Clinical Biochemistry 46 (2013) 335–340

Contents lists available at SciVerse ScienceDirect

Clinical Biochemistry

journal homepage: www.elsevier.com/locate/clinbiochem

The Ala16Val MnSOD gene polymorphism modulates oxidative response to exercise

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ARTICLE INFO

Article history:
Received 4 June 2012
Received in revised form 20 November 2012
Accepted 22 November 2012
Available online 5 December 2012

Keywords:
MnSOD polymorphism
Gene expression
Enzyme activity
Redox balance
Physical exercise

ABSTRACT

Objectives: In humans, the manganese-superoxide dismutase (MnSOD) gene contains a polymorphism (Ala16Val) that has been related to several metabolic dysfunctions and chronic diseases. However, the obtained results suggest that risks related to this polymorphism are directly influenced by environmental factors. Because few studies have analyzed this possible influence, we performed a controlled study to evaluate if the oxidative stress caused by exercise is differentially modulated by the Ala16Val MnSOD polymorphism.

Design and methods: Fifty-seven males were previously genotyped and 10 subjects per genotype were selected to perform a bout of controlled intense exercise. MnSOD mRNA expression, protein content, enzyme activity, and total glutathione and thiol content from peripheral blood mononuclear cells were evaluated before and 1 h after a bout of intense exercise.

Results: The AA genotype participants showed increased post-exercise MnSOD mRNA expression and enzyme activity compared to baseline values. Conversely, MnSOD mRNA expression did not change but protein thiol content decreased significantly after the bout of exercise in VV carriers. A comparison of the genotypes showed that the AA genotype presented a higher MnSOD protein content than VV volunteers after exercise; while a dose-effect for the A allele was found for enzyme activity.

Conclusion: This study supports recent evidence that genotypes of key antioxidant enzymes may be associated with differential oxidative stress modulation and the hypothesis that the risk of disease associated with the MnSOD Ala16Val gene polymorphism may be controlled by environmental factors.

1. Introduction

Cells express a nucleus-encoded, mitochondrial localized manganese-superoxide dismutase (MnSOD) to protect mitochondria from superoxide (O_2^-)-mediated damage. Previous studies confirmed that MnSOD is essential for life as illustrated by its neonatal lethality in knockout mice [1,2]. A single nucleotide polymorphism (SNP) has been identified in the MnSOD encoding gene, with genetic variants found to contain a structural mutation of a thiamine (T) substituted for a cytosine (C) in the exon 2. The substitution affects the 16th codon, mutating a valine (GTT) into an alanine (GCT) (Ala16Val). This protein modification produces a β-sheet secondary structure instead of the expected α-helix structure [3]. The Ala16Val polymorphism has been associated with several diseases, such as breast and prostate cancers and cardiovascular dysfunctions, and risk factors, such as hypercholesterolemia [4–8].

However, the obtained results are still contradictory, suggesting environmental influences on risks of disease related to the Ala16Val MnSOD polymorphism [9], and investigations with controlled conditions of environmental variables and this polymorphism are still incipient. Because intense or unaccustomed physical exercise increases oxygen consumption, enhances reactive oxidative species (ROS) production and thus causes oxidative stress [10,11], exercise may be an environmental variable used as an *in vivo* model to investigate acute oxidative stress effects on humans [10,12].

The relevance in the analysis of an exercise effect is based on evidence that shows that mitochondria are particularly susceptible to oxidative damage from O_2^- generated by metabolic pathways activated during exercise [10,13]. Because ROS are reported to induce endogenous antioxidant enzymes [14], exercise-related ROS production is a reliable method to study the molecular pathways related to the MnSOD modulation during oxidative stress.

Therefore, we analyzed the influence of the MnSOD Ala16Val gene polymorphism on gene expression, protein content, enzyme activity, and the redox status of biochemical markers after a controlled bout of intense exercise in young men.

