prominent cause of NOS uncoupling is the loss of the critical NOS cofactor BH<sub>4</sub> either by oxidation or by decreased expression of the recycling enzyme dihydrofolate reductase (DHFR) [43].

Depletion of the NOS substrate L-Arg as well as BH<sub>4</sub> depletion and oxidative stress can result in NOS uncoupling. L-Arginase is the final enzyme of the urea cycle and competes with NOS for L-Arg thus reciprocally regulating NOS activity. L-Arginase inhibition in old rats restores eNOS coupling and reverses vascular stiffness by increasing the availability of L-Arg [9]. It has been demonstrated in these studies that increased L-Arginase activity in aging was due to iNOS-dependent s-nitrosylation of L-Arginase, thus increasing its activity and resulting in NOS uncoupling.

Direct administration of BH<sub>4</sub> appears to be a very effective therapy to recouple NOS. However, the salvage pathway can be enhanced to increase bioavailable levels of BH<sub>4</sub> by stimulating DHFR with dietary folic acid. Enhanced DHFR expression and activity from folic acid result in increased NO and BH<sub>4</sub> bioavailability while decreasing superoxide production in endothelial cells. These effects are consistent with NOS recoupling [44].

#### **2-3-4 Endogenous NO synthases inhibitors**

The proteolysis of proteins containing methylarginine residues leads to the release of free methylarginine residues into the cytoplasm. Free asymmetric-dimethyl-L-Arginine (ADMA) is actively degraded by intracellular enzymes: dimethylarginine dimethylaminohydrolases (DDAHs). Pharmacological inhibition of DDAHs by S-2-amino-4(3-methyl guanido) butanoic acid leads to the accumulation of free ADMA in endothelial cells, indicating that the enzyme is an important determinant of local levels of methylarginines within cells and tissues [45].

Asymmetric methyl-L-Arginines inhibit NO synthesis in vivo by competing with L-Arg at the active site of NO synthases. The synthesis of NO is selectively inhibited by guanidino-substituted analogs of L-

Arg, such as N<sub>G</sub>-monomethyl-L-Arg (L-MMA) or N-nitro-L-Arg (L-NNA), which act as competitive inhibitors at the active site of the enzyme [46]. L-MMA and ADMA act as endogenous inhibitors of NO synthase [47]. ADMA belongs to a group of naturally occurring methyl L-Args, which are by-products of protein degradation in cells (proteolysis of post-translationally methylated tissue proteins). ADMA is continuously formed during the intracellular turnover of proteins. Methylated L-Arg residues are released into the cytoplasm from the breakdown of proteins that have been acted on by the enzymes protein-L-Arg methyl transferase (PRMT) 1 and 2, methylation being an important mechanism in biology [48]. In mammalian cells, these enzymes have been classified into type I (PRMT1, 3, 4, 6, and 8) and type II (PRMT5, 7, and FBXO11) enzymes, depending on their specific catalytic activity. Both types of PRMT, however, catalyze the formation of monomethyl-L-Arg (MMA) from L-Arg. In a second step, type I PRMTs produce ADMA, while type II PRMT catalyzes symmetric dimethyl-L-Arg (SDMA). Proteins containing methyl-L-Arg groups lead to the release of free methyl-L-Arg into the cytoplasm. Free methyl-L-Args are cleared from the plasma by renal excretion and hepatic metabolism. MMA and ADMA can be degraded to Citrulline and dimethylamines (DMA) by DDAHs [49]. ADMA can be exported from the cell to the circulation via cationic amino acid transporters (CAT), which also mediate uptake by other cells or organs, thereby facilitating transport of ADMA [50]. Whole blood contains ADMA incorporated in protein-L-Arg. The role of erythrocytes in the storage and generation of the endogenous NOS inhibitor ADMA has been investigated. There is fast bidirectional traffic of ADMA across the plasma membrane of the erythrocyte, leading to equilibrium between intra- and extracellular ADMA. Upon lysis of erythrocytes, proteolytic activity leads to a substantial release of free ADMA from methylated proteins but with no appreciable contribution from hemoglobin. The erythrocyte serves both as a reservoir and a source of free ADMA [50].

