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Pharmacogenetic influence of *eNOS* gene variant on endothelial and glucose metabolism responses to L-arginine supplementation: Post hoc analysis of the L-arginine trial

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ABSTRACT

Objective. To evaluate whether variants of the *eNOS* gene are associated with endothelial and metabolic responses to L-arginine (L-arg) supplementation.

Material and Methods. We examined a single nucleotide polymorphism of the *eNOS* gene (rs753482-A > C) to investigate the effects of this variant on endothelial function (EF), colony-forming unit–endothelial cell (CFU-EC) number, asymmetric-dimethylarginine (ADMA) level, insulin sensitivity index (ISI), and insulin secretion (IS) in a post hoc analysis of the L-arg trial. The L-arg trial (6.4 g/day for 18 months) was a single-center, randomized, double-blind, parallel-group, placebo-controlled, phase III trial in individuals with impaired glucose tolerance and metabolic syndrome. followed by a 12-month extended follow-up period after termination of the study drug (NCT 00917449).

Results. At baseline, EF, CFU-EC numbers, ADMA levels, and ISI were impaired in subjects carrying minor allele C (both heterozygotes, AC and homozygotes, CC) as compared to subjects carrying major allele A (homozygotes, AA) ($p < 0.01$). Compared to placebo, L-arg increased EF, CFU-EC numbers, and ISI, and improved ADMA levels and IS ($p < 0.01$). The greatest improvements were found in AA subjects treated with L-arg, while the worst results were found in AC + CC subjects treated with placebo. In the placebo-treated subjects, EF, CFU-EC, ISI, and IS were significantly lower and ADMA was significantly higher in AC + CC subjects than in AA subjects.

Abbreviations: T2DM, Type 2 diabetes mellitus; NGT, normal glucose tolerance; IGT, impaired glucose tolerance; MS, metabolic syndrome; CAD, coronary artery disease; *eNOS* gene, endothelial nitric oxide synthase gene; NO, nitric oxide; L-arg, L-arginine; EF, endothelial function; CFU-EC, colony forming unit–endothelial cell; EPC, endothelial progenitor cells; ADMA, asymmetric-dimethylarginine; ISI, insulin sensitivity index; IS, insulin secretion; HR, hazard ratio; CI, confidence interval; HOMA, homeostasis model assessment; OGTT, oral glucose tolerance test; IGI, insulinogenic index; FM, fat mass; FFM, fat free mass.

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Conclusions. Treatment with L-arg induced similar improvements in EF, CFU-EC numbers, ADMA levels, ISI, and IS in both AA subjects and AC + CC subjects. The presence of minor allele resulted in the worst prognosis in terms of EF, CFU-EC numbers, ADMA levels, ISI, and IS during the 30-month observation period.

1. Introduction

Type 2 diabetes mellitus (T2DM) is considered to be heterogeneous with regard to its clinical presentation, features, and pathogenesis. Patients with T2DM are generally treated in a similar manner, independent of underlying differences in pathophysiology and genetic risk factors that might affect therapeutic response. Research over the past several decades has identified that insulin secretion and cellular responses to insulin are mediated by numerous receptors, transporters, ion channels, enzymes, functional regulators, transcription modifiers, and structural elements. Variations in the expression or function of these mediators undoubtedly represent important determinants of T2DM heterogeneity that may result in varying effects on β-cell growth and development, insulin secretion, or changes in insulin responsiveness in specific or multiple tissues. Determining T2DM heterogeneity would require direct measurement of these factors from samples of β-cells or insulin-target tissues, which unfortunately cannot be routinely obtained from T2DM patients.

We recently demonstrated that L-arginine (L-arg; 6.4 g/d) and lifestyle modifications for 18 months significantly increased regression to normal glucose tolerance (NGT) in a clinical trial involving patients with impaired glucose tolerance (IGT) and metabolic syndrome (MS) [1]. Although the incidence of diabetes was not significantly reduced, the increase in the number of subjects with NGT after L-arg therapy was explained by improvements in endothelial function, insulin sensitivity, and insulin secretion. Improvements were detected during the 18-month treatment period or during the 12-month extended follow-up period [1].

