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Research paper

Endothelial nitric oxide synthase genotypes modulate peripheral vasodilatory properties after myocardial infarction

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ABSTRACT

Background: Studies in population genetics suggest an important relationship between the eNOS G894T polymorphism and occurrence of acute myocardial infarction (AMI), with little known on its influence on the post-AMI period.
Aim: To investigate the association of allelic variants produced by the G894T transversion in eNOS (rs1799983) with post-AMI variables.
Methods: Cross-sectional analyses of anthropometric, clinical and laboratory assessments obtained within the first 24 h and after 5 and 30 days of the AMI event across T carriers and G homozygotes of eNOS in 371 consecutive cases of AMI with ST-segment elevation admitted to a Brazilian emergency service in cardiology. Genotypes were determined by polymerase chain reaction followed by enzymatic restriction.
Results: Despite no difference between genotypic groups on aspects as Killip–Kimbal classification scores, extension of infarcted mass, lipid profile or pattern of medication use, an increase in serum nitric oxide from admission to day 5 was higher for T carriers ($p < 0.001$). Thirty days post-AMI, peripheral blood flow reserve was larger among T carriers either by flow- ($p = 0.037$) and nitrate-mediated ($p = 0.040$) dilation testing.
Conclusion: Our results suggest an association of the eNOS 894T allele with an apparent improvement in late arterial function in post-AMI patients.

1. Introduction

According to projections by the World Health Organization for the coming decades, cardiovascular diseases (CVDs) will expand their leadership as the major cause of death worldwide (WHO, 2014). In Brazil, even though mortality rates due to circulatory disorders have been reduced in recent years, CVDs remain responsible for over one third of all deaths (Baena et al., 2013). Within the large spectra of CVDs, ischemic heart diseases are the most common, among which acute myocardial infarction (AMI) stands out as the absolute, leading cause of deaths in men and women (Baena et al., 2012).

The incidence of acute coronary events increases about 12-fold in the first, subsequent year after a primary event of AMI (post-AMI) (Soares et al., 2009). However, the clinical and laboratory characteristics that influence this elevated risk are not well understood. In fact, there are few prospective cohort studies specifically devoted to the topic, and most of the existing knowledge comes from brief records and cross-sectional or case–control studies.

In the last decade, there has been an increased interest in the vascular endothelium as model of a tissue that bears intrinsic antiplatelet, vasodilatory and atheroprotective properties, mainly through release of active or signaling agents (Bonetti et al., 2003; Strijdom et al., 2009). In this context, nitric oxide (NOx) produced by the vascular endothelial cells is a key mediator of vascular physiology due to its prominent vasodilator activity (Schulz et al., 2008; Otani, 2009). As a factor

Abbreviations: AMI, acute myocardial infarction; ANCOVA, analysis of covariance; BMI, body mass index; CK-MB, creatine kinase isoenzyme MB; C NMR, cardiac nuclear magnetic resonance; CRP, C-reactive protein; CVD, cardiovascular diseases; eNOS, endothelial nitric oxide synthase; FMD, flow-mediated dilation; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; MI, myocardial infarction; NMD, nitrate-mediated vasodilation; NOx, nitric oxide; PCI, percutaneous coronary interventions; SD, standard deviation; SPSS, Statistical Package for Social Sciences.

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with short half-life, the availability of peripheral NOx is mainly provided by the post-transcriptionally regulated endothelial nitric oxide synthase (eNOS).

Variations in expression and activity of eNOS are potential candidates to explain, at least in part, the pathophysiology of cardiovascular diseases (Dietz et al., 1997). Studies in population genetics have demonstrated an important relationship between the G894T polymorphism at exon 7 of the eNOS gene (also known as the Glu298Asp variation, or rs1799983) with the occurrence of AMI (Zigra et al., 2013; Luo et al., 2014). It was described that this variation does not alter functional properties of the isoenzymes under physiological conditions, with equivalent intracellular specific activity and cytolocalization (McDonald et al., 2004). Nonetheless, the variation appears to produce different transcriptional rates (with post-transcriptional implications) when the alleles are submitted to stressing conditions, with the substitution of Glu298 (GAG) for Asp (GAT) yielding enhanced eNOS expression (Joshi et al., 2007).

Nonetheless, some controversy on the risky allele for cardiovascular disorders remains (Andrikopoulos et al., 2008), and this inconsistency could be attributed to environmental factors, allelic architectures, interaction among genes and variability in clinical phenotypes, what may reflect in differences in morbidity and mortality among genetically homogeneous groups (Dipple and McCabe, 2000; Wang and Wang, 2000; Dias et al., 2009). This scenario of heterogeneity and inconsistency in the results produced so far represents an open field for conduction of studies that seek developing a prospective follow-up of post-AMI patients as well as to perform statistically and experimentally controlled analyses on variables of alleged influence on the clinical course of the post-AMI.

The aim of the study was to investigate the association of allelic variants produced by the Glu298Asp substitution in the eNOS gene with clinical and biochemical variables representative of the post-infarction period.

