

Nitric Oxide and the Cardiovascular System: Cell Activation, Vascular Reactivity and Genetic Variant

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Summary

Nitric oxide (NO), primarily identified as an endothelium-derived relaxing factor, is a free radical that signals different biological processes. The identification of NO synthase (NOS) isoforms and the subsequent characterization of the mechanisms of cell activation of the enzymes permitted the partial understanding of both the physiological interactions and of the mechanisms of the diseases in which NO is involved. Mainly expressed in the vascular endothelium, the endothelial NOS isoform (eNOS) plays an important role in the regulation of vascular reactivity and in the development and progression of atherosclerosis. The purpose of this review is to contextualize the reader about the eNOS structure and its mechanisms of cell activation. In view of the advances in molecular biology, we will also address the known mechanisms of gene expression regulation and the role of variants on the genetic code of eNOS associated with cardiovascular phenotypes. Although the importance of NO as an atheroprotective molecule is recognized, our focus will be the review of the literature on NO and its participation in the modulation of the muscle vasodilatation phenotype.

Introduction

The primary evidence that the endothelium is an indispensable component in the regulation of the vascular tone emerged from experimental analyses demonstrating that, in the absence of this single layer of squamous epithelium, acetylcholine-induced vasodilatation did not occur. In that moment, Furchgott and Zawadzki¹ documented the fact that, when stimulated, the endothelium had the capacity to release a vasoactive substance, which was named endothelium-derived relaxing factor (EDRF). Some years later, EDRF was

identified by Ignarro et al² as nitric oxide (NO), a compound characterized in 1977 by Ferid Murad which caused smooth muscle cell relaxation, when released by nitrates. For this finding, Robert F. Furchgott, Ferid Murad and Louis J. Ignarro were awarded the Nobel Prize of Physiology or Medicine in 1998 (Figure 1). A series of studies was responsible for the demonstration that the endothelium releases other EDRF such as prostacyclin (PGI₂), and the endothelium-derived hyperpolarizing factor (EDHF), in addition to other endothelium-derived contracting factors (EDCF) such as endothelin (ET-1), cyclooxygenase pathway products such as thromboxane A₂ (TXA₂) and reactive oxygen species such as the superoxide anion (O₂⁻)³. In addition to characterizing the endothelium as a biological sensor capable of detecting any mechanical, physical or chemical stimulus - and respond to it - these findings raised the endothelium to the position of a multifunctional tissue that plays an important role in the homeostasis of all physiological systems.

Nitric oxide and the cardiovascular system

NO, a gaseous molecule that acts in the signaling of different biological processes, is a free radical presenting an unpaired electron in the last layer and a half-life of 4 to 8 seconds in oxygenated aqueous medium^{4,5}. It is described as a labile gas, capable of free diffusion across cell membranes, a characteristic that enhances its high biological activity⁶. The multiple NO actions were further broadened by the confirmation that the vascular endothelium is an active organ and that its integrity promotes beneficial effects, such as antioxidant, anti-inflammatory, anticoagulant, and pro-fibrinolytic actions; inhibition of leukocyte adhesion and migration; inhibition of smooth muscle cell migration and proliferation; and inhibition of platelet aggregation and adhesion⁷. Thus, this atheroprotective setting is characterized by a harmony between substances released by the endothelium, in which NO is cited as one of the most relevant vasoactive compounds. Characterized as a systemic disorder that precedes atherosclerosis and its complications, endothelial dysfunction in atherosclerotic coronary arteries was first described by Ludmer et al⁸ and later related to the change in NO bioavailability⁹.

In the current literature, it is agreed that the reduced NO biological activity, caused both by synthase reduction and by increased degradation due to oxidation stress, has been identified as the most relevant mechanism in the multifactorial process in endothelial dysfunction and in the participation in the main cardiovascular dysfunctions¹⁰. Thus, reductions in NO bioavailability and subsequent endothelial

Keywords

Nitric oxide; nitric oxide synthase type III; polymorphism, genetic.

