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The bradykinin β_2 receptor (*BDKRB2*) and endothelial nitric oxide synthase 3 (*NOS3*) genes and endurance performance during Ironman Triathlons

Colleen J. Saunders[2], Stavroulla L. Xenophontos[3], Marios A. Cariolou[3], Lakis C. Anastassiades[4], Timothy D. Noakes[2] and Malcolm Collins[1,2,*]

[1]MRC/UCT Research Unit for Exercise Science and Sports Medicine of the Medical Research Council of South Africa and [2]Department of Human Biology, University of Cape Town, South Africa , [3]Molecular Genetics Department B, Laboratory of Forensic Genetics, The Cyprus Institute of Neurology and Genetics, Nicosia, Cyprus and [4]Cardiovascular Diagnostic Centre, Nicosia, Cyprus

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We have previously shown that the insertion allele of the angiotensin-converting enzyme (*ACE*) gene was over-represented in the fastest South-African-born finishers of the South African Ironman Triathlons. As ACE is a component of the skeletal muscle kallikrein-kinin system (KKS), the aim of this study is to determine if there are any further associations between polymorphisms within the *BDKRB2* and *NOS3* genes, which encode for the KKS components, bradykinin β_2 receptor and nitric oxide synthase, respectively, and ultra-endurance performance during the Ironman Triathlons. Four-hundred and forty-three male Caucasian triathletes who completed the 2000 and/or 2001 South African Ironman Triathlons and 203 healthy Caucasian male control subjects were genotyped for the functional −9/+9 polymorphism within exon 1 of the *BDKRB2* gene and the G894T *NOS3* gene polymorphisms. The *BDKRB2* −9/−9 genotype occurred at a significantly higher frequency when the triathlete group (27.0%) was compared with the control group (19.3%, $P = 0.035$). When divided into tertiles, there was also a significant linear trend for the *NOS3* GG genotype distribution among the fastest (35.0%), middle (40.4%) and slowest (46.9%) finishers ($P = 0.039$). The overall finishing times of the triathletes with an *NOS3* GG genotype together with a *BDKRB2* +9 allele were significantly slower than those with other genotype combinations ($P = 0.001$). The *NOS3/BDKRB2* genotype ($\beta = -0.150$, $B = -31.48$, $P = 0.002$), together with body mass index and age, accounted for 14.6% of the variance in the overall race time for the triathlon. In conclusion, both the *NOS3* and *BDKRB2* genes are associated with the actual performance during the Ironman Triathlons.

INTRODUCTION

Many factors including physical build, physiological, biochemical, biomechanical, psychological and environmental all play a role in determining an individual's athletic ability (1). There is, however, an increasing body of evidence which suggests that athletic ability is also partly determined by an individual's genetic makeup. Indeed, an individual's genetic makeup determines their physical build, physiological, biochemical and biomechanical characteristics (2). The insertion (I allele) and deletion (D allele) of a 287 bp Alu repeat sequence within intron 16 of the angiotensin-converting enzyme (*ACE*) gene have been shown to be associated with endurance performance (3–8) and power activities (3,7,9) respectively.

ACE is a key component of both the systemic and tissue renin-angiotensin systems (RAS) in which it catalyses the production of angiotensin II from angiotensin I (10). Initially,

*To whom correspondence should be addressed at: UCT/MRC Research Unit for Exercise Science and Sports Medicine, PO Box 115, Newlands 7725, South Africa. Tel: +357 27216504574; Fax: +357 27216867530; E-mail: mcollins@sports.uct.ac.za

circulating or systemic RAS was thought to exert its influence on athletic ability by affecting cardiorespiratory fitness (11). However, various studies have not shown any associations of the *ACE* gene with factors influencing cardiorespiratory fitness and performance (12,13). There is evidence to suggest that the *ACE* genotype, working through the local RAS in skeletal muscle, is more likely to exert its influence on athletic ability through differences in muscular efficiency (5,14,15). Although some investigators have suggested that a local RAS in skeletal muscle is involved in athletic ability (16), genes encoding other components of the RAS, such as angiotensinogen and the angiotensin receptors, have not been shown to be associated with athletic ability (4,6). These genes, together with the *ACE* gene, have however been shown to be associated with several cardiovascular pathologies in which the RAS has been implicated (17).

ACE is also a component of the skeletal muscle kallikrein-kinin system (KKS) where it degrades bradykinin into inactive fragments (18,19). It is, therefore, possible that ACE may influence performance through this system rather than RAS. It has been suggested that bradykinin acts via the bradykinin β_2 receptor, which is encoded for by the *BDKRB2* gene, to increase skeletal muscle glucose uptake during exercise (18,20). In addition, the activation of the bradykinin β_2 receptor results in the production of the vasodilator, nitric oxide (NO) from arginine by the enzyme nitric oxide synthase (NOS) (19,21). It has been suggested that under physiologic conditions, NO regulates mitochondrial metabolism to optimize the ratio between oxygen consumption and energy production (21).