2. Material and methods

2.1. Patients

The sample consisted of participants in the ongoing Brasilia Heart Study Group (since May 2006) which follows consecutive cases of MI patients admitted with ST-segment elevation and treated at the emergency service of the *Hospital de Base do Distrito Federal* for a minimum period of 1 month by a multiprofessional team. For our analyses, inclusion criteria were as follows: (i) less than 24 h after the onset of AMI symptoms, (ii) ST segment elevation of at least 1 mm (frontal plane) or 2 mm (horizontal plane) in two contiguous leads, and (iii) evidence of myocardial necrosis by increased creatine kinase isoenzyme MB (CK-MB) and troponin above the reference limit (25 U/L and 0.04 ng/mL, respectively), with any cognitive impairment that hampers verbal response to medical queries as the main exclusion criterion. Additional exclusion criteria were neoplastic disease, chronic renal failure on dialysis, severe liver disease or heart failure (based on clinical and radiological evaluation). Genotypes of the rs1799983 SNP were taken into account as an inclusion or exclusion factor.

The project from which this study is part of was approved by the Ethics Committee in Research of the State Department of Health of the Brazilian Federal District, official letters 082/2006 and 354/2011. All participants signed an informed consent.

2.2. Anthropometric, clinical and biochemical analyses

In the first 24 h after the onset of the AMI symptoms, the body mass index (BMI, kg/m^2) and waist circumference (WC, cm) were assessed, along with information on the subject's history of diabetes, hypertension, exercise, smoking and previous AMI as well as family history of coronary disease. To assist these assessments, blood samples were taken after a 12 h fasting period for specific measurements of glycated hemoglobin, glucose, triglycerides, HDL cholesterol (HDL-C), LDL-cholesterol (LDL-C), creatinine and creatine kinase MB fraction (CK-MB). Hypertension was defined as a repeatedly elevated blood pressure exceeding 140 (systolic) and/or 90 (diastolic) mm Hg during in-hospital treatment or regular use of drugs to treat hypertension prior to AMI. Definition of type 2 diabetes included self-reported previous diagnosis of the condition and/or fasting glycosylated hemoglobin (Hb1Ac) $\geq$6.5%, following guidelines from the American Diabetes Association (ADA, 2005) as existent at the study's onset. Sedentary were those who did not practice physical activities for more than 30 min over at least four days a week (DHHS, 2009), whereas smokers were those who reported consumption of more than 100 cigarettes over the lifetime (Backinger et al., 2008). Dyslipidemic was rendered the patient with any of the following lipid profiles: triglycerides $\geq$ 150 mg/dL, LDL-C $\geq$ 130 mg/dL and/or HDL-C < 40 mg/dL. The MDRD formula was used to determine the estimated glomerular filtration rate (eGFR).

On the fifth day post-AMI, new fasting sampling was performed and the specific measurements consisted of blood glucose and of lipid profile (triglycerides, HDL-C and LDL-C). Also on the fifth day post-AMI, medical records were revised to assess in-hospital parameters of clinical evolution for each patient, enclosing the Killip–Kimball classification score, the medications used in the period and occurrence of thrombosis and/or percutaneous coronary interventions (PCI). If the patient evolved to death within 30 days from the infarct, his/her data would be deleted from the database for this analysis. All biochemical analyses were performed in the same clinical laboratory certified by the Accreditation Program of Clinical Laboratories of the Brazilian Society of Clinical Pathology.

2.3. Glu298Asp polymorphism analysis of the eNOS gene

Buffy coat samples were obtained at admission and stored at −80 °C. DNA purification used QIAamp DNA Mini Extraction Kit (Qiagen, Brazil). Analyses of the rs1799983 SNP (the Glu298Asp variation) of the eNOS gene were performed by the polymerase chain reaction followed by specific enzymatic restriction (PCR-RFLP), based on the protocol described elsewhere (Pulkkinen et al., 2000) and adapted for this study with primers eNOS-F1 (5′-AAGGCAGGAGACAGTGGATG GA-3′) and eNOS-R1 (5′-CCCAGTCAATCCCTTTGGTGCTCA-3′). Each PCR reaction (25 μl) was composed of 100 ng of DNA, 10 mM of Tris–HCl (pH 8.8), 25 mM of KCl, 1.5 mM of $MgCl_2$, 0.2 mM of each primer, 0.2 μM of each dNTP, 0.25 mg/mL of ovalbumin and 0.5 U of Taq polymerase (Phoneutria, Minas Gerais, Brazil). After 1 min of hot start at 80 °C, the amplification program was composed of an initial denaturation step at 96 °C for 2 min, followed by 35 cycles of denaturation at 96 °C for 30 s; annealing at 56 °C for 1 min, extension at 72 °C for 1 min, and a final extension step at 72 °C for 5 min in an PE GeneAmp 9700 thermocycler (Applied Biosystems, CA, USA). Products (248 base pairs) were subjected to enzymatic digestion (37 °C, overnight) with the *Mbo*I restriction endonuclease (Jena Bioscience, Germany) in a total volume of 15 μL and using 0.5 U. Products were subjected to electrophoresis on 2.2% agarose gel, with carriers of the T-allele identified by two fragments (190 and 58 base pairs) and G-carriers by an intact product. Every sample was analyzed twice at least, and further checked if the genotypes indicated by the first two productive reactions were in conflict. Productive, template-related amplification was confirmed by use of a blank (negative) control within each set of reactions.

2.4. Measurement of total NOx/nitrite/nitrate

For quantification of total serum levels of nitric oxide mediator (NOx), an enzymatic assay was employed based on the conversion of nitrate to nitrite (NO^{2-}) by the action of nitrate reductase followed by colorimetric detection by the Griess diazotization reaction, according to the manufacturer's instructions (R&D Systems Inc., MN, USA). The readings