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Clinical and pharmacogenetic impact of endothelial nitric oxide synthase polymorphisms on cardiovascular diseases

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ABSTRACT

Nitric oxide (NO) is a vasoactive substance synthesized from L-arginine by neuronal (NOS1), endothelial (NOS3), and inducible (NOS2) nitric oxide synthases. NOS3 is the most important NO synthase isoform in the vascular endothelium and therefore it exerts critical roles in the cardiovascular system. NOS3 is encoded by *NOS3* gene, which displays a large number of genetic polymorphisms such as single nucleotide polymorphisms (SNPs), variable number of tandem repeats (VNTRs), microsatellites, and insertions/deletions. Interestingly, NOS3 regulation and NO production are affected by some *NOS3* polymorphisms. Given these functional consequences and the protective role of NOS3 against cardiovascular diseases, many studies have investigated whether *NOS3* polymorphisms affect the susceptibility to cardiovascular diseases and the responses to drugs that affect NOS3 activity in the cardiovascular system. In addition, a growing body of evidence shows the effects of combinations of *NOS3* polymorphisms within haplotype blocks on NO bioavailability and disease susceptibility. In this review, we discuss the basic biochemical mechanisms of NOS3 regulation and the clinical and pharmacogenetic impact of *NOS3* polymorphisms on cardiovascular diseases.

1. Introduction

Cardiovascular diseases are responsible for significant morbidity, mortality, and health-care costs worldwide [1]. In this regard, the endothelium (a monolayer of cells lining the lumen of blood vessels) plays a critical role in maintaining the cardiovascular homeostasis by regulating the vascular tone, structure, and function. This is achieved particularly by regulated release of vasoactive substances [2]. One of the most important substances produced by endothelial cells is nitric oxide (NO), a small gaseous and lipophilic molecule synthesized from L-arginine by three different synthases: neuronal (NOS1), endothelial (NOS3) and inducible (NOS2) NO synthases [3].

NOS3 is the dominant NO synthase in the vasculature and plays a critical role in the cardiovascular system, as consistently shown in clinical and experimental studies [4]. Indeed, pharmacological inhibition of NOS3 promotes hypertension and impairs the cardiovascular system [5]. Accordingly, NOS3 knockout mice show increased blood pressure and other cardiovascular alterations [6]. Given the protective role of NOS3 activity against cardiovascular disorders, a large number of studies has examined how commonly found genetic variants in *NOS3* gene modify the susceptibility to cardiovascular diseases and the responses to drug therapy. The present review focused on how *NOS3* genetic polymorphisms may affect NOS3 regulation and endogenous NO formation, as well as the effects of these polymorphisms on the susceptibility to cardiovascular diseases and responses to drugs affecting the cardiovascular system.

2. Roles of NO in the cardiovascular system

NO is a small gaseous and lipophilic molecule that plays critical roles in the regulation of cardiovascular homeostasis [7]. Importantly, one of the most important roles played by NO is controlling the vascular tonus, and this is mediated by activation of soluble guanylate cyclase (sGC), a heme-containing heterodimeric enzyme expressed in vascular smooth muscle cells [8–10]. In response to NO, sGC activity enhances more than 200 fold leading to rapid

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conversion of guanosine triphosphate into cyclic guanosine monophosphate (cGMP) [11], which in turn activates protein kinase G-1 (PKG-1), an enzyme that promotes multiple phosphorylation of cellular protein targets. This results in lower intracellular free calcium concentrations and vascular relaxation [12]. Targets for PKG-1 phosphorylation in vascular smooth muscle cells include large-conductance calcium-activated potassium channels [12–14], L-type calcium channels [15], as well as 1,4,5 inositoltriphosphate receptor-associated cGMP kinase substrate and phospholamban in the sarcoplasmic reticulum [16,17].

NO also indirectly regulates the vascular tonus by interfering with vascular redox environment [17]. This effect involves antioxidant proprieties of NO attributed to its scavenging of superoxide anion [17], as well as upregulation of the antioxidant enzyme superoxide dismutase, which catalyzes the dismutation of superoxide anion to hydrogen peroxide [18]. In contrast, NO reaction with superoxide anion leads to peroxynitrite production [19], which is usually limited by relatively high concentrations of superoxide dismutase [20]. However, when NO levels increase, for example as a consequence of NOS2 upregulation [21,22], the formation of peroxynitrite may prevail and impair the vascular homeostasis, thus leading to endothelial dysfunction, a major feature in the pathophysiology of hypertension [21,22].

In addition to controlling the vascular tonus, endogenous NO exerts antithrombotic effects as a consequence of its diffusion across platelet membrane and sGC activation, which inhibits platelet aggregation [17]. Moreover, NO regulates leukocyte adhesion to the vascular endothelium by inhibiting nuclear factor Kappa-B, which up-regulates the vascular endothelial expression of chemokines and adhesion molecules [23]. Endogenous NO also exerts antiproliferative effects by cGMP-dependent and independent mechanisms [24,25]. The cGMP-dependent effects involve the inhibition of calcium influx [24], as this ion induces proliferation of vascular smooth muscle cells [26]. However, the cGMP-independent effects are attributed to inhibition of arginase and ornithine decarboxylase activity, thereby decreasing the production of polyamides needed in DNA synthesis [25].