ADMA is metabolized extensively by the kidneys and liver, which are the principal sites of DDAH-1 expression. Some ADMA is excreted by the kidney. DDAHs have been highly conserved throughout evolution. Two isoforms of DDAH, encoded by genes located on chromosomes 1 (DDAH-1) and 6 (DDAH-2), have been identified. The two isoforms have distinct tissue distributions, but both have been identified within the cardiovascular system [51].

The predictive value of plasma ADMA concentrations is of fundamental importance in subjects with pathologies in which the risk of heart disease and coronary events remains high. From a prospective cohort of patients with acute myocardial infarction (MI), the aim of studies in our laboratory was to identify the determinants of ADMA levels and to analyze the predictive value of ADMA on mortality at long-term follow-up [6]. Our findings suggest the predictive value of ADMA for long-term mortality after MI, goes beyond that obtained with baseline determinants of prognosis. In Finnish middle-aged men elevated levels of plasma ADMA have been initially shown to be associated with the risk of cardiovascular (CV) events [52]. Studies are being developed to establish the true role of asymmetric dimethylarginine as a marker and mediator of CV diseases, with possible therapeutic applications [53].

## 3 Arginine and cardiovascular function

### 3-1 Endothelium and heart aspects

#### 3- 1- 1 NO: a ubiquitous endogenous cardiovascular agent?

Endothelial dysfunction is a common trait of essentially all cardiovascular risk factors. Impaired NO-mediated endothelial function is characteristic of cardiovascular diseases, correlates with risk factor profiles and is an independent predictor of adverse cardiac events [54, 55]. In organ bath experiments, eNOS<sup>-/-</sup> mice demonstrated expected impairment of endothelial-derived relaxation to NOS-dependent stimuli, such as acetylcholine. These mice are also hypertensive. Experimental studies in eNOS<sup>-/-</sup> mice demonstrated that eNOS has necessary roles in vascular disease pathophysiology [56]. When eNOS<sup>-/-</sup> mice are crossed with the hyperlipidemic ApoE<sup>-/-</sup> mouse line, a lack of eNOS expression increases both diet-induced atherosclerosis and injury-induced neointima formation [57]. Recent studies have addressed the degree of NOS uncoupling seen in in vivo disease models. ApoE<sup>-/-</sup> mice have both a reduced production of NO, compared with matched wild-type mice, and an overproduction of NOS-derived ROS species, as demonstrated by their inhibition with L-NAME treatment [58].

Cardiovascular diseases associated with oxidative stress have been linked with reduced L-Arg transport. In our laboratory, we investigated the role of NOS modulation on 1) the evolution of functional parameters and the level of post-ischemic recovery and 2) the amount of free radical species released during a sequence of global myocardial ischemia and reperfusion, using L-Arg as a substrate or N<sub>G</sub>-nitro-L-Arg methyl ester (L-NAME) as an inhibitor, in comparison with their D-specific enantiomers [59]. Under our experimental conditions, it was not possible to determine differences in cardiac function between D- or L-Arg- treated hearts. Many authors have described beneficial effects of L-Arg treatment on the recovery of myocardial function during reperfusion [60] whereas others have shown a harmful effect of L-Arg administration [61]. These discrepancies can be attributed to different experimental models but also to the critical timing of the administration of this amino acid. Moreover, a species difference has been reported concerning the effectiveness of endogenous NO to protect hearts against reperfusion-induced dysfunction [62].

In normal vascular physiology, NO is one of the most potent endogenous vasodilators and, by its anti-inflammatory, anti-proliferative and anti-thrombotic properties, is widely recognized as an endogenous vasoprotective agent in various pathologies. Endothelial dysfunction with reduced NO production and/or bioavailability may contribute to the development and progression of vascular complications in patients. However, large amounts of NO produced by inducible NO synthase (iNOS) and/or peroxynitrite (ONOO<sup>-</sup>), due to the reaction of NO with superoxide anion, are involved in pro-inflammatory reactions and tissue damage. This implies that NO is a Janus-faced molecule [63]. Cardiomyocytes are specialized cells whose primary function is contraction in order to provide the motor force that drives cardiac output and generates arterial pressure. There is mounting evidence that cardiomyocytes also respond to danger signals with a complex inflammatory and functional response. In this field, sepsis induces a primary depression of cardiac contractility, depending on the timeframe. It has been demonstrated that this inflammation is necessary to limit tissue damage and

initiate the healing process in the myocardium [30, 64-66]. However, prolonged, less-specific, uncontrolled inflammation is associated with iNOS over-expression and leads to chronic myocarditis and heart failure. This over-expression of iNOS occurs early in sepsis, takes place in nearly all the vital organs and is associated with oxidative stress [67]. Patients with sepsis show increased NO production and elevated nitrite and nitrate levels, which have been correlated with the severity of illness. The negative role of iNOS in sepsis is also indicated by the fact that the administration of the iNOS substrate arginine results in a poorer outcome in sepsis [68]. All these data are not consistent with the notion that NO is a ubiquitous endogenous cardioprotective agent.

