The Relationship between Insulin Resistance and Polymorphisms of the Endothelial Nitric Oxide Synthase Gene in Patients with Coronary Artery Disease

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Nitric oxide (NO) regulates endothelial function and is believed to prevent atherogenesis. In endothelial cells, endothelial nitric oxide synthase (eNOS) is expressed constitutively, and regulates NO synthesis. A mutation of the eNOS gene has been associated with the development of coronary artery disease (CAD). The development of CAD is also influenced by insulin resistance, and recent studies suggest that NO might affect cellular insulin activity. We investigated the association between eNOS polymorphisms and insulin resistance in patients with CAD. We screened 45 patients with a history of myocardial infarction (MI), angina pectoris (AP), or coronary spasm. Genotypes were determined by polymerase chain reaction-restriction fragment-length polymorphism analysis. We examined two polymorphisms of the eNOS gene (The T⁻⁷⁸⁶→C variant and the missense Glu298Asp variant). Insulin resistance was measured by determining the plasma immunoreactive insulin concentration at the 120 min time point (IRI 120) of a 75 g oral glucose tolerance test. The IRI 120 of the T⁻⁷⁸⁶→C variant group was higher than that for the control group ($p<0.05$). This finding demonstrates that the T⁻⁷⁸⁶→C mutation in the eNOS gene decreases insulin sensitivity. *J Atheroscler Thromb*, 2003; 10: 43-47.

Key words: Myocardial infarction, Missense, Genetic variants, Nitric oxide

Introduction

Nitric oxide (NO) is synthesized from L-arginine by endothelial NO synthase (eNOS), and is responsible for maintaining basal vascular tone. In addition to relaxing vascular smooth muscle cells, NO inhibits platelet and leukocyte adhesion to vascular endothelium, inhibits vascular smooth muscle cell migration and growth, and limits the oxidation of atherogenic low density lipoprotein [1-5]. Experiments with eNOS knockout mice have shown decreased NO synthesis and insulin resistance [6].

Recently, several polymorphisms of the eNOS gene have been described in humans. One of the most extensively studied mutations is the missense Glu298Asp variant. This is a G to T conversion of nucleotide 894 in exon 7. This common variant has been associated with a higher incidence of myocardial infarction (MI), coronary spasm, and hypertension [7-10]. The other mutation is a T⁻⁷⁸⁶→C variant. This mutation occurs in the 5'-flanking region of the eNOS gene, and is associated with coronary spasm, MI, and cerebral circulation [11, 13].

Insulin resistance has also been suggested to contribute to the pathogenesis of coronary artery disease (CAD) [14, 15]. However, no other investigators have described the relationship between eNOS gene polymorphisms and insulin resistance. To further elucidate the role of mutations of the eNOS gene in insulin resistance in patients with CAD, we examined the relationship between eNOS

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Received August 26, 2002.

Accepted for publication October 30, 2002.

polymorphisms (T⁻⁷⁸⁶→C variant, missense Glu298Asp variant) and insulin resistance in a Japanese population. Insulin resistance was measured using the plasma immunoreactive insulin concentration at the 120 min time point (IRI 120) of a 75 g oral glucose tolerance test. We found an effect of eNOS gene variations on insulin resistance.

Subjects and Methods

Study patients

The study included 45 patients with coronary artery disease [28 with myocardial infarction (MI), 11 with angina pectoris (AP), 6 with coronary spasm] admitted to Saga Medical College hospital from September 1998 to November 1999 (Table 1). Myocardial infarction was diagnosed by the presence of typical symptoms, electrocardiographic changes, serum creatine kinase-MB isozyme (CK-MB) elevations, and cardiac catheterization. Angina pectoris was diagnosed by symptoms, electrocardiographic changes, and angiographic evidence of coronary artery stenosis. Coronary spasm was angiographically documented during cardiac catheterization. We excluded patients taking medications that influence insulin sensitivity, β -blockers, or troglitazone. The study was approved by our institutional review board, and informed consent was obtained from each patient before inclusion in the study.

Identification of eNOS gene variants by polymerase chain reaction-restriction fragment-length polymorphism (PCR-RFLP)

To identify possible mutations of the eNOS gene, we performed PCR-RFLP. Genomic DNA was prepared from blood leukocytes by established methods.

eNOS -786T→C

A set of primers was designed to amplify the 159 base pair (bp) fragment encompassing the mutation -786 T→C (the sense and antisense primers were 5'-TGAAGTGCCTGGAGAGTGCT-3', and 5'-ACGCACGCTTCCCGGGTCCGCAGGTCAGCA-3', respectively). The PCR fragments were digested with the restriction enzyme Hae III and separated by agarose gel electrophoresis.

eNOS 894G→T

A specific set of primers and restriction enzyme were used as described previously [16].

Oral glucose tolerance test (OGTT)

After a 12h overnight fast, patients underwent a 75 g OGTT after a 12 h overnight fast.

Statistical analysis

Statistical analysis was performed using the Mann-Whitney U test. StatView J 4.5 software (Abacus Concepts, CA) was used for the analysis. A value of $p < 0.05$ was considered statistically significant.

Table 1. Clinical characteristics of study patients

Patients with CAD (n=45)	
Age (y)	62.6 ± 9.8
Male/female	33/12
MI/AP/spasm	28/11/6
DM/IGT/NGT	12/13/20
FBG (mg/dl)	101 ± 23
FIRI (μ /ml)	8.1 ± 3.7
HbA _{1c} (%)	5.7 ± 1.2

CAD, coronary artery disease; MI, myocardial infarction; AP, angina pectoris; DM, diabetes mellitus; IGT, impaired glucose tolerance; NGT, normal glucose tolerance; FBG, fasting blood glucose; FIRI, fasting immunoreactive insulin. Data are reported as the mean ± SD.