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Manuscript received February 12, 2009; revised manuscript received June
26, 2009; accepted August 14, 2009.

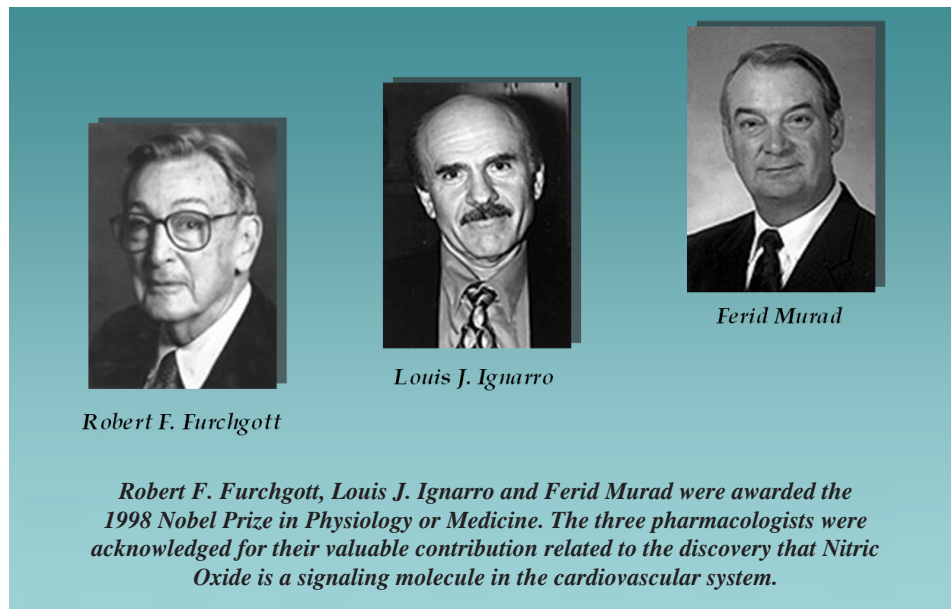


Figure 1 - 1998 Nobel Prize winners in Physiology or Medicine. Source: Available at: <<http://nobelprize.org>>.

dysfunction trigger, in the vascular environment, events such as tone alterations, thrombotic dysfunctions, smooth muscle cell (SMC) proliferation and migration, as well as leukocyte adhesion¹¹. When endothelial dysfunction occurs, there is also an increased production of reactive oxygen species (ROS)¹², and these can reduce endothelial NO availability via different pathways: direct NO inactivation by superoxide with peroxynitrite (ONOO⁻)¹³ formation; reduction in NO synthase expression and activity due to changes in their substrate or cofactors, and in the increase in asymmetric dimethylarginine (ADMA) levels¹⁴; and also endothelial NOS uncoupling caused by increased tetrahydrobiopterin (BH₄) oxidation¹⁵.

The understanding of the complexity of endothelial function has improved, and the difficulty in studying each one of its components separately has been overcome. In this context, animal models able to reproduce endothelial dysfunction have been developed, thus enabling the system to work under conditions of low or high NO bioavailability, for instance. Additionally, studies *in vivo* in humans, using intra-arterial infusion of compounds with the potential of modulating endothelial-dependent or independent function, permitted the investigation of modulatory mechanisms on the vascular function under different physiological conditions and more prevalent diseases.

Nitric oxide synthases

NO enzymatic production from the amino acid L-arginine is mediated by a family of three nitric oxide synthases (NOS) codified by distinct genes¹⁶. The isoforms share 50%-60% homology in the amino acid sequence in the oxidase and reductase domains¹⁷. These isoforms have distinct characteristics that reflect their specific functions *in vivo*¹⁸.