2. Methods

2.1. Participants and experimental design

Fifty-seven males (20.6 ± 1.8 yr) participated in the study, which was approved by the Ethics Committee of the University of León and carried out according to the Declaration of Helsinki. Written inform consent to enroll in the study was then obtained from participants

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0009-9120/$ – see front matter
http://dx.doi.org/10.1016/j.clinbiochem.2012.11.020

who were not taking inflammatory, hormonal, or antioxidant supplements.

After obtaining a blood sample for MnSOD Ala16Val genotyping and full medical screening, the participants were weighed, had their height measured, and had their body fat calculated according to a previously described equation [15]. A VO_2max test until exhaustion was carried out on a cycle ergometer with electromagnetic brakes (Ergoline, GmH-Lindestrable, Bitz, Germany). Participants warmed up for 5 min with 25 W workload. After, the workload was increased by 25 W/min^{-1} until exhaustion. Achievement of VO_2max was considered to be the attainment of at least two of the following criteria: (a) participants' volitional exhaustion, (b) a *plateau* in oxygen consumption (VO_2), or (c) a heart rate (HR) ±10 beats min^{-1} of age-predicted maximal HR (Polar S810, Polar Electro OY, Kempele, Finland). Ventilatory and gas exchange variables were continuously monitored and collected breath-by-breath using an automated and open-circuit system (CPX plus, Medical Graphics, St. Paul, MN, USA). The data were averaged every 5 s, and the metabolic cart was calibrated with calibration gas mixtures of known O_2 and CO_2 concentrations (accuracy 0.01%) as provided by the manufacturer (Medical Graphics, St. Paul, MN, USA). The highest VO_2 obtained during the incremental exercise test was taken as the VO_2max whereas the intensity obtained just before exhaustion is referred to as peak power (W_{max}).

A bout of intense exercise was performed two weeks after the all-out test. Participants had to maintain a pedaling rate of 70 r·min^{-1} for 40 min at 75% of the VO_2max obtained on the previous test. The W_{max} at 75% was adjusted by extrapolation depending on the VO_2max obtained during the all-out test. The VO_2 was registered along the bout of intense exercise: 1) during the first 5 min; 2) from 10 to 15 min; 3) 20 to 25 min; and 4) 30 min until the end of the test. The HR was continuously monitored along the bout of exercise. Blood samples were obtained immediately before and 1 h after the exercise bout. The participants consumed 500 mL of commercial mineral water between blood sampling to avoid dehydration. A small amount of blood sample was used as a hematocrit measurement (Haemofuge A, Heraeus Sepatech, Germany). The VO_2, HR, and W were measured using the same devices along the whole study.

2.2. MnSOD Ala16Val genotyping

A peripheral blood sample (5 mL) was drawn from the antecubital vein using EDTA as anticoagulant. Genomic DNA was isolated from leukocytes using a GFX Genomic Blood DNA Purification Kit (Amersham Biosciences Inc., Uppsala, Sweden), and MnSOD genotyping was performed according to Gottlieb et al. [6].

2.3. Dietary intake

A 24 h self-administered dietary recall was used to assess food intake prior to the bout of intense exercise. The food composition present in the records was calculated using the Nutriber® software, version 1.1.3 (Spain), and the values are presented as the mean ± standard error of means (SEM).

2.4. Analytical procedures

Blood samples (35 mL) were obtained from the antecubital vein and immediately centrifuged at 1500 ×*g* for 10 min at 4 °C. The peripheral blood mononuclear cells (PBMC) were separated as previously described [17] and used since they are reliable cells to investigate exercise-related oxidative stress and inflammation [16,17].

