Endothelial nitric oxide synthase g894t (rs1799983) gene polymorphism in Polish athletes

Research Article

Jerzy Eider¹, Krzysztof Ficek¹, Mariusz Kaczmarczyk², Agnieszka Maciejewska-Karłowska¹, Marek Sawczuk¹, Paweł Cięszczyk¹,³ *

¹University of Szczecin, Department of Physical Culture and Health Promotion, 71-065 Szczecin, Poland
²Pomeranian Medical University, Department of Clinical and Molecular Biochemistry, 70-111 Szczecin, Poland
³Gdansk University of Physical Education and Sport, Department of Health Promotion, Faculty of Tourism and Recreation, 80-336 Gdansk, Poland

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Abstract: The NOS3 gene has been associated with athletic endurance performance and elite power athletic status. With respect to NOS3 G894T and its relation to athletic performance or status, results across various studies have not been consistent. Therefore, the lack of consistency among previous studies prompted us to design a case-control study in a Polish Caucasian population to examine the relationship between the NOS3 G894T polymorphism and athletes’ status, i.e. type and intensity of exercise performed (power-oriented, “mixed” power/endurance activity, endurance-oriented) and the possible association between the G894T variant and athletic performance. The case-control study was performed in a group of 360 Polish athletes (cases) of the highest nationally competitive standard (male n = 156 and female n = 67) and 191 unrelated, sedentary control subjects. The G894T genotype and allele distributions differed significantly between power-oriented (P = 0.009, P = 0.003), “mixed” (P = 0.021, P = 0.009), endurance (P = 0.043, P = 0.014) athletes when compared to control subjects (P values for genotypes and alleles, respectively). There were no significant differences between elite and sub-elite athletes in any group. The over-representation of the GG genotype and G allele in all athletes suggests that the G894 allele may favour all types of sports, however, the strongest predisposition was seen among power-oriented athletes.

Keywords: Nitric oxide • Genotype • Genetic Variation • Athletes • Physical Fitness

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1. Introduction

Nitric oxide (NO) is a gaseous free radical that serves as a multifunctional messenger [1]. NO mediates crucial features of neuronal communication, blood vessel modulation and immune response [2]. It is a factor in common pathological conditions such as hypertension and atherosclerosis [3]. Nitric oxide is generated from arginine by a family of three distinct calmodulin-dependent nitric oxide synthase (NOS) enzymes (isoforms): endothelial (eNOS), neuronal (nNOS, or brain bNOS) [4] and inducible (iNOS) [2] and each of the isoforms is encoded by distinct genes on different chromosomes, NOS3, NOS1, and NOS2, respectively [5].

Blood flow is essential for the delivery of nutrients, maintenance of fluid balance, and the removal of metabolites; the delivery of blood flow to skeletal muscle is, therefore, a prerequisite for maintaining physical activity [6,7]. Nitric oxide has been shown to be one of the most important intrinsic factors in regulating basal vascular tone, a balance between constrictor and dilator influences, however, the results regarding the increase in muscle blood flow with exercise (hyperamia) are less concordant [8]. Moreover, NO has been shown to exert several other distinct effects on various aspects

* E-mail: cieszychczyk@poczta.onet.pl
of skeletal muscle structure and function, such as excitation-contraction coupling [9], skeletal muscle fiber type conversion (faster-to-slower fiber type) [10], mitochondrial energy production [11], glucose metabolism [12], and autoregulation of blood flow [13].

Two NOS isoforms have been identified in skeletal muscle: nNOS and eNOS [14]. The primary NOS isoform found in skeletal muscle is nNOS, specifically, the splice variant of neuronal NOS, termed nNOS mu [13,15], whereas eNOS is mainly present in endothelial cells [5]. Animal studies have demonstrated that both eNOS and nNOS contribute to control of vascular tone, however, the relative contributions of eNOS- and nNOS-derived NO to exercise hyperemia remain unclear [8].