### **3-1-2 L-Arginine supplementation**

As previously mentioned, one of the main factors in the regulation of L-Arg transporters is substrate availability. It has been reported that the exogenous administration of L-Arg restores NO bioavailability [69]. However, it has not been possible to demonstrate in all of the studies that L-Arg supplementation improved endothelial function in cardiovascular disease such as heart failure or hypertension [70]. Clinical studies with L-Arg have also shown inconsistent effects on endothelial function. In some clinical studies, acute and subacute L-Arg administration improved NO-dependent vasodilatation in patients with coronary artery disease or hypercholesterolemia. In contrast, the VINTAGE MI study demonstrated that in patients assigned to receive L-Arg (goal dose of 3 g 3 times a day) or matching placebo for 6 months, did not improve vascular stiffness measurements or ejection fraction and may have been associated with higher post-infarction mortality [71]. The conclusion of this study was that L-Arg therapy should not be given to patients following myocardial infarction. It neither alters noninvasive measurements of vascular stiffness nor improves left ventricular function. L-Arg therapy in older patients with diffuse atherosclerosis may worsen clinical outcomes. In addition to these negative results, one can postulate that in these pathologies it is not clear that endothelial dysfunction was related to L-Arg deficiency.

Results of recently published studies indicate that dietary L-Arg supplementation, even if started in adult life, markedly increases mitochondrial biogenesis and brown adipose tissue mass in obese animals and humans [72]. Emerging evidence shows that dietary L-Arg supplementation reduces adiposity in experimental models (rats, sheep and pigs) [11, 73-75]. Results of human studies indicate that L-Arg supplementation may be a novel therapy for obesity and metabolic syndrome, acting via decreased plasma levels of glucose, fatty acids, triglycerides, as well as improved whole-body insulin sensitivity [11] (see: L-Arginine and adipose tissue).

### **3-1-3 BH<sub>4</sub> supplementation**

Experimental studies have shown positive effects of BH<sub>4</sub> supplementation in reducing plaque size and leukocyte infiltration in the ApoE<sup>-/-</sup> model of atherosclerosis [34, 76]. These positive effects are assumed to be mediated through effects on eNOS, as they closely mimic the effects of endothelial specific GCH1 overexpression. Studies using this mouse model have shown that increasing endothelial cell BH<sub>4</sub> is protective in models of diabetic vascular dysfunction and atherogenesis [77, 78]. It has been shown that a reduction in BH<sub>4</sub> levels, through the use of the synthesis inhibitor

DAHP, alters vascular function *ex vivo* causing relaxation of arterial rings to occur through hydrogen peroxide production. This effect was reversible by a BH<sub>4</sub> analogue [79].

BH<sub>4</sub> is the predominant form of biopterin in normal plasma or cells. It is prone to oxidation to 7,8-dihydrobiopterin (BH<sub>2</sub>) by intracellular ROS. Inactive BH<sub>2</sub> is reduced back to BH<sub>4</sub> by regeneration enzymes to replenish cellular BH<sub>4</sub> levels. The BH<sub>4</sub>/BH<sub>2</sub> ratio as well as BH<sub>4</sub> levels determines the fate of NO [80]. BH<sub>4</sub> can be oxidized by ROS, leading to a reduction in vascular BH<sub>4</sub>, an increase in vascular BH<sub>2</sub> availability and a decrease eNOS function thus inducing an increase in ROS production associated with a decrease in NO production. Recently, it has been reported that dietary co-supplementation with BH<sub>4</sub>, L-Arg, and Vitamin C acted synergistically to decrease oxidative stress, increase NO and improve blood flow in response to acute hind-limb ischemia. In rats co-supplementation with BH<sub>4</sub> + L-Arg + Vitamin C resulted in increased eNOS activity and NO concentration as well as greater foot blood flow recovery than in rats that received normal chow or either agent separately [81]. Impaired endothelial function in coronary artery disease (CAD) patients is associated with reduced BH<sub>4</sub> levels in vascular tissue, and with increased systemic levels of BH<sub>4</sub> in plasma, which correlate with markers of systemic inflammation, such as plasma hsCRP [82]. Acute inflammatory stimulation impairs endothelial function but increases plasma BH<sub>4</sub>, in parallel with increased IL-6 [83], demonstrating that inflammation leads to opposing changes in systemic vs. endothelial BH<sub>4</sub> effects. Genetic variants of GTP-CH1, in which GTP-CH1 expression is reduced render subjects unable to upregulate BH<sub>4</sub> levels following systemic inflammation [84].