Total RNA was isolated from PBMCs by using a RiboPure™ Blood Kit (Ambion Inc., Austin, TX, USA) and quantified by the fluorescent method Ribogreen RNA Quantification Kit (Molecular Probes, Leiden, The Netherlands) as described elsewhere [18]. TaqMan primers and probes for MnSOD (Genbank M36693.1 and Hs00167309) and 18S (housekeeping gene) rRNA (Genbank X03205.1 and Hs 99999901_s1) were designed from the commercially available TaqMan® Assays-on-Demand Gene (Applied Biosystems, Foster City, CA, USA). Relative changes in gene expression levels were determined using the $2^{-\Delta\Delta ct}$ method as described previously [19].

For Western blot analysis, PBMCs were homogenized with 150 μl of 0.25 mM sucrose, 1 mM EDTA, 10 mM Tris and a protease inhibitor cocktail [20]. Samples containing 50 μg of protein were separated by SDS-polyacrylamide gel electrophoresis (9% acrylamide) and transferred to PVDF membranes. Non-specific binding was blocked by preincubation of the PVDF in PBS containing 5% bovine serum albumin for 1 h. The membranes were then incubated overnight at 4 °C with appropriate antibodies. Bound primary antibody was detected using a peroxidase-conjugated secondary antibody (Amersham Inc., Piscataway, NJ, USA). The blot was stripped in 6.25 mM Tris, pH 6.7, 2% SDS and 100 mM β-mercaptoethanol at 50 °C for 15 min and probed again with anti-beta-actin antibodies (Sigma-Aldrich, St. Louis, MO, USA) (42 kDa) to verify equal protein loading in each lane.

MnSOD activity was determined in PBMCs by following the MnSOD inhibition of the reaction of O_2^- with nitroblue tetrazolium (NBT) as previously described [21] and expressed as U/mg protein. The automated glutathione recycling method described elsewhere [22] was used to assess total glutathione content (tGSH) using a microtiter plate reader (Synergy™ HT Multi-Mode Microplate Reader, Bio-Tek Instruments Inc., Winooski, VT, USA). The protein thiol content (−SH) was analyzed with a spectrophotometric method adapted for use on a 96-well plate reader [23]. Total glutathione is expressed as μmol GSH/mg protein and -SH groups are expressed as nmol SH/mg protein.

2.5. Statistical analysis

Data were expressed as the mean ± SEM. Allele frequencies were estimated using the gene-counting method. Chi square analysis was used to estimate the Hardy–Weinberg equilibrium. The results for RT-PCR and western blotting were presented as percentages from baseline values. Student's *t*-test was used to determine significant differences between the mean for the individual response to the bout of exercise. A one-way analysis of variance (ANOVA) with the factor genotype was also used. Bonferroni *post hoc* analysis was applied where appropriate. SPSS 15.0 (Statistical Package for Social Sciences, Chicago, IL, USA) was used, and statistical significance was set at $P<0.05$.

3. Results

The genotype and allelic frequencies of the initial samples were calculated, and 25% AA ($n=14$), 33% VV ($n=19$), and 42% AV ($n=24$) were observed. Calculated allelic frequencies of the A and V alleles were of 0.456 and 0.544, respectively. The gene frequencies were all in Hardy-Weinberg equilibrium for the groups investigated ($P=0.273$), indicating the samples were homogenous, and avoiding possible genetic variations on the measurements performed.

Table 1 presents results from the anthropometric and functional parameters, and Table 2 depicts food intake data. No differences between genotypes were observed. VO_2 data and respiratory quotient did not significantly differ among genotypes during exercise (Table 3). Mean and maximal HR assessed during the bout of exercise and baseline and post-exercise hematocrit levels were also similar among genotypes (Table 4).

Fig. 1 depicts MnSOD mRNA levels, protein content and activity for each genotype. Heterozygous and VV homozygous participants did not show significant changes in mRNA expression from baseline to post-exercise values. The AA homozygous participants registered a significant increase in mRNA gene expression after the bout of intense exercise ($P=0.038$). No significant differences were found between genotypes. The AV, AA, and VV genotypes showed no differences between baseline and post-exercise values for MnSOD protein content.