Endothelial NO synthase (eNOS or NOS III; 7q35-36) and neuronal nitric oxide synthase (nNOS or NOS I; 12q24.2) have a mechanism of constitutive activation (cNOS). The inducible isoform (iNOS or NOS II; 17cen-q12) is expressed in abnormal cell processes such as heart failure¹⁹, after induction by cytokines and other inflammatory agents, which results in a high NO flow^{20,21}. Endothelial NOS is mainly located in endothelial cell compartments named caveolae²², and is essential for keeping the baseline vascular tone. This tone is partly mediated by NO synthesis, a vasoactive compound that participates in blood flow regulation in the different vascular beds and, particularly, in the coronary blood flow²³. The subcellular location of NO synthesis has a great influence on its biological activity. In the 1990's, the initial identification of eNOS location in the caveolae in the cell plasma membrane provided the structural basis for the recognition of compartmentalization in the mechanisms of cell signaling promoted by NO. The subsequent observation that eNOS interacts directly with structural proteins of the caveola - the caveolins, provided biochemical evidence of the interaction between eNOS and caveola and its implication with numerous signaling molecules concentrated in this environment of the cell membrane²⁴. A great number of evidences revealed that the caveolae are capable of recruiting numerous signaling molecules and to regulate their activities instead of acting as a simple support for cell exchange and transportation²⁵. Thus, it has been described that eNOS is located inside the caveolae and is kept in a less active state via its interaction with caveolin-1²⁶.

eNOS structure

eNOS functions as a dimer consisting of two identical monomers, which, in turn, can be functionally and structurally

divided into two major domains: a C-terminal reductase domain, homologous to cytochrome P450 which contains binding sites for NADPH, flavine mononucleotide (FMN) and flavine adenine dinucleotide (FAD); and an N-terminal oxidase, which subtracts one electron from the L-arginine substrate and has binding sites for heme iron, for the tetrahydrobiopterin cofactor (BH₄) and for L-arginine^{20,21,27} (Figure 2). The catalysis reaction of constitutive NOS involves two oxidation stages: L-arginine hydroxylation into N^G-hydroxy-L-arginine, followed by oxidation of this intermediate compound with utilization of one NADPH electron, thus forming L-citrulline and NO²⁸. This reaction consumes 1.5 mol of NADPH and 2 moles of oxygen per mol of L-citrulline formed^{16,29,30}. Cofactors such as heme iron, BH₄ and L-arginine have been studied in depth, and their low bioavailability induces the phenomenon of dysfunctional eNOS³¹⁻³³. Heme iron is essential for dimerization of the three isoforms³⁴: low concentrations or absence of L-arginine catalyze oxygen reduction in superoxide (O₂⁻)³⁵, and decreased levels of BH₄ lead to the simultaneous production of NO e O₂⁻, products that react with each other forming peroxynitrite (ONOO⁻)³⁶.

Regulation of eNOS activity and gene expression

Ever since it was verified that the endothelial cells contain a baseline concentration of eNOS protein, the eNOS gene was considered constitutively expressed. Interestingly, further studies demonstrated that stable mRNA concentrations are subject to a modest level of regulation³⁷. The eNOS gene promoter was cloned, and it was demonstrated that it has a complex regulatory mechanisms of gene expression. Like the promoter of constitutively expressed genes, the eNOS gene promoter does not contain the TATA box sequence. However, it has multiple cis-regulatory DNA sequences, including CCAT box, Sp1 sites, GATA motifs, CACCC box, AP-1 and AP-2 sites, p53 binding sites, and NF-1 elements, in addition to sequences responsive to sterol elements and shear stress³⁸. Bovine and human promoter sequences have a homology of 75%, thus suggesting a high evolutionary preservation of the gene transcriptional regulation. Located in the proximal promoter, the positive regulatory domains I and II (PRD I and PRD II) are involved in the baseline regulation of gene