Other studies have found that by increasing the bioavailability of nitric oxide (NO) by using orally administered L-arg, it is possible to decrease the levels of the endogenous competitive inhibitor of NO synthase (NOS), asymmetric dimethylarginine (ADMA), and improve endothelial function in patients with hypertension [2,3]. This finding could explain, at least in part, the beneficial effects of L-arg on endothelial function. In fact, it is well known that ADMA inhibits vascular NO production, thereby altering vascular tone and systemic vascular resistance [4]. Thus, ADMA has been recently identified as a novel cardiovascular risk factor [5].

Since L-arg is the substrate for endothelial NOS (*eNOS*), we postulated that the efficacy of L-arg supplementation with regard to endothelial and metabolic outcomes could be affected in subjects carrying *eNOS* gene polymorphisms. The gene encoding *eNOS* is located on human chromosome 7q36, a genetic region previously linked to MS and cardiovascular risk factors [6–8]. Due to the importance of NO production in the endothelium and its regulation of vascular function [9,10], the *eNOS* gene has been considered a logical target for DNA sequence variations that may contribute to the pathophysiology of cardiovascular diseases. Of the polymorphisms identified thus far in the *eNOS* locus, we previously reported the genetic association of *eNOS* gene variant rs753482-A > C with T2DM and MS in a small cohort of subjects [11]. This minor allele was consistently enriched in T2DM patients, suggesting a functional association between *eNOS* variants and T2DM. In that study, healthy carriers of the minor allele C showed typical features of MS, including insulin resistance, hyperinsulinemia, and increased fasting NO levels [11]. Moreover, we observed that T2DM patients with critical limb ischemia and carrying minor allele, C, (both heterozygotes, AC and homozygotes, CC), showed a reduction in the number of circulating endothelial progenitor cells (EPC). This finding was associated with an increased cumulative risk of restenosis, amputation, ulceration, and death at 6 months after peripheral angioplasty (hazard ratio [HR] 5.3, 95% confidence interval [CI]: 1.41–19.5, $p < 0.02$), compared to the corresponding risks in patients carrying the major allele, A [12].

More recently, our research group demonstrated that an unreported transcript variant skipping exons 20–21 is prevalent in carriers of the minor allele C and is effectively translated into a novel truncated form of *eNOS* (Δ20–21 eNOS) [13]. In *in vitro* experiments, truncated *eNOS* was associated with increased basal NO production, insensitivity to calcium stimulation, and upon heterodimerization with the full-length *eNOS* protein, a dominant negative effect on NO production. A similar phenotype was found in humans; indeed, both patients with coronary artery disease (CAD) and healthy carriers of the CC genotype showed increased basal NO levels in peripheral blood and platelets, and negatively responded to oral glucose load by failing to show an increase in NO synthesis following an insulin wave. As an additional marker of endothelial function, forearm vasodilation after reactive hyperemia was dramatically impaired in CC carriers, who are more at risk of early restenosis. These data indicated that subjects carrying the CC genotype express a novel, stable, truncated form of *eNOS* with altered enzymatic activity that influences NO production and endothelial function. The Δ20–21 eNOS polypeptide is 92 kDa in length and quite stable. It interrupts the canonical sequence to amino acid 775 by adding an inauthentic tail, 61 amino acids in length. This subsequently affects the reductase domain through the loss of its C-terminal region, which includes the FAD and NADPH regulatory domains. However, the FMN domain is maintained and can interact with the L-arg domain [13]. To the best of our knowledge, no studies have been conducted to determine the pharmacogenetic implications of *eNOS* rs753482-A > C gene polymorphism on endothelial and glucose metabolism responses to long-term L-arg supplementation in subjects with IGT and MS. Therefore, the aim of the present study was to perform a post hoc analysis of the L-arg trial to evaluate whether L-arg supplementation helps subjects with a genetic variant of *eNOS* to overcome endothelial and metabolic alterations.