Supplementation with BH<sub>4</sub> or its physiological precursor, sepiapterin, improved endothelial function in vessels from animal models of hypercholesterolemia and diabetes [85]. In light of experimental findings, modulation of the L-Arg–NO pathway by BH<sub>4</sub> and L-Arg has been developed in clinical studies; this modulation being beneficial for ameliorating vascular insulin resistance in obesity and diabetes. A clinical study has shown that concomitant intra-arterial infusion of BH<sub>4</sub> in type 2 diabetic patients improved endothelium-dependent vasodilation, demonstrating the therapeutic potential of upregulating BH<sub>4</sub> bioavailability [86]. But the beneficial results may have limited long-term benefits. Chronic BH<sub>4</sub> supplementation may result in endothelial BH<sub>2</sub> accumulation and thus induce eNOS uncoupling [87]. More than 400 reports have been published to date on studies that tried to improve vascular and heart dysfunction by treatment with BH<sub>4</sub>. However, there have been few clinical trials [64, 88].

### **3-1-4 L-Citrulline supplementation**

It has been reported that L-Citrulline, a byproduct of NO formation from L-Arg and oxygen, is effectively recycled to L-Arg via the enzymes argininosuccinate synthase and argininosuccinate lyase of the Citrulline-NO cycle, and plays an important role in the metabolism and regulation of NO [89]. A clinical trial in healthy volunteers showed that oral intake of L-Citrulline dose-dependently increased the plasma level of L-Arg more effectively than an equivalent dose of L-Arg [90]. Recently, it has been demonstrated that, in an Alzheimer mouse model, Citrulline supplementation in the diet of aged rats led to a substantial improvement in the composition of membrane rafts in the hippocampus [91]. These effects are potentially of great significance, since rafts are specialized in signal transduction. In synaptic lipid rafts, consistent differences in raft protein levels have been observed between young and aged rat brains [92]. Interestingly, biochemical studies showed that 1) both L-Arg and

L - Citrulline were effective hypocholesterolemic and hypolipidemic agents 2) L-Citrulline is to some extent more effective than L-Arg [93-95].

### **3-2. Arginine and red blood cells (RBCs)**

RBCs possess a functional NOS enzyme located in the cell membrane and cytoplasm [96]. L-Arg uptake in erythrocytes is mediated by membrane transport systems. Arginine uptake by intact RBCs occurs through transport channels. RBC contain CATs which transport arginine as well as other amino acids [97]. CAT activity is known to be accelerated in certain disease states and could be responsible for arginine depletion in RBC [98]. Recently, it has been reported that the membrane  $\gamma^+$  amino acid transporter contributed to arginine depletion in stored packed RBC transfusion. The uptake of arginine and synthesis of ornithine in packed RBC from different donors at the same duration of storage vary considerably [99]. CAT1 seems to be highly expressed in relatively mature erythroid cells and is possibly involved in late (or terminal) erythroid maturation via the modulation of NO formation [100]. Recently, the role of free heme in the dysregulation of arginine metabolism observed in malaria and other hemolytic diseases has been investigated. It appears that free heme compromises L-Arg transport into the RBC mainly through inhibition of the carrier system “ $\gamma_L$ ” and promotes intracellular L-Arg degradation by the activation of arginase I [101]. It has previously been observed that shear stress acting on RBC activates NOS and causes enhanced NO export [102, 103]. An important role has been attributed to RBC hemoglobin in the critical balance between the generation and scavenging of NO which determines its local bioavailability and influences vascular function. RBC–NO interaction is an important determinant of local NO availability in the vasculature [29, 104].