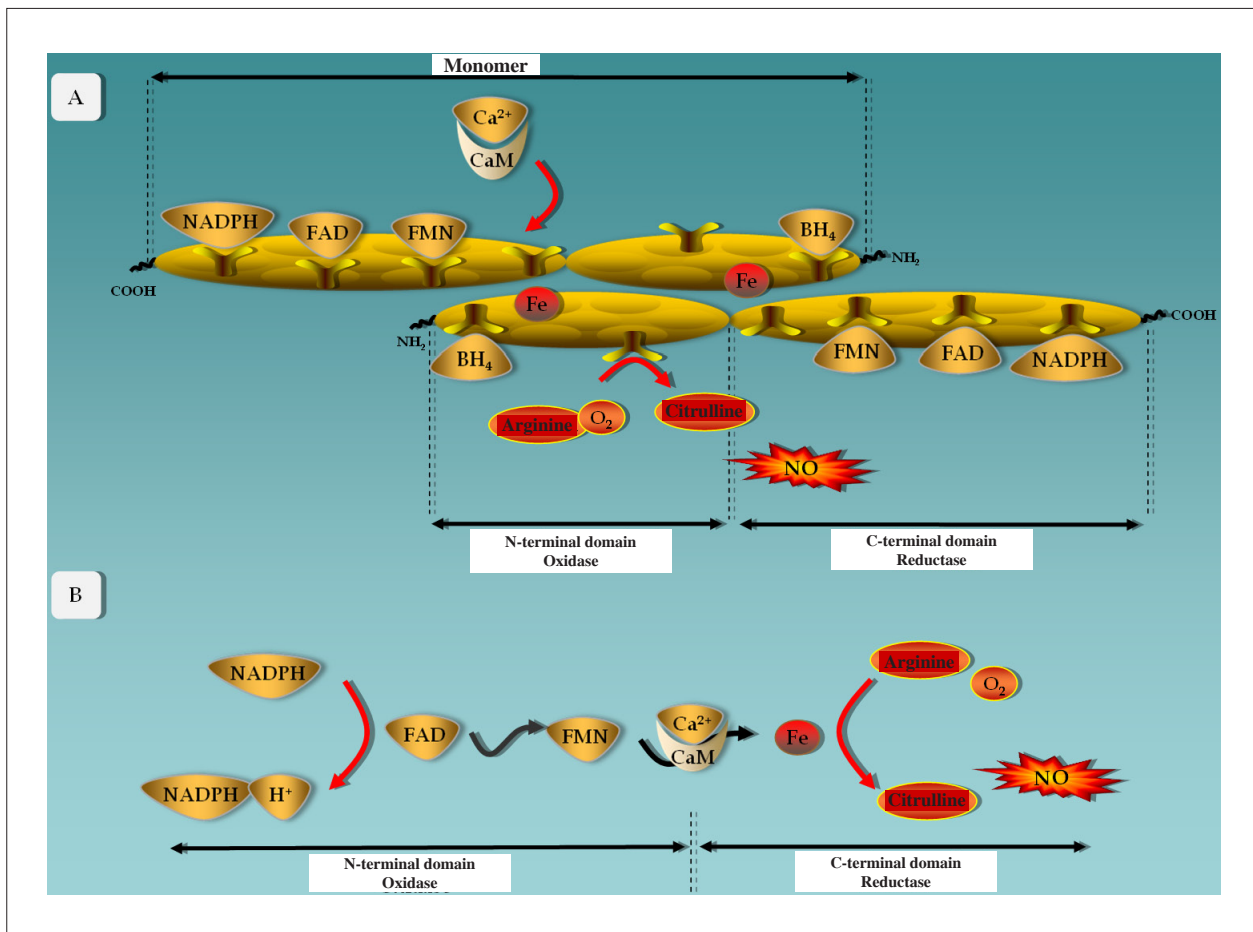


Figure 2 - A) Model proposed for the dimeric eNOS structure. **B)** Electron transfer between cofactors and substrates of the enzyme structure. The electron flows in the direction NADPH → FAD → FMN in the reductase domain of one monomer to Fe in the oxidase domain of the contralateral monomer. In figure A, observe that electron flow and arginine catalysis are shown in only one side of the enzyme. Electron transfer from one domain to another is mediated by calmodulin, which justifies the need for its linking to the recognition site for enzyme activation and subsequent NO synthesis. FAD - flavine adenine dinucleotide; FMN - flavine mononucleotide; BH₄ - tetrahydrobiopterine; Fe - heme iron; CaM - calmodulin.

