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Molecular mechanisms of Endothelial Dysfunction: from the nitric oxide synthesis to ADMA inhibition

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Short Title: MOLECULAR MECHANISM OF ENDOTHELIAL DYSFUNCTION

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Abstract

Endothelial dysfunction symbolize several pathological conditions, including altered anticoagulant and anti-inflammatory properties of the endothelium, impaired modulation of vascular growth, and dysregulation of vascular remodeling. Nevertheless, this term has been used commonly to refer to an impairment of endothelium-dependent vasorelaxation caused by a loss of nitric oxide bioactivity.

The clinical and scientific relevance of Nitric Oxide synthesis and bioavailability in endothelial dysfunction is based on the fact that it is a common factor in the pathogenesis of cardiovascular diseases. These alterations have been demonstrated in both animal models and humans, in the scope of dangerous pathological conditions as cigarette smoking, hypertension, hypercholesterolemia, aging, diabetes and heart failure.

A decline in Nitric Oxide bioavailability may be caused by decreased expression of the endothelial NO synthase, a reduction of substrate or cofactors for this enzyme, alterations of cellular signaling, enzyme inhibition by asymmetric dimethyl arginine and, finally, accelerated Nitric Oxide degradation by reactive oxygen species.

The knowledge of the processes related to these alterations becomes of remarkable importance for the understanding and of the generation of innovating and effective therapeutic strategies for cardiovascular diseases.

Key Words: Nitric Oxide, Nitric Oxide Synthase, Endothelial Dysfunction, Cardiovascular Diseases.
o is a nitrogen-centered free radical produced exclusively by the nitric oxide synthase action (NOS). Three isoforms of this enzyme have been described which are highly homologue in their primary structure. Inducible nitric oxide synthase (iNOS) is expressed in the phagocytic cells by some pro-inflammatory stimuli like cytokines. The two other isoforms are constitutively expressed in nervous tissue (nNOS) and the endothelial cells (eNOS).

Endothelial nitric oxide synthase (eNOS) synthesizes NO from L-arginine through an oxidation process that involves five electrons transfer by means of the intermediary NG-hydroxy-L-arginine.

The substrates used by this enzyme are the amino acid L-arginine and molecular oxygen, and the cofactors required are flavine adenine mononucleotide, tetrahydrobiopterin (BH4), flavine adenine mononucleotide (FMN) and flavine adenine dinucleotide (FAD), nicotinamide adenine dinucleotide (NADPH). Besides, the last two isoforms contain binding sites for hem group and calmodulin, being both of them essential for its activity. After calcium-calmodulin complex union to eNOS (between the COOH-terminal reductase domain and the NH2-terminal oxydase domain) the electrons are yielded by the NADPH and then transported to oxigenase domain which contains the hem group, which results in citrulin and NO formation.

A) Reduction of Nitric Oxide production

• eNOS transcription Alteration

Although the term “inducible” has been restricted for iNOS, the expression of eNOS is also regulated by a variety of stimulus. There is extensive evidence about factors which decrease eNOS expression, among which the tumor necrosis factor alpha (TNF-α) is included, which unstabilizes eNOS ARNm, apparently through regulatory proteins affinity increase to the 3' domain of iNOS ARNm molecule. Other stimuli that have been reported as ARNm stability reducers include lipopolysaccharide, hypoxia, and high concentrations of oxidized low density lipoproteins (LDLox) (Figure 3).

• Alteration in eNOS activity

Decrease in BH4 intracellular concentrations and Uncoupling phenomena

Under some circumstances eNOS can generate superoxide anion instead NO as a consequence of BH4 concentration decline; this process is known as NADPH oxidation/NO synthesis uncoupling phenomenon. Superoxide radical production is mediated through hem group in eNOS oxygenase domain when arginine and BH4 concentration is relatively low.
Mammals cells can generate BH4 through guanosine triphosphate (GTP) cyclohydrolase I (GTPCH I) enzymatic action. Physiological studies have shown a significant GTPCH I and BH4 activity decrease in various pathological states like insulinresistance, cigarette smoking, and hypercholesterolemia, probably through a LDL increase, as well as an expression arise in some pro-inflammatory cytokines (TNF-α and the interleukin-1β) (Figure 4). Furthermore, clinical and experimental studies have confirmed that BH4 acute administration improves endothelial dysfunction related to hypercholesterolemia, atherosclerosis, hypertension and smoking. These mechanisms expose an important link between pathophysiological states involved in endothelial dysfunction development.