Several polymorphic sites have been identified within the NOS3 gene, including single nucleotide polymorphisms: T-786C (rs2070744), G894T (Glu298Asp, rs1799983), along with sequence repeat polymorphisms: 27 base pairs (bp) repeat in intron 4 (4A/4B) (intron 4 VNTR), microsatellite (CA) repeats in intron 13 [16], and others. Although some controversy exists, there is a growing body of evidence in favour of a functional effect of T-786C, G894T and intron 4 VNTR [17-20]. Given the potential role of nitric oxide in regulating tolerance to physical exercise and recovery mechanisms, these NOS3 genetic variants have been tested and/or associated with various training or exercise response phenotypes, such as: cardiovascular hemodynamics traits, e.g. blood pressure [21,22] and heart rate responses [23]; cardio-biochemical parameters [24]; adaptation to parasympathetic modulation and the level of oxidative stress induced by aerobic exercise training [21,25]; vascular reactivity [26,27], and exercise-induced adaptation to hypoxia [28].

It has been suggested that an increase in NO production may facilitate oxygen and nutrients delivery to working muscles, thereby improving exercise tolerance and recovery [29]. Hence, the NOS3 gene, due to its central role in the production of NO in the vascular endothelium [6], became a candidate gene for association with athlete status or athletic performance. Indeed, NOS3 has been associated with endurance performance, endurance elite status [8,30], power elite athlete status [31,32] and elite soccer player’s status [33], being an example of “mixed” exercise phenotype requiring both endurance and power abilities.

With respect to NOS3 G894T and its relation to athletic performance and/or athletes’ status, results across various studies have been inconsistent. Saunders et al. [30] reported an excess of the G894 allele (combined with a DKRB2 -9/-9 genotype) in the fastest finishing Caucasian Ironman triathletes when compared with control subjects. However, Wolfarth et al. [6] in the Genathlete study found no difference in allele and genotype frequencies of the G894T between elite endurance athletes and controls. Cieszczyk et al. [34] found no evidence of association between G894T and endurance performance in elite Polish rowers. Likewise, no differences in allele and genotype frequencies of the Glu298Asp were found by Buxens et al. [35] between elite Spanish Caucasian elite endurance and power-oriented athletes.

The lack of consistency among the aforementioned studies prompted us to design a case-control association study in a Polish Caucasian population to examine, firstly, the relationship between the NOS3 G894T polymorphism and athletes’ status and secondly, the possible association between the G894T variant and athletic performance.

2. Experimental Procedures

2.1 Subjects

The study was carried out in a group of 360 Polish athletes (male n=273 and female n=87) of the highest competitive standard. The group of athletes was comprised of three subgroups: endurance (END), power-oriented (PWR) and “mixed” athletes (MXD). Endurance athletes (n=114) were characterized by predominantly aerobic energy production (duration of exertion over 5 minutes, intensity of exertion moderate to high) and included triathletes (n=18), race walkers (n=6), road cyclists (n=20), marathon runners (n=12), 0.8-10 km runners (n=35) and 800-1500 m swimmers (n=23). Power-oriented athletes (n=116) were characterized by predominantly anaerobic energy production (duration of exertion < 1 minute, intensity of exertion submaximal to maximal), including 100-400 m runners (n=36), powerlifters (n=36), weightlifters (n=20), throwers (n=8) and jumpers (n=16). In the third subgroup (n=130) there were athletes with mixed aerobic and anaerobic energy production (football players=44, wrestlers=26, boxers=23, judokas n=19, fencers n=18).

Among 360 athletes, 168 were classified as elite, 32 of whom were top-elite (gold medallists in the World and European Championships, World Cups or Olympic Games) and 136 who were silver or bronze medallists in the World and European Championships, World Cups or Olympic Games. The others (n=192) were classified as sub-elite (participants in international competitions). Control group (C) consisted of 191 (male n=124 and female n=67) unrelated, sedentary volunteers (students of the University of Szczecin, aged 19–23).
All athletes and controls were Caucasian to minimize the risk of population bias and stratification.

The procedures followed in the study were conducted in accordance with the principles of the World Medical Association Declaration of Helsinki and ethical standards in sport and exercise science research. All procedures were approved by the Pomeranian Medical University Ethics Committee and all participants gave informed consent to genotyping with the understanding that it was anonymous and obtained results would be confidential.