transcription, showing an affinity with the transcription factors Sp-1, Sp-3, Ets-1, Elf-1, YY1 and MYC-associated zinc finger protein³⁹. Studies in vitro demonstrated that eNOS promoter responsiveness to shear stress is dependent on sequences located between -1,000 and -975, a region relative to transcription start^{40,41}. Besides, the binding of NF- κ B subunits p50 and p65 to the responsive element GAGACC (-990; -984) - located upstream of the transcription start site, is involved in shear stress activation of the promoter⁴². Also, transcription in bovine endothelial cells subject to laminar flow showed a nine-fold increase in mRNA. This effect was mediated by two distinct mechanisms: 1) transient increase in gene transcription, and 2) subsequent prolonged mRNA half-life⁴³.

The mechanism of eNOS activation has been described as the most elaborated of the free isoforms, reflecting the complexity of the physiologic control of the different vascular beds^{17,44}. The classical mechanism of constitutive isoform activation is calcium (Ca^{++})-dependent, whereas iNOS is independent of the elevation of intracellular Ca^{++} concentrations, because of the high affinity of the enzyme binding with calmodulin^{31,39,44}.

The complexity of the eNOS post-transcriptional regulatory mechanisms has been considered in the dimerization of the protein subunits and in the role of the protein caveolin in the formation of the caveolar structure⁴⁵. Endothelial NOS is located inside the caveola and is kept in a less active state via its interaction with caveolin-1²⁶. The mechanisms migration from the cell membrane to the Golgi apparatus and Akt, PKA, and AMPK-kinase-dependent phosphorylations are described as responsible for the activation of this NOS isoform. Thus, the interaction keeps eNOS inactive, and calmodulin acts directly on the competition with caveolin in the promotion of the calcium-dependent enzyme activation¹⁵. Endothelial NOS activity is well known in the vascular environment, and is regulated by six mechanisms after its translation: lipid inclusion; calcium/modulin-dependent mechanism; direct protein-protein interactions; several phosphorylations; glycosylation; and substrate and cofactor availability. Thus, eNOS can interact with several proteins in their "less active" or "more active" states. The plasmalemma-bound eNOS N-myristoylation and palmitoylation processes are required, which, in this state, is associated with caveolin-1 and HSP 90. The protein that interacts with HSP70, named "CHIP", interacts with both HSP70 and HSP90, and downregulates eNOS traffic to the Golgi apparatus, in contrast to "NOSIP" and "NOSTRIN" which can downregulate eNOS location in the plasma membrane. The main eNOS activation mechanism occurs via phosphorylation of the amino acid serine at position 1177⁴⁶ by the Akt-kinase enzyme (or kinase protein B), which increases eNOS sensitivity to baseline Ca^{++} /calmodulin concentrations⁴⁷. Tonic or phasic eNOS activation in response to blood flow is independent of Ca^{++} concentration changes and constitutes the shear stress. Dimmeler et al⁴⁸ demonstrated that the exchange of the serine^{1177/1179} residue for the amino acid alanine makes eNOS incapable of responding to phosphorylation and activation by the Akt enzyme, a phosphatidylinositol-3-kinase (PI-3K)-dependent pathway. Although phosphorylation of the serine¹¹⁷⁷ residue plays a pivotal role in eNOS enzyme activation, its regulation is

known to be also dependent on the phosphorylation pattern of other currently well-characterized sites⁴⁹. Phosphorylation of the serine⁶³³ residue, located in the binding domain of flavin mononucleotide (FMN) also increases eNOS activity and seems to be particularly important to keep NO synthesis after serine¹¹⁷⁷ residue phosphorylation and activation by Ca^{++} /calmodulin. Phosphorylated by protein kinase C (PKC), the threonin⁴⁹⁵ residue interferes with the calmodulin binding domain, thus downregulating NO synthesis.