The hypothesis that S-nitrosothiols (RSNOs) possess many of the biological activities of NO, possibly acting as a form of NO storage and transport, has stimulated a wealth of research into the biological significance of these compounds. Two main hypotheses describe the role of hemoglobin in the regulation of NO bioavailability. It has been suggested that hemoglobin interacts with circulating NO to form Fe-nitrosyl hemoglobin and then S-nitrosothiols, which deliver NO extracellularly. Alternatively, the existence of diffusional barriers that protect NO from hemoglobin-mediated degradation has been proposed [105]. An important point in this field was the demonstration of a specific NOS within RBC (RBC-NOS). NOS activity in RBCs (0.3 pmol/pg/min) is comparable to that in endothelial cells (ECs) (0.7 pmol/pg/min). Taking into account that the total protein content of both cell types is also comparable in human beings (1.1 kg protein in RBCs and 1.5 kg protein in ECs), the relevance of RBC NOS is more than conceivable [96]. In this context, therapeutic interventions to enhance RBC–NOS activity, such as HMG-CoA reductase inhibitors (statins) or erythropoietin, might gain importance in the therapy of diverse diseases including cardiovascular diseases. A considerable number of studies demonstrated the potential modulatory role of statins on endothelial nitric oxide synthase (eNOS), a key enzyme involved in the regulation of cardiovascular function by generating NO [106]. However, the precise mechanisms involved in the cardiovascular defensive potential of statins have not completely been elucidated. Among their pleiotropic effects, statins exert antioxidant and anti-inflammatory properties [107].

NO synthesis by RBC should still be considered an important factor contributing to local hemodynamics, since it has been shown that NO generated by RBC regulates RBC deformability [108]. In the microcirculation, the relatively fast increase in intracellular NO concentration at physiological levels of shear stress would be expected to improve RBC deformability and blood flow accordingly. The improvement in RBC deformability by NO is a phenomenon that may have significant hemodynamic consequences. This effect is greater under low oxygen tension, implying that NO plays a more important role in hypoxic conditions [109].

### **3-3: Arginine, white blood cells and inflammation**

In myeloid cells, arginine is mainly metabolized either by iNOS or by arginase 1, enzymes that are stimulated by T helper 1 and 2 cytokines, respectively [110]. Thus, the activation of iNOS or arginase (or both) reflects the type of inflammatory response in a specific disease process [111]. Under resting conditions, little arginine is used by myeloid cells due to a lack of expression of high-affinity cell membrane transporters. In addition, in the absence of immune stimulation, myeloid cells do not express the major arginine metabolizing enzymes, iNOS and arginase 1 [112]. Thus, dietary L-Arg supplementation cannot enhance myeloid cell function in the absence of disease. It is only after stimulation that L-Arg transport into the myeloid cell is greatly increased, mainly as a result of increased expression of the high-affinity CATs. As we reported, CATs are highly regulated and are co-induced with arginine-metabolizing enzymes. It has been proposed that the pathological production of arginase 1 in myeloid suppressor cells could cause sufficient arginine depletion, resulting in compromised T-cell function and NO production and ultimately lead to increased susceptibility to infection [110, 111]. Investigators demonstrated that under certain circumstances, such as cancer or after trauma, myeloid cells expressing arginase 1 are upregulated leading to the depleting of the arginine necessary for normal physiological processes such as NO production and/or T-cell function [113].

The initiation of inflammation appears to be associated with a multifactorial process. Once activated by injury, endothelial and smooth muscle cells become transcriptionally active and synthesize proinflammatory proteins. Chemokines and cell adhesion molecules (CAMs) attract circulating inflammatory cells to the arterial surface and facilitate their attachment to and incorporation into the endothelial cells and vascular smooth muscle cells of vessel walls. Then, cytokines activate inflammatory cells and transform monocytes into macrophages associated with sequential steps during extravasation from blood into tissues [114]. Several studies have also reported temporal dissociations of leukocyte adhesion and/or emigration from increases in permeability [115]. Leukocyte migration is a critical step in white cell mobilization from vessel lumen to sites of tissue infection. Openings at endothelial junctions and/or damage to vascular walls during leukocyte migration were assumed to cause increased transport of fluid and large molecules. Blocking various adhesion molecules on monocytes or endothelial cells prevented the monocytes from reaching junctions. In response to a variety of stimuli, neutrophils release large quantities of ROS through respiratory bursts. This is essential to kill invading microorganisms but, in contrast, can also cause tissue damage. The displacements of leukocytes are controlled by internal and external signals which activate complex signal transduction cascades to mediate chemotactic responses [116]. These processes during ischemia-reperfusion [117-119] and cardiopulmonary bypass (CPB) or hemorrhagic