Various methods were used to obtain the samples, including: targeting national teams and providing information to national coaching staffs and athletes attending training camps.

2.2 Procedures

The buccal cells donated by the subjects were collected in Resuspension Solution (Sigma, Germany) with the use of Sterile Foam Tipped Applicators (Puritan, USA). DNA was extracted from the buccal cells using GenElute Mammalian Genomic DNA Miniprep Kit (Sigma, Germany) according to the manufacturer’s protocol. For the selected samples, the presence of extracted DNA was confirmed by electrophoresis.

Genotyping

Genotypes of the NOS3 G894T polymorphism (rs1799983) were determined by polymerase chain reaction (PCR) followed by digestion with Ban II. The PCR was performed in standard buffer and each 20 µL PCR test contained 100 ng genomic DNA, 0.2 mmol L⁻¹ each primer, 200 mmol L⁻¹ each dNTPs and 0.5 U Taq polymerase. The reactions were incubated at 94°C for 3 min, 60°C for 1 min and 72°C for 1 min, followed by 35 cycles of 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 45 s and finally one cycle of 72°C for 10 min. The PCR product was digested with 5 U (10 U µL⁻¹) of Ban II at 37°C for 4 h. The resulting fragments were separated on 2.5% acrylamide gel and visualized under UV light after ethidium bromide staining.

2.3 Statistical analyses

Any differences in genotype and allele frequency were analyzed using Chi² tests. Odds ratios (OR) with 95% confidence intervals (95% CI) were calculated. All calculations were performed using STATISTICA (StatSoft, Inc. (2011). STATISTICA (data analysis software system), version 10. www.statsoft.com.), except Hardy-Weinberg equilibrium, and FDR (False Discovery Rate) adjusted P values (P_adj), which were tested or obtained with the programming language and environment R (http://www.r-project.org) using “genetics” and “fdrtool” packages, respectively. P values <0.05 were considered statistically significant.

3. Results

The G894T genotype distribution met Hardy-Weinberg proportions amongst all athletes: power-oriented (PWR), P=0.586; mixed endurance/power activity (MXD), P=0.338; endurance-oriented (END), P=0.802; as well as amongst sedentary controls (C), P=0.746.

The genotype distribution and allele frequencies amongst the whole cohort (n=360): 60.3% GG, 33.6% GT, 6.1% TT, the G allele frequency 77.1%, differed significantly from control subjects (n=191): 43.5% GG, 46.1% GT, 10.5% TT, the G allele frequency 66.5%; P=0.0006 (Chi²=14.7, df=2), P=0.0002 (Chi²=14.3, df=1), for genotype and allele frequencies respectively.

When analysed separately according to athlete status, the G894T genotype and allele distribution in all athlete groups: PWR, MXD, END, also differed significantly from those in control subjects (Table 1).

<table>
<thead>
<tr>
<th></th>
<th>Power (PWR) (n=116)</th>
<th>“Mixed” (MXD) (n=130)</th>
<th>Endurance (END) (n=114)</th>
<th>Controls (C) (n=191)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG genotype, % (n)</td>
<td>62.9 (73)</td>
<td>60.0 (78)</td>
<td>57.9 (66)</td>
<td>43.5 (83)</td>
</tr>
<tr>
<td>GT genotype, % (n)</td>
<td>31.9 (37)</td>
<td>33.1 (43)</td>
<td>36.0 (41)</td>
<td>46.1 (88)</td>
</tr>
<tr>
<td>TT genotype, % (n)</td>
<td>5.2 (6)</td>
<td>6.9 (9)</td>
<td>6.1 (7)</td>
<td>10.5 (20)</td>
</tr>
<tr>
<td>P</td>
<td>0.003 (0.009*)</td>
<td>0.014 (0.021*)</td>
<td>0.043 (0.043*)</td>
<td>---</td>
</tr>
<tr>
<td>G allele, % (n)</td>
<td>78.9 (183)</td>
<td>76.5 (199)</td>
<td>75.9 (173)</td>
<td>66.5 (254)</td>
</tr>
<tr>
<td>T allele, % (n)</td>
<td>21.1 (49)</td>
<td>23.5 (61)</td>
<td>24.1 (55)</td>
<td>33.5 (128)</td>
</tr>
<tr>
<td>P</td>
<td>0.001 (0.003*)</td>
<td>0.006 (0.009*)</td>
<td>0.014 (0.014*)</td>
<td>---</td>
</tr>
</tbody>
</table>