Results

Screening for the T⁻⁷⁸⁶→C variant in the 5'-flanking region of the eNOS gene.

Fig. 1A shows a representative agarose gel loaded with PCR products after digestion with Hae III from 3 different patients. The eNOS/TT, TC, and CC genotypes were present in 38 (84.4%), 7 (15.6%), and 0 (0%) of 45 patients with CAD, respectively.

Screening for the missense Glu298Asp variant of the eNOS gene.

Fig. 1B shows a representative agarose gel loaded with PCR products after digestion with Mbo I from 3 different patients. The eNOS/GG, GT, and TT genotypes were present in 38 (84.4%), 5 (11.1%), and 2 (4.4%) of patients with CAD.

The relationship between insulin sensitivity and eNOS genotype.

All patients were divided into three groups: wild type (eNOS -786TT and eNOS 894GG), T⁻⁷⁸⁶→C variant (eNOS -786TC/CC), and missense Glu298Asp variant (eNOS 894GT/TT). Table 2 summarizes the clinical characteristics of each group. No significant differences were observed between the three groups with respect to age, gender and a body mass index. The plasma immunoreactive insulin concentration at the 120 min time point (IRI 120) of a 75 g oral glucose tolerance test (75 g OGTT) was higher in the T⁻⁷⁸⁶→C variant group (Table 2, $p < 0.05$). A similar tendency was observed in the missense Glu298Asp variant group, but this difference was not significant.

Discussion

It has been recognized that the insulin resistance syndrome is one of the major predictors of atherosclerotic disease. The relationship between insulin resistance and cardiovascular risk, particularly the risk of coronary artery

disease, has been well established in many prospective studies [17]. Insulin itself affects arterial smooth muscle cells, macrophages, and endothelial cells and may accelerate atherosclerosis [18]. Some studies have suggested that endothelium-dependent vasodilator function may be impaired in patients with atherosclerosis [19]. Furthermore, an association between endothelial nitric oxide production and insulin resistance has been reported [20].

In this study, we found a possible association between the T⁻⁷⁸⁶→C eNOS gene polymorphism and insulin resistance. Furthermore, we hypothesize that low NO concentrations, caused by a defect in NO synthesis, may play a role. Experiments using transgenic eNOS knockout mice have confirmed that the absence of eNOS activity results in abnormal endothelium dependent vasodilation [21, 22] and accelerated atherosclerosis [23, 24]. Recent data show that eNOS knockout mice are insulin resistant, as evidenced by fasting hyperinsulinemia and decreased glucose infusion rates during euglycemic clamp studies [6]. These findings are thought to be mediated at the level of eNOS expressed in skeletal muscle tissue, where it may

regulate insulin sensitivity [25].

The T⁻⁷⁸⁶→C mutation has been reported to be associated with coronary spasm [12] and acute myocardial infarction in the absence of organic coronary artery stenosis [12]. It has been hypothesized that the -786T→C mutation suppresses eNOS gene transcription, reducing the production of endothelial NO and increasing vasoreactivity.

The incidences of the T⁻⁷⁸⁶→C and Glu298Asp variants in patients with CAD were 30 to 70% [12, 26], and 21.1 to 64% [7, 8, 16, 26], respectively, in previous studies. Compared with these previous studies, the incidences in our study were lower (15.6%, and 15.6%, respectively, Table 2). CAD represents a state of insulin resistance, and insulin resistance is considered a common factor for various atherogenic risk factors, including hypertension, glucose intolerance, and dyslipidemia. [27] Our patients represented a skewed group because of the influence of insulin resistance. That is why we could not find a positive association between the presence of the Glu298Asp variant and the development of CAD in the present study. Furthermore, our patient population was too small to iden-

Fig. 1A. Use of PCR-RLFP analysis to screen for the T⁻⁷⁸⁶→C mutation in the 5'-flanking region of the eNOS gene. Shown is a representative agarose gel loaded with wild type (TT), heterozygote (TC), and homozygote (CC) PCR product after digestion with Hae III, respectively.

Fig. 1B. Use of PCR-RLFP analysis to screen for the missense Glu298Asp mutation at nucleotide position 894 of the eNOS gene in exon 7. Shown is a representative agarose gel loaded with wild type (GG), heterozygote (GT), and homozygote (TT) PCR product after digestion with Mbo I, respectively.

Table 2. Comparison of clinical characteristics

	Wild type	T ⁻⁷⁸⁶ →C variant	Missense Glu298Asp variant
Age (y)	62.3 ± 10.6	66.5 ± 9.3	61.3 ± 6.1
Male/female	23/8	5/2	5/2
MI/AP/spasm	18/8/5	4/2/1	4/2/1
BMI (kg/m ²)	23.1 ± 2.6	23.6 ± 3.1	23.4 ± 2.4
IRI120 (μ/ml)	55.8 ± 31.25	78.4 ± 26.7*	69.6 ± 38.4

SI, insulin sensitivity index; IRI120, immunoreactive insulin concentration 120 min after 75 g OGTT. Data are reported as the mean ± SD.

* $p < 0.05$ for variant vs. wild type.

tify a statistically significant association.

Our data demonstrate the relationship between the T⁻⁷⁸⁶→C eNOS polymorphism and insulin sensitivity. However, this tendency is not sufficient to conclude that the eNOS polymorphism is solely responsible for insulin resistance. These results demonstrate that insulin sensitivity is not affected by eNOS polymorphisms directly, but through changes in endothelial function and eNOS production in skeletal muscle.

In conclusion, the present study suggests that T⁻⁷⁸⁶→C eNOS polymorphisms are associated with the development of insulin resistance.

Acknowledgments: We thank the staff of the cardiology division in Saga Medical school for their clinical assistance and suggestion.

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