shock have been well studied. CPB triggers a generalized inflammatory response that is largely mediated by the activation of polymorphonuclear neutrophils, their adhesion to endothelial cells, and the subsequent release of cytotoxic products. It has been known for several years that the inflammatory response to extracorporeal circulation underlies the occasional development of postoperative organ dysfunction [120, 121]. In these pathological situations, the effects of L-Arg were studied. L-Arg infusion during resuscitation offers a significant functional, metabolic, and survival benefit after severe hemorrhagic shock. The mechanism seems to be by activation of NO synthesis with benefits to local perfusion and inflammation after global reperfusion [122].

In the context of ischemia-reperfusion, *in vivo*, it has been shown that supplementation with L-Arg at different time points can either improve or reduce cardiac function during the course of ischemia-reperfusion (I/R). In the early stage of reperfusion, treatment with L-Arg reduced the myocardial nitrotyrosine content, partially reversed the impaired cardiac function induced by reperfusion injury and resulted in a decrease in infarct size. In contrast, when L-Arg was supplemented at a later stage, the formation of toxic peroxynitrite was further increased and cardiac dysfunction was further aggravated with an increase in infarction size [123]. Previous results demonstrated that L-Arg administered at different time points during ischemia/reperfusion exerted different effects on post-ischemic myocardial injury, and suggest that the stimulation of eNOS reduces nitrative stress and decreases apoptosis, whereas stimulation of iNOS increases nitrative stress and exacerbates myocardial reperfusion injury [124]. We investigated *in vitro*, using isolated rat hearts, the role of NOS modulation on 1) the evolution of functional parameters and the level of post-ischemic recovery and 2) the amount of free radical species released during a sequence of global myocardial ischemia and reperfusion, using L-arginine as a substrate or *N*<sub>G</sub>-nitro-L-arginine methyl ester (L-NAME) as an inhibitor, in comparison with their D-specific enantiomers [59]. In contrast, some studies have described beneficial effects of L-Arg treatment on the recovery of myocardial function during reperfusion [60, 125, 126]. We demonstrated that the administration of 3 mM D- or L-Arg during the pre-ischemic perfusion period slightly decreased (11%) coronary flow. During post-ischemic reperfusion, coronary flow was dramatically impaired and recovered to -25% of its initial value. Under our experimental conditions, it was not possible to determine differences in cardiac function between D- or L- Arg - treated hearts. Authors have described the beneficial effects of L-Arg treatment on the recovery of myocardial function during reperfusion [127].

A method of indirectly evaluating the L-Arg-nitric oxide pathway is to study leukocyte adhesion to endothelial cells or to measure circulating levels of endothelial adhesion molecules such as ICAM-1, VCAM-1, and E-selectin. A study investigated the effect of oral L-Arg supplementation on increased monocyte adhesion secondary to cigarette smoking. Monocyte-endothelial cell adhesion and endothelial ICAM-1 expression were measured in eight smokers and eight age- and sex-matched controls. At baseline, these parameters were higher in smokers than in controls. Subjects were then given a one-time dose of 7 g of oral L-Arg, and blood samples were obtained 2 h later. After the L-Arg dose, monocyte-endothelial cell adhesion and ICAM-1 expression were both reduced in smokers. No such changes were noted for VCAM-1 or E-selectin. L-Arg had no effect on the control subjects [128]. Other studies in this field, reported that intravenous infusion of L- Arg (30 g Arg-HCl over 45 min) normalized coronary vasomotion in long-term smokers and 3-day oral administration of L-Arg (7 g/day) prevented smoking-induced impairment of endothelial function in young adults [129, 130]. The mechanisms by which L-Arg administration may prevent cardiovascular dysfunction include



restoring endothelial NO synthesis, decreasing superoxide production and inhibiting platelet adherence and leukocyte adherence to the endothelium.