**Competitive inhibition of eNOS by asymmetric dimethylarginine**

Vallance et al. first described in 1992 asymmetric dimethylarginine (ADMA) as a NOS endogenous inhibitor. Since then, the role of this molecule in the regulation of endothelial NO synthesis has increasingly attracted attention. This aminoacid is synthesized in endothelial cells from arginine by an enzyme belonging to arginine protein methyl transferase (PRMTs) family, specifically PRMT-1. Earlier experimental evidence showed that supplementation with L-arginine improves endothelium-dependent vasodilatation impairment in rabbits with hypercholesterolemia and atherosclerosis, diminishes platelet aggregation, inhibits monocyte adhesion and vascular smooth muscle proliferation, which is markedly in contrast with both, experimental studies that showed a fail in L-arginine endothelium-dependent vasodilatation stimulation in isolated arterial rings, and in vitro studies showing eNOS saturation at physiological L-arginine concentrations and a failure of exogenous L-arginine administration in achieve an enzyme’s activity increment, confirming a lack of L-arginine vasodilator effect in isolated arterial rings. This discrepancy between findings observed in intact animals versus in vitro assays was termed the “L-arginine paradox” that can be explained by the existence of an L-arginine competitive inhibitor now known as ADMA. Thus, administration of L-arginine at high doses should displace ADMA from the eNOS catalytic site and restore NO production to physiological levels in intact experimental models and humans beings.

The expression and activity of PRMT-1 and in consequence ADMA synthesis is modulated in endothelial cells by a variety of stimuli. For example, an expression increase in this enzyme had been observed in response to LDL molecules and shear stress, conducing to intracellular ADMA concentration. On the other hand, PRMT-1 activity can be blocked by the suppression of a group of protein kinases called kappa beta inhibitors kinase (κβ-I) that phosphorylates a group of proteins known as kappa beta inhibitors (κβ-I), which once phosphorylated are unable to retain the κβ nuclear factor (κβ-NF) in the cytoplasm, which, then becomes free to translocate to the nucleus, site where exerts its functions as transcription factor, suggesting a regulatory interplay by cytokines in the increase of its activity. Besides, multiple pathogenic factors like hypercholesterolemia, hyperglycemia, pro-inflammatory cytokines and hyperhomocysteinemia can diminish the activity of dimethylarginine dimethylaminohydrolase (DDAH), an enzyme responsible of hydrolyzes ADMA degradation, causing a significant intracellular elevation of this aminoacid (Figure 5). Thus, the nexus between cardiovascular disease risk factors, ADMA and endothelial dysfunction are becoming evident in conjunction with the essential role of oxidative stress in atherosclerosis generation.

**Figura 4:** Desacoplamiento de eNOS. Para la síntesis del NO por parte de la eNOS son necesarias concentraciones adecuadas de BH4 y de su sustrato la L-arginina. Cuando alguno de estos factores se encuentra disminuido en cantidad suficiente, la enzima no es capaz de generar NO, produciendo en su lugar un radical libre al torno de la orina, que la L-arginina es un aminoácido que circula en el plasma, es excretado por la orina, y es encontrado en células y tejidos. Cuando las concentraciones de la primera (ADMA) exceden las de la segunda (L-arginina), se produce una inhibición competitiva de la síntesis de NO por parte de eNOS que conduce a la génesis de la disfunción endotelial.

**Figura 5:** ADMA y Disfunción. La dimetilarginina aislizada (ADMA) al igual que la L-arginina es un aminoácido que circula en el plasma, es excretado por la orina, y es encontrado en células y tejidos. Cuando las concentraciones de la primera (ADMA) exceden las de la segunda (L-arginina), se produce una inhibición competitiva de la síntesis de NO por parte de eNOS que conduce a la génesis de la disfunción endotelial.