3. Nitrite concentration as a possible biomarker to reflect endogenous NO production

Given that NO has a very short half-life, there are no simple methods available to assess endogenous NO production *in vivo*, and thus NO oxidation products (nitrite and nitrate) [27] are usually measured because they may reflect NO formation [28,29]. While the measurement of nitrate in plasma after an overnight fast has been widely used as a parameter of endogenous formation of NO, a large number of studies has now clearly shown that assessing circulating nitrite concentrations provides much improved information [30–35]. This is because plasma nitrate concentrations are affected by several interfering factors including diet, clinical conditions, medications, smoking status, and other environmental factors [28]. In contrast, the circulating levels of nitrite sensitively reflect endothelial NO formation [30]. In addition to nitrate and nitrite, plasma cGMP levels have also been suggested as a less reliable marker of NO formation [29].

More recently, nitrate and nitrite have been valued as major sources of NO, independent of the classical enzymatic NO formation from L-arginine, and this now constitutes a complementary pathway known as nitrate-nitrite-NO pathway [36,37]. Dietary nitrate enters the circulation and is secreted into the oral cavity (by salivary glands), where it is converted into nitrite by oral commensal bacteria [36,37]. Swallowed nitrite achieves the stomach and generates NO and other bioactive nitrogen species under the acidic conditions of the gastric cavity [36–38]. Nitrite is also absorbed from the intestine into the circulation and converted to bioactive NO in blood and tissues by enzymes with nitrite reductase activity, particularly under certain conditions [39]. Interestingly, these processes have been implicated in the cardiovascular effects of exogenously nitrite [38,40–43]. Altogether, these findings highlight the idea that accumulated nitrite correspond to an important NO reservoir.

4. An overview of NOS3 regulation

Although NOS3 was firstly described in endothelial cells [44,45], it is expressed in non-endothelial cell types including platelets [46], cardiomyocytes [47], and neurons [48]. In this regard, NOS3 expression and activity are regulated by transcriptional, post-transcriptional, and posttranslational mechanisms.

At transcriptional level, the promoter region in the *NOS3* gene (which encodes NOS3) plays a critical role in the regulation of NOS3 expression [49]. The human *NOS3* promoter exhibits two regulatory regions that are particularly important to basal NOS3 transcription: a positive regulatory domain I, which corresponds to a high-affinity Sp1 transcription factor recognition site and binds three nucleoproteins identified as Sp1 and two variants of Sp3, and a positive regulatory domain II, which forms nucleoprotein complexes with the transcription factors Ets-1, Elf-1, YY1, Sp1, and MYC-associated zinc finger protein (MAZ) [49]. While Sp1, Sp3, Ets-1, Elf-1 and YY1 positively regulate human *NOS3* promoter activity, MAZ seems to inhibit the *NOS3* promoter [49]. In addition to these regulatory regions, methylation of *NOS3* promoter also affects NOS3 transcription [50]. Indeed, methylation of *NOS3* promoter resulted in impaired promoter activity in mammalian cells, and this effect was associated with decreased ability of Sp1, Sp3 and Ets-1 to transactivate the *NOS3* promoter [50]. These findings suggest that DNA methylation is involved in cell-specific expression of the human NOS3 gene [51].

Complex mechanisms may affect posttranscriptional NOS3 regulation and include alterations of mRNA stability, nucleocytoplasmatic transport, and subcellular localization [52]. These alterations are mediated by *cis*-acting RNA elements located in the 5′- and 3′- mRNA untranslated regions (5′-UTRs and 3′-UTRs) [53]. Interestingly, studies focusing on *cis* elements in the NOS3 3′-UTR report that certain sequences at the origin of the 3′-UTR are important for the binding of cytosolic proteins and change its configuration to enhance its susceptibility to RNase activity [54–56]. In addition to *cis* elements, NOS3 3′-UTR is a target for micro (mi)RNAs, which are approximately 22-nucleotide endogenous small RNAs that negatively modulate gene expression and induce mRNA degradation or translational repression [57,58]. Interestingly, genetic knockdown of Dicer (an enzyme required to miRNA maturation) resulted in increased NOS3 expression [59], thus suggesting that miRNAs promote NOS3 downregulation at posttranscriptional level.

Posttranslational mechanisms are also relevant for NOS3 activity, as demonstrated by the effects of its interaction with caveolin-1 [60]. In resting endothelial cells, a strong and direct interaction of NOS3 with caveolin-1 in the caveolae inactivates the enzyme [60]. Conversely, binding of calcium-activated calmodulin to NOS3 shifts caveolin-1 and activates NOS3 [61]. Moreover, as active NOS3 enzyme is a homodimer and its homodimerization depends on the availability of the essential cofactor tetrahydrobiopterin (BH4), mechanisms affecting BH4 synthesis and consumption also control NOS3 activity [62]. In fact, suboptimal levels of BH4 decrease NO formation and uncouple NOS3, thus resulting in NOS3-mediated reduction of oxygen with generation of superoxide anion instead of NO [61]. Additionally, NOS3 activity is regulated upon phosphorylation [63–65]. While there are several phosphorylation sites