Table 1. Genotype distribution as well as allele frequencies of the G894T polymorphism in Polish athletes and control group. Comparisons with controls.

* the FDR adjusted P values; FDR: False Discovery Rate
Owing to the low number of TT homozygotes (n=6, n=9, n=7, for PWR, MXD, END groups respectively) we combined homozygotes and heterozygotes for the minor allele T together (TT+GT) and compared them with homozygotes GG (dominant mode of inheritance for minor allele). In general, the GG homozygotes were significantly over-represented in athletes, when each group of athletes was compared with sedentary controls. Specifically, when compared with the GG genotype, the chance of being a power-oriented athlete was 2.21 times higher (95% confidence interval, 1.34-3.65, P=0.0009, P\textsubscript{adj}=0.0027), the chance of being a mixed power/endurance athlete was 1.95 times higher (95% confidence intervals, 1.21-3.15, P=0.004, P\textsubscript{adj}=0.006), and the chance of being an endurance athlete was 1.09 times higher (95% confidence intervals, 1.09-2.94, P=0.014, P\textsubscript{adj}=0.014) than for the carriers of T variant allele (TT+GT). We also found a linear trend – decreasing frequency of G allele (78.9%, 76.5%, 75.9%, for PWR, MXD, END groups respectively) parallel to the increasing aerobic component of physical performance – though it was not significant (Chi\textsuperscript{2}=0.588, P=0.443 for linear trend). Next, each group of athletes (PWR, MXD, END) was stratified by level of competition into elite and sub-elite categories. The distribution of the G allele in power-oriented, mixed activity athletes, and endurance athletes with respect to elite status in comparison with control subjects is presented in Figure 1.

There were no significant differences between elite and sub-elite athletes within the power-oriented group (79.3% vs. 78.5%, P=0.872), mixed power/endurance group (77.9% vs. 75.0%, P=0.588) or endurance-oriented athletes (77.5% vs. 75.0%, P=0.674).

4. Discussion

The main finding of the present study is a significant over-representation of the GG genotype and the G894 allele irrespective of athletes’ status, i.e. type and intensity of exercise performed (power-oriented, endurance-oriented, or “mixed”). Also, the genotype and allele frequencies differed significantly between the whole athlete cohort and sedentary control subjects. This suggests that the G894T polymorphism may be associated, at least in the Polish population, with overall physical fitness and physical ability, no matter what type of sports activity the athletes are involved in. Additionally, there was a trend, although not significant, towards an increase in both the GG genotype and the G allele frequency in relation to a smaller aerobic component of physical ability. This suggest that the G allele promotes power-oriented sport events. Several other common genetic variants have been described in Polish athletes including insertion/deletion polymorphism in the ACE gene [36] or C1780T in the alpha-2 adrenergic receptor (ADRA2A) gene [37].