Recent studies have evaluated the capacity of certain drugs, such as antioxidants and renin-angiotensin system blockers, to reduce endothelial dysfunction by means mechanisms of eNOS activation via phosphorylation of specific sites such as the serine¹¹⁷⁷ residue. This phosphorylation may be affected by the subcellular enzyme location, such as the caveolae, intercellular junctions, Golgi apparatus, and cytosolic compartments, as well as by protein kinases and phosphatases associated with these structures. Recently, our group⁵⁰ demonstrated that Telmisartan, an angiotensin II receptor blocker, promotes reduction of endothelial dysfunction by means of eNOS activation via phosphorylation of specific sites, such as serine¹¹⁷⁷ and serine⁶³⁵ residues.

Nitric oxide, vascular tone and muscle vasodilatation

After confirmation that NO is synthesized by endothelial cells and that it participates in the cardiovascular hemodynamic regulation, interest was focused on the quantification of its participation in this homeostasis of this system. Studies in vivo in healthy individuals demonstrated that the intra-arterial administration of N^G -monomethyl-L-arginine (L-NMMA) - an unspecific NOS activity blocker, reduces local blood flow by 25% to 50%⁵¹. Although the baseline vascular tone is the product of vasoconstrictor *versus* vasodilator forces, these results demonstrate that NO is, at least in part, the modulator of the phenotype at issue.

During exercise and mental stress conditions, in addition to the tachycardic response and blood pressure increase, vasodilatation in the skeletal muscle bed is also observed as part of the physiological responses of body adjustment. It was hypothesized that part of this muscle vasodilator response would be modulated by a neural component, and this was later evidenced by the existence of cholinergic sympathetic fibers in the skeletal muscles of some mammal species, except for primates and humans. Electrical stimulation of the sympathetic nerve was verified to lead to vasodilatation in the human muscle bed when presynaptic norepinephrine release was inhibited by intra-arterial drug infusion. However, this vasodilator response was attenuated when a muscarinic antagonist was administered^{52,53}. Later, it was evident that NO is, at least in part, the modulator of the vasodilator response verified when cholinergic sympathetic fibers are stimulated⁵⁴. In fact, Blair et al⁵⁵ had already shown that forearm vasodilatation in humans during physiologic maneuvers is mediated by a neural component. During application of mental stress, blood flow in the sympathectomized limb did not change in comparison to the blood flow in the control limb. In addition, intra-arterial atropine infusion in the control limb reduced

the increase in blood flow by approximately 50%. At that moment, using indirect evidence, the authors suggested the existence of cholinergic sympathetic enervation in the skeletal musculature of humans. Mimicking animal experiments, Dietz et al's studies^{56,57} later showed that part of the muscle vasodilator response, as measured in the forearm during exercise or mental stress, is attenuated by the intra-arterial administration of L-NMMA. The mechanisms through which acetylcholine and NO are synthesized and released during the body defense reactions in humans are still not fully understood. Evidences obtained with pharmacological blockade permit only the suggestion of the existence of cholinergic sympathetic fibers in the skeletal musculature. Due to these limitations, the authors do not rule out the possibility of vasodilatation being caused by a combination between circulating and local factors. A small part of endothelial cells could synthesize and release acetylcholine⁵⁸. In addition, activation of β_2 -adrenergic receptors located in the vascular smooth muscle would result in relaxation of this tissue and, consequently, in vasodilatation. However, Majmudar et al⁵⁹ verified that part of the vasodilatation resulting from β_2 -adrenoceptor activation is mediated by NO. Although the authors do not explain the mechanism responsible for this phenomenon, approximately 25% of the vasodilatation observed in the forearm with infusion of Ritodrine (a selective β_2 -adrenergic agonist) was attenuated with the concomitant administration of L-NMMA. These results suggest the existence of β_2 -adrenoceptors in the vascular endothelium, thus contributing to the increase in eNOS activity. In addition, increased mechanical stimulation of the vascular endothelium would result in increased NO synthesis via PI-3K-Akt kinase, with subsequent serine¹¹⁷⁷ residue phosphorylation.