### **3-4: Arginine and adipose tissue**

Adipose tissue is no longer considered an inert tissue functioning solely as an energy store, but is emerging as an important factor in the regulation of many pathological processes. In mammals, adipose tissue occurs in two forms: white adipose tissue and brown adipose tissue. Most adipose tissue in mammals is white adipose tissue and this is thought to be the site of energy storage. Recent evidence indicates that periadventitial adipose cells are a functional component of the vasculature, exerting paracrine influence on blood vessel contractility, local inflammation and immunity [131, 132]. In different experimental studies, it has been demonstrated that perivascular adipose tissue showed increased superoxide production and NADPH oxidase activity, as well as adipocyte hypertrophy, associated with inflammatory cell infiltration [133, 134]. Recent data suggest that in a rodent model, metabolic syndrome is associated with perivascular adipose inflammation and oxidative stress, hypertrophic resistance artery remodeling, and endothelial dysfunction, the latter a result of decreased NO and enhanced superoxide generated by uncoupled endothelial NO synthase [135]. Recently, it has been reported that perivascular adipose tissue controls insulin-induced vasoreactivity in the microcirculation through secretion of adiponectin and subsequent AMPK $\alpha$ 2 signaling. Perivascular adipose cells from obese mice inhibit insulin-induced vasodilation, which can be restored by inhibition of JNK [136]. Studies in isolated adipocytes demonstrated that leptin induced eNOS phosphorylation with a resulting increase in NO production [137, 138]. It is now well established that adipose tissue in humans expresses the whole gene set that codes for the enzymatic system responsible for the biosynthesis and degradation of ADMA and that methylarginine is released by adipocytes in culture [139]. The enzymes, that are responsible for the posttranslational modification of arginine residues in regulatory proteins, exist in several human tissues but the precise function of ADMA in fat cells remains unknown [140]. Adipose tissue is an active player in systemic inflammatory processes and a relevant component in the control of insulin sensitivity [141, 142]. Different mechanisms may be involved. Growing evidence indicates that physiological levels of L-Arg promote the oxidation of glucose and long-chain fatty acids. Additionally L-Arg supplementation increases lipolysis and inhibits lipogenesis by modulating the expression and function of key enzymes involved in anti-oxidative response and fat metabolism in insulin-sensitive tissues [75, 143].

The results of recent studies have shown that dietary L-Arg supplementation reduces white adipose tissue in both genetically obese rats and diet-induced obese rats and markedly increases brown adipose tissue (BAT) mass [75, 144, 145]. The main objectives of a recent review were to highlight a regulatory role for L-Arg in BAT growth and development to reduce white adipose tissue deposition. The authors proposed the underlying molecular and cellular mechanisms [146]. It was suggested that L-Arg reduces adiposity through regulating gene expression and enzyme activity, and enhancing mitochondrial biogenesis, BAT development, blood flow, lipolysis, and the oxidation of fatty acids and glucose in the whole body. First, L-Arg supplementation reduces mRNA levels for fatty-acid binding protein 1, glycogenin, caspases 1 and 2, and hepatic lipase, whereas it increases expression

of PPAR gamma, heme oxygenase 3, glutathione synthetase, insulin-like growth factor II and sphingosine-1-phosphate receptor [143].

Studies have been conducted to explore the nutritional and/or therapeutic role of L-Arg to treat a wide array of human cardiovascular diseases. In fact, numerous studies have examined short-term effects of infusions of these agents in patients with heart disease, hypertension, diabetes, and hypercholesterolemia. Although the results of oral L-Arg supplementation in animals have generally shown beneficial effects, data in humans are more varied [8]. As we previously reported, L-Arg offers promise as a therapy for metabolic diseases such as metabolic syndrome. Some studies indicate that L-Arg supplementation may be a novel therapy for obesity and metabolic syndrome [11]. The question of the minimal effective dose of L-Arg to be administered is rather important because it has been demonstrated that high doses of L-Arg supplementation could paradoxically contribute to the formation of atherosclerotic lesions via mechanisms involving lipid oxidation, peroxynitrite formation, and NO synthase uncoupling [147, 148]. Concerning dietary L-Citrulline supplementation, recent studies suggest that, in humans, L-Citrulline supplementation may improve cardiovascular function [149, 150]. However, all of these studies on the effects of L-Arg or L-Citrulline have several limitations: the number of patients studied was small and the intervention periods were short. More studies are required to evaluate the effects observed in a larger number of patients for a longer period. Finally, the utility of arginine supplementation in patients remains highly controversial. In contrast, if a specific disease condition is indeed due to arginine deficiency, it should be improved or reversed by supplementation with arginine or its precursor Citrulline, but the efficacy of enteral arginine supplementation is limited by gastrointestinal discomfort following ingestion. It has been reported that L-Citrulline did not present these disadvantages [151].