**Lipids and Caveolae Metabolism**

Studies conducted worldwide have indicated that the eNOS location into the cell determines its enzymatic activity. A place of particular importance in the cell for the function of this enzyme is the caveolae. Caveolae is a structure constituted by specialized inclusions in the plasmatic membrane whose main components are cholesterol, glycoesphingolipids,
and a structural protein called caveolin. At present, all evidence indicates that numerous extracellular stimuli exert its signals transduction through this structure. In this sense, eNOS location in caveole determines its enzymatic activity inhibition by caveolin-1 linkage that cause a blockade in eNOS interaction with the calmodulin when the intracellular calcium levels are low. Also, high levels of oxidized LDL cause a decrease of cholesterol content of caveolae, resulting in caveolin-1-eNOS complex translocation to the cytoplasm, and in consequence, inhibition of its activity. Likewise, there is evidence that hypercholesterolemic serum and native LDL are capable to up regulate caveolin-1 concentration, increasing heterocomplexes formation between eNOS and this protein, diminishing NO production. Furthermore, it is known that some proatherogenic lipids, such as lysophosphatidylcholine and LDL, interfere with signal transduction from receptors that activate eNOS (acytcholine receptors, bradykinin, serotonin, histamine and others) (Figure 6). This process enlightens other mechanism which correlates prepathogenic and/or pathological conditions with the decrease of NO levels, and therefore, endothelial dysfunction.

B) Decrease in NO Bioavailability

- **Increase in Arginase activity**
  
  Arginase is a key enzyme involved in arginine to ornithine (and urea) conversion. This enzyme has two isoforms, arginase I, which is constitutively expressed in the endothelial cells, and arginase II which can be induced by lipopolysaccharides and interferon-γ. Thus, systemic or local infectious processes could generate a significant increase on arginase levels causing an important decrease in arginine and consequently a lack in eNOS substrate bioavailability and consequently a failure in NO synthesis.

- **Oxidative stress**
  
  Even in presence of an adequate NO generation, some circumstances avoid this molecule to reach its biological targets due to a decrease in its bioavailability, as a consequence of the interaction with some chemical compounds. There is abundant experimental evidence indicating the role of NO oxidative inactivation as mediator of endothelial dysfunction and a prepathogenic vascular phenotype. For example, in hyperlipidemia, excessive LDL synthesis entails a concomitant formation of LDL, which results in oxidative stress that causes NO conversion to peroxynitrite (chemical specie without the biological effects of NO) by a reaction that proceeds at 6.7 x 10⁷ M⁻³s⁻¹. This velocity is approximately three times higher than the reaction between superoxide and superoxide dismutase (SOD). So that, in a compartment with NO, superoxide and SOD, the superoxide anion is able to react with any of the two other compounds. The results of different studies support the role of the superoxide as essential element in the decrease of NO bioavailability in oxidative stress conditions. In rabbits with aortic atherosclerosis, a remarkable decrease in endothelium-related relaxation was seen (despite an NO synthesis increase up to three times greater in relation to the NO synthesis level in healthy rabbits), which was corrected by SOD treatment. Likewise, ascorbic acid infusion improves the vascular response to the acetylcholine in smokers, diabetic and patients with high blood pressure.

- **Hyperglycemic Stress**
  
  Hyperglycemia increases oxygen-derived free radicals production via arachidonic acid metabolism arises. In human aortic endothelial cells, despite the extended exposition to high glucose concentration increases eNOS expression, a concomitantly superoxide anion elevation (probably from NADH/NADPH oxidase) result in NO inactivation. Besides, an extended hyperglycemic stress causes advanced glycosilation endproducts (AGES) accumulation, which is able to inactivate NO. In fact, the alteration in the capacity of endothelium-depending vessels relaxation in diabetic rats can be partially restablished by aminoguanidine (an AGES) administration. Thus, it is easy to deduce the importance of this mechanism as an remarkable connection between diabetes and cardiovascular diseases.

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**Concluding Remarks**

Endothelial dysfunction and more specifically, alteration in NO synthesis or action constitutes an essential step in the pathophysiology of most prevalent cardiovascular diseases. Due to essential NO functions (antiatherogenic, antithrombotic, antiproliferative agent), important changes are produced in the endothelial physiology when its biodisponibility is distorted. The multiple processes related to the reduction of synthesis and bioavailability of NO are far from being completely clarified. Although now days, a significant and growing body of information is being conformed, however, studies devoting to comprehension of biology of eNOS and its pathological modifications in the course of endothelial dysfunction are required. This fact will allow new pharmacological strategies generation in order to treat coronary heart disease since their beginnings, and consequently, preventing most feared complications in a safer and efficient approach.

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