eNOS polymorphisms and functional studies on the G894T variant

Genotyped and sequenced in 1993 by Marsden et al³⁸ (GenBank D26607), eNOS is located in chromosome 7q35-36, and variations in its sequence have been described in the promoter, exons and introns²⁷. The gene (21-22 kbp) comprises 26 exons and 25 introns with 133 kDa. The polypeptide sequence generated contains 1,203 amino acids³⁸. The existence of three single nucleotide polymorphisms (SNP) in the promoter, in non-binding locations of transcription factors has already been described in the literature³⁹. SNP were found in introns 2, 11, 12, 18, 22 and 23⁶⁰, and repeat sequence polymorphisms in introns 2, 4, 8, and 13^{38,61}. Of the polymorphisms found in exons 6 and 7, replacement of the nitrogenous base guanine for thymine (G→T) at position 894 located in exon 7 results in a change of the amino acid glutamate (GAG) to aspartate (GAT) at position 298 of the polypeptide sequence⁶². It is suggested that polymorphisms located in the gene promoter have an influence on mRNA transcription, whereas polymorphisms located in coding regions may result in changes in enzymatic activity⁶³.

Residue 298 is located outside in the oxidase domain of the enzyme, binding sites for L-arginine or BH_4 . Enzymatic studies using recombinant eNOS showed no difference in Michaelis constant (k_m) or in V_{max} between the two enzyme forms⁶³.

Although the enzymatic activity is seemingly not affected by the Asp²⁹⁸ form of the enzyme, Tesouro et al⁶⁴ showed that this variant is more susceptible to proteolytic cleavage into 100 and 35 kDa fragments, more precisely in position Asp²⁹⁸-Pro²⁹⁹, in comparison to the Glu²⁹⁸ variant. However, Fairchild et al⁶⁵ demonstrated that this proteolytic susceptibility occurred because of an artifact of the sample preparation method. The inconsistency of these results does not rule out the possibility that an unknown proteolytic mechanism or even a change in post-transcriptional regulation may be modulated by the Asp²⁹⁸ variant of the enzyme *in vivo*.

Association between the G894T variant of the eNOS gene and cardiovascular phenotypes

Several diseases have been associated with abnormalities in NO biosynthesis, and many of these conditions are related to autonomic dysfunction. Population genetics studies have demonstrated a significant association of eNOS gene G894T polymorphism with coronary artery disease (CAD)⁶⁶⁻⁶⁸ and also with acetylcholine (Ach)-induced coronary vasospasm⁶⁹. Among the cardiovascular dysfunctions, the G894T variant was also demonstrated to be associated with hypertension^{70,71}, although this association had not been verified in other populations^{72,73}.

The correlation between genotype and clinical phenotype varies both quantitatively and qualitatively, and the inconsistent association between eNOS polymorphism and several clinical phenotypes is a phenomenon commonly observed in other phenotype-associated genes²⁷. This inconsistency has been attributed to environmental factors, independent alleles, interaction between genes and variability in clinical phenotypes. The importance of environmental factors in the genesis of diseases is reflected in morbidity and mortality differences between genetically homogeneous groups, however with different life styles.

A genetic variation may not be relevant to a determined population, thus reflecting differences in the frequency of allele distribution. One example is the frequency of the allele 894T, which is significantly higher in white populations in comparison to the Japanese population²³. The inconsistent association between eNOS polymorphism and vascular changes is still being attributed to the variation in eNOS distribution in different organs. Arteries of specific organs are subject to different hemodynamic pressures, determining the vessel wall response and subsequent degree of endothelial dysfunction²⁷.