#### **4 Conclusions: therapeutic potential of L-Arginine supplementation**

Animal model data are positive; most of the evidence from experimental models shows that oral L-Arg supplementation has a beneficial effect. L-Arg appears to preserve endothelial function. Long-term randomized clinical trials are needed to determine whether oral L-Arg supplementation could really be advantageous for vascular health. Several possible hypotheses exist for the varied results in human subjects. The patient populations in human studies were diverse and included patients with various pathologies as well as smokers, postmenopausal women and healthy subjects. Randomized long-duration clinical trials are required to really elucidate the effects of L-Arg supplementation on health. Long-term studies are needed to determine whether there is a difference in the availability of dietary L-Arg or L-Citrulline when it is given over the short or long term. Clinical trials need to continue to determine the optimal concentrations and combinations of L-Arg with other protective compounds such as BH<sub>4</sub>, and/or antioxidants to combat the oxidative stress that drives down NO production in humans.

## 5 References

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## Figure Legends

### Figure 1. Pathways of L-Arginine synthesis and metabolism in mammals.

The L-Arginine biosynthesis pathway represents a regulated and highly localized source of substrate for nitric oxide (NO) production. L-Citrulline, which is co-produced with NO, can be recycled to L-Arginine. (1) OCT: ornithine carbamoyltransferase; (2) ASL: argininosuccinate lyase, (3) ASS: argininosuccinate synthase

### Figure 2. Molecular structures of the nitric oxide synthase (NOS) dimer. (oxygenase domain and reductase domain)

The N-terminal catalytic domain binds the heme prosthetic group as well as the redox cofactor, tetrahydrobiopterin (BH<sub>4</sub>) associated with a regulatory protein, calmodulin (CaM). The C-terminal reductase domain has the binding sites for FMN, FAD, and NADPH. NOS produces NO from L-Arginine (L-Arg) in the presence of O<sub>2</sub> and NADPH. Zn is responsible for connecting the monomers to the heme groups. NADPH-oxidase donates an electron, which is ultimately used to convert L-Arginine and oxygen to the reaction products L-Citrulline and nitric oxide (NO). Asymmetric dimethylarginine (ADMA) inhibits NO synthesis by competing with L-Arg at the active site of NOS.

FMN, flavin mononucleotide; FAD, flavine adenine dinucleotide.

### Figure 3. Metabolism of asymmetric dimethylarginine (ADMA)

Methylated arginine residues are released from the breakdown of proteins that have been acted on by protein-arginine methyl transferases (PRMTs). PRMT type I and II catalyze the formation of monomethylarginine (L-MMA) from L-Arginine. In the second step, type I PRMTs produce ADMA, while type II PRMTs catalyze symmetric dimethylarginine (SDMA). ADMA can be degraded to L-Citrulline and dimethylamine (DMA) by dimethylarginine dimethylaminohydrolases (DDAH 1 and 2)

### Figure 4. This schema presents the sources and consequences of the production of nitric oxide (NO), asymmetric dimethylarginine (ADMA) and reactive oxygen species in intracellular and extracellular compartments. (See details in the text)

ADMA, asymmetric dimethylarginine; BH<sub>4</sub>, tetrahydrobiopterin; CATs, cationic amino acid transporters; DDAHs, dimethylarginine dimethylaminohydrolases; DMA, dimethylamines; L-Arg, L-arginine; DMGV,  $\alpha$ -keto- $\delta$ -(N(G),N(G)-dimethylguanidino)valeric acid; LDL, low-density lipoprotein; L-MMA, N<sub>G</sub>-monomethyl-L-arginine; L-NNA, N-nitro-L-arginine; NADPH, nicotinamide dinucleotide phosphate NO, nitric oxide; NOHA, N-omega-hydroxy-L-arginine; PRMT, protein arginine methyl transferase; SDMA, symmetric dimethylarginine;  $\gamma$ -LAT transporters, system  $\gamma$ -L AA transporters.