Previous studies provided inconsistent results on the association between the G894T NOS3 polymorphism and athlete status or athletic performance. Some case-
control studies have failed to find any association between this variant and endurance athlete status [6,34,35]. In the Geneathlete cohort, Wolfarth et al. [6] did not find any difference in allele frequency and genotype distribution of G894T polymorphism between 316 male Caucasian endurance athletes and 299 sedentary male control subjects. In contrast, Saunders et al. found a significant association of this variant with endurance performance in triathletes [30]. There were no significant differences in the frequencies of the genotype and allele distributions of the NOS3 gene G894T polymorphism between groups of triathletes classified according to finishing time into fastest (n=148), middle (n=147), slowest (n=148) and control subjects (n=203), however, the triathletes with a GG NOS3 genotype were significantly slower in the swimming and cycling phases than those possessing a 894T allele [30]. In contrast to Saunders et al. [30], we did not find an association of G894T with athletic performance. There were no differences in the G894T allele frequencies in athletes stratified by the level of competitiveness (elite versus sub-elite) regardless of the athletes’ status (power, “mixed” and endurance). Ahmetov et al. [38] suggested that the difference in allelic frequencies between athletes within the same event category (for example strength/power sports), but with different levels of achievements may be explained by selection pressure. Specifically, the athletes possessing a “desired” allele or its combination are more likely to become elite. Our results indicate a lack of selection pressure associated with the G allele of the G894T polymorphism, because the athletes carrying the G allele were not favoured and were not more likely to become elite, which contrasts with the findings reported by Saunders et al. [30]. However, it should be emphasised, that the athletes’ competitiveness was defined differently. We used the classification proposed by Druzhuzevskaya et al. [39] which is based on the individual achievements in national and international competitions, while Saunders et al. used a cross-sectional approach with finishing time as a phenotype [30]. This might explain the discordance in results between the two studies.

In addition, Saunders et al. [30] observed a significant linear trend of increasing frequency of the GG genotypes among triathlon finishers from the fastest, through the middle, to the slowest. One might speculate that those triathletes with the slowest finishing time were the least predisposed to endurance disciplines. In this regard, the results of Saunders et al. are in broad agreement with our results. Naturally power-oriented athletes, according to a “trade-off” hypothesis [40], are the least predisposed to endurance-oriented activities. Therefore, the finding of an increasing frequency of the GG genotype among triathlon finishers from the fastest, through the middle, to the slowest, seems to be, at least in part, supported by our study, where we found a linear trend of increasing frequency of the GG genotype among athletes from endurance-oriented, through “mixed”, to power-oriented groups.

Although we found significant differences in the frequencies of the genotype and allele distributions of the NOS3 gene G894T polymorphism when all three athlete groups (power-oriented, endurance-oriented, and “mixed”) were compared to controls, the predisposition was the strongest among power-oriented athletes. In support of this, for carriers of the GG genotype, the lowest OR (1.09) was seen in endurance athletes, intermediate (1.95) in mixed power/endurance oriented athletes, and the highest in the power-oriented athlete group (2.21).

In fact, to our knowledge, the present study is the first showing an association between the G894T NOS3 polymorphism and a predisposition to power-oriented sports. Although a controversy exists over whether or not the G894T polymorphism is functional [17,41], Wang et al. [17] showed that eNOS enzyme activity increased with the presence of the G allele. Hence, one may hypothesise that the predisposition to strength/power sports may be dependent on enhanced nitric oxide bioavailability. This hypothesis seems to be supported, indirectly, in two studies by Gomez-Gallego et al. [31] and Sessa et al. [32], who found a significant association between another NOS3 variant, an intronic polymorphism T-786C, and elite performance in strength/power sports [31]. The T-786 allele, associated with both higher mRNA and eNOS protein concentrations [17], was significantly more common in power-oriented athletes when compared with endurance athletes as well as sedentary control subjects [31]. It is worth noting, that those two NOS3 polymorphism, T-786C and G894T, in several studies [42-44], but not in all [17], were found to be in linkage disequilibrium. Indeed, in Caucasians, the most common haplotype is T-786/ G894 [44], reflecting the high degree of linkage between the T-786 and G894 alleles. Nonetheless, it would be wrong to draw reasonable conclusions from the linkage between two markers only. In fact, Buxens et al. [35] obtained discordant results comparing the T-786C and G894T genotypes between elite endurance and elite power-oriented athletes.