Philip et al⁶² studied patients undergoing surgical revascularization and observed that vascular reactivity to phenylephrine (PE) infusion is influenced by the G894T variant of the eNOS gene. The dose-dependent vasoconstrictor response to PE was significantly greater for the TT and GT alleles in comparison to the homozygote GG group, thus indicating that vascular reactivity to vasoconstrictor drugs may be influenced by eNOS polymorphism in humans. The greater dose-dependent response of patients with the 894T variant suggests reduced NO biosynthesis. Systemic

administration of *N*^G-nitro-L-arginine methyl ester (L-NAME) leads to hypertension in humans^{74,75}. According to Frandsen et al⁷⁶, the administration of 4mg/kg of L-NAME in humans reduces eNOS activity by 67%. The possibly reduced NO bioavailability subsequent to eNOS gene variations is an important candidate to the susceptibility to the development of endothelial dysfunction^{62,63} and changes in modulation of the sympathetic nervous activity on the vessel⁷⁷.

Studies demonstrating the association between the G894T variant of the eNOS gene and cardiovascular phenotypes point to a possible reduction in NO bioavailability, thus suggesting that eNOS transcribed from the mutant allele has an abnormal enzymatic activity. The lack of evidence supporting this rationale encouraged us to test the functionality of the genetic variant on the muscle vasodilatation phenotype⁷⁸. If the G894T variant of the eNOS gene could reduce the enzymatic activity, individuals with the mutant allele (T) would show a smaller increase in muscle blood flow in response to the handgrip isometric exercise. In order to test this hypothesis, 287 individuals were genotyped; from these, 33 healthy individuals were selected to represent three genotypes: GG (wild type), GT and TT. The results showed that attenuated reflex muscle vasodilatation in response to exercise occurred only in the TT genotype, since vasodilatation among heterozygotes (GT genotype) was similar to that observed among homozygotes for the G allele (GG genotype). These results suggest that the presence of the G allele is sufficient to overcome a possible T allele deficiency. Subsequent analyses *in vivo* proved that the attenuated muscle vasodilatation observed in the TT genotype is a consequence of the reduced eNOS-mediated vasodilatation, since intra-arterial L-NMMA infusion did not change the vasodilator response to exercise in this genotype. In contrast, L-NMMA significantly reduced the vasodilator response to exercise in the GG genotype to values similar to those of the TT genotype. Also, our study demonstrated that the attenuated muscle vasodilatation observed in the TT genotype may not be accounted to a possibly increased vasoconstrictor sympathetic tone. In fact, the muscle sympathetic nervous activity, as measured directly in the fibular nerve using the microneurography technique, increased in a similar fashion among the genotypes during exercise. Although these results do not confirm the functionality of the T allele in the alteration of the enzymatic activity (this variant may be in linkage disequilibrium with other functional variant on the same gene or on a nearby gene on the same chromosome), they may be used as a marker of the dysfunction observed

in the muscle vasodilatation phenotype. This is the first demonstration that the G894T variant of the eNOS gene is functionally associated with reduced eNOS-mediated vasodilatation. Also, these results suggest that the reduced endothelium-dependent vasodilatation may anticipate vascular dysfunction in individuals with the TT-genotype.

Conclusion

NO synthases were proven to be constitutively expressed (eNOS and nNOS) and NO plays an important role in the regulation of cardiovascular activities. These facts aroused the interest for the understanding of the molecular and cellular mechanisms that regulate their functionality. Although these mechanisms are complex and difficult to access, some of them have already been elucidated, thus permitting partial knowledge of the NO biology. In this context, the advances in molecular biology techniques enabled the identification of variants in the human genetic code that could, at least in part, explain the phenotypical response variation between individuals. Genetic association studies are not easy to understand. A single gene may have a small to moderate participation in the regulation of a multigenic phenotype and, in this situation, a determinate functional genetic variant in this gene would explain only a small part of the phenotype response variation. The screening of candidate genes, that is, the identification of multiple genes and their respective polymorphisms which trigger variations in the cardiovascular function leads to the moment when part of the diagnosis and management adopted for the treatment will be based on genomic medicine.

Potential Conflict of Interest

No potential conflict of interest relevant to this article was reported.

Sources of Funding

This study was funded by FAPESP.

Study Association

This article is part of the thesis of doctoral submitted by Rodrigo Gonçalves Dias, from *Universidade Estadual de Campinas e Instituto do Coração - InCor (HCFMUSP) de São Paulo*.

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