As mentioned previously, according to a “trade-off” hypothesis [40], athletes would be predisposed to either sprint/power or endurance events, not to both. In this context, the over-representation of the GG genotype and the G894 allele in all athletes irrespective of their classification according to athletes’ status, seems to
resulting from the subdivision according to elite status. Hence, we can not completely exclude the effect of the G894T polymorphism on athletic performance. Although we have taken care to eliminate possible confounding factors, such as population stratification, which is a serious concern, especially in case-control association studies [51], this may be simply unavoidable. Berger et al. showed that population substructures can be detected even in a seemingly homogenous population [52]. The effect of population admixture has been shown in the study by Saunders et al. A significant difference in the GG genotype distribution between fast and slow triathletes appeared when only South-African-born athletes were considered [30]. Our athletes were all Caucasians, and came from a relatively small geographic area, yet the ethnic admixture can not be completely ruled out.

In conclusion, we have shown that the variation in the NOS3 gene is associated with athlete status. The over-representation of the GG genotype and G allele in all athletes irrespective of their classification according to athlete status (power-oriented, endurance-oriented, and “mixed”) suggests that the G894 allele may favour all types of sports, however, the strongest predisposition was seen among power-oriented athletes. These findings support the hypothesis that increased NO bioavailability may facilitate exercise-induced skeletal muscle hypertrophy, an important component of an athletes’ power or strength [31]. More case-control studies in independent cohorts as well as cross-sectional association studies with power and endurance phenotypes are needed.

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concur with this hypothesis. However, the biological plausibility of the G894T polymorphism’s functionality may offer a possible explanation for this phenomenon. In fact, the genes encoding nitrite oxide synthase (NOS) were primarily thought to be potential candidate genes for endurance performance [6]. This belief was based on the following premises: nitric oxide is the major endothelium-derived relaxing factor causing vasodilation [45], some NOS3 variants were shown to have functional relevance [17], and finally, reduced local muscle blood flow seems to be an important constraint for endurance performance [31]. The exercise-induced increase in muscle blood flow (hyperemia) begins immediately following the first brief contraction of exercise, can be massive and long-lasting, and appears to be matched to current muscle metabolic demand [8]. There are several types of vasodilatory mechanisms that involve nitric oxide. NO may contribute to steady-state exercise hyperemia, the dynamic adjustments of muscle blood flow to exercise and the modulation of sympathetic vasoconstriction in exercising muscles. Interestingly, it appears that the role of nitric oxide in these categories is modest [8]. On the other hand, NO exerts several distinct effects on various aspects of skeletal muscle structure and function that could possibly be favourable in strength/power sports, such as excitation-contraction coupling [9], skeletal muscle fiber type conversion (faster-to-slower fiber type) [10], modulation of mitochondrial energy production [11] and glucose metabolism [12]. Further, animal studies provided evidence that nitric oxide may promote exercise-induced muscle hypertrophy [14,46-48], stretch-induced proliferation of myoblasts [49] and prevent muscle atrophy [50]. Indeed, Gomez-Gallego et al. [31] proposed that the association of T-786C polymorphism with elite power status may be attributed, at least in part, to the role that NO plays on muscle hypertrophy.

The weakness of our study may come mainly from the small sample sizes, especially the subgroups resulting from the subdivision according to elite status. Hence, we can not completely exclude the effect of the G894T polymorphism on athletic performance. Although we have taken care to eliminate possible confounding factors, such as population stratification, which is a serious concern, especially in case-control association studies [51], this may be simply unavoidable. Berger et al. showed that population substructures can be detected even in a seemingly homogenous population [52]. The effect of population admixture has been shown in the study by Saunders et al. A significant difference in the GG genotype distribution between fast and slow triathletes appeared when only South-African-born athletes were considered [30]. Our athletes were all Caucasians, and came from a relatively small geographic area, yet the ethnic admixture can not be completely ruled out.

In conclusion, we have shown that the variation in the NOS3 gene is associated with athlete status. The over-representation of the GG genotype and G allele in all athletes irrespective of their classification according to athlete status (power-oriented, endurance-oriented, and “mixed”) suggests that the G894 allele may favour all types of sports, however, the strongest predisposition was seen among power-oriented athletes. These findings support the hypothesis that increased NO bioavailability may facilitate exercise-induced skeletal muscle hypertrophy, an important component of an athletes’ power or strength [31]. More case-control studies in independent cohorts as well as cross-sectional association studies with power and endurance phenotypes are needed.

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