Recently, Williams *et al*. (22) have shown that the absence (-9), rather than the presence ($+9$), of a 9 bp repeat sequence in exon 1 of the *BDKRB2* gene, which has been mapped to chromosome 14q32 (23), is associated with efficiency of muscular contraction and running distance in Olympic track athletes. The -9 allele is associated with increased bradykinin β_2 receptor activity (24,25).

The *NOS3* gene, which encodes endothelial constitutive NOS (ecNOS), has been mapped to chromosome 7q36 (26) and contains a missense Glu298Asp (G894T) polymorphism within exon 7 (27). Evidence suggests that the T allele is associated with reduced ecNOS activity, reduced basal NO production and vascular disease in several populations (28,29). In contrast, others have shown that there is no association of this allele with NO levels and furthermore, no association with impaired NO-mediated endothelial vasomotor function, nor any effect on endothelium-dependent vasodilation (30–32). The reported NO levels can be influenced by the different methods used for NO level measurement as well as ethnic differences and various confounding factors, such as diet and smoking, which interact with this *NOS3* polymorphism (29,30,33). Nevertheless, the inheritance of the wild-type GG genotype should, in theory, be more advantageous with respect to endurance performance than any other genotype at this polymorphic locus.

Previous work from our laboratory has shown that the I allele of the *ACE* gene was associated with performance in the 2000 and 2001 South African Ironman Triathlons (8). If this association is a result of *ACE* genotype influencing the KKS, we can expect to find additional associations between other components of the KKS and performance in this cohort. The aim of this study, therefore, is to determine whether there are any additional associations between polymorphisms within the *BDKRB2* ($-9/-9$ genotype) and/or *NOS3* (GG genotype) genes, which encode for components of the KKS, and ultra-endurance performance during the 2000 and 2001 South African Ironman Triathlons.

RESULTS

Subject characteristics

The entire field of 701 male finishers completed the event within 16.6 h (745 ± 96 min, ranging from 508 to 998 min) as previously described (8) (Fig. 1). The 148 athletes in the Fast Triath group finished either the 2000 ($n = 30$) or 2001 ($n = 118$) event within 11.8 h (656 ± 42 min, ranging from 521 to 709 min), whereas the 147 athletes in the Mid Triath group ($n = 106$ for 2001) finished within the middle of the field during the next 1.5 h (752 ± 26 min, ranging from 709 to 799 min). The 148 athletes in the Slow Triath group ($n = 115$ for 2001) completed the event within the last 2.8 h (860 ± 46 min, ranging from 799 to 968 min). There was no significant difference in the average finishing times of the male athletes who completed the 2000 and 2001 events ($P = 0.121$). In addition, there was a strong correlation ($r = 0.859$) between the 2000 and 2001 finishing times of the 115 triathletes who completed both events. The 2001 times for these athletes, were, therefore included in the analysis of this study.

As shown in Table 1, the Fast Triath, Mid Triath, Slow Triath and Con groups were equally matched for height. All three triathlete groups were significantly older than the control group ($P < 0.001$), whereas the Fast Triath group was significantly younger than the Slow Triath group ($P = 0.046$). As expected, the Fast Triath group had a significantly lower body weight and body mass index (BMI) than the Mid Triath (weight, $P = 0.003$; BMI, $P < 0.001$), Slow Triath ($P < 0.001$) and control groups ($P < 0.001$). The control group was also significantly heavier ($P < 0.001$), with a corresponding higher BMI ($P = 0.002$) than the Mid Triath group. The percentage South-African-born subjects in the control group was significantly higher than those in the Fast Triath ($P < 0.001$), Mid Triath ($P = 0.002$) and Slow Triath ($P < 0.001$) groups. There were also significantly more South-African-born subjects in the Mid Triath group ($P < 0.001$) and Slow Triath group ($P < 0.001$) when compared with the Fast Triath group. The athletes born outside South Africa (734 ± 104 min) finished the triathlon on average faster than their South-African-born (772 ± 81 min) counterparts ($P < 0.001$). Although there were significant differences in percentage South-African-born individuals in the four groups, similar results in the general physiological characteristics of the groups were obtained when only the South-African-born individuals were included in the analysis (data not shown).

Except for age, where the *BDKRB2* heterozygous individuals ($-9/+9$) were significantly younger (31.1 ± 9.0 years, $n = 310$) than the homozygous individuals ($-9/-9$: 33.3 ± 8.8 years, $n = 152$, $P = 0.043$ and $+9/+9$: 33.3 ± 8.9 years, $n = 153$, $P = 0.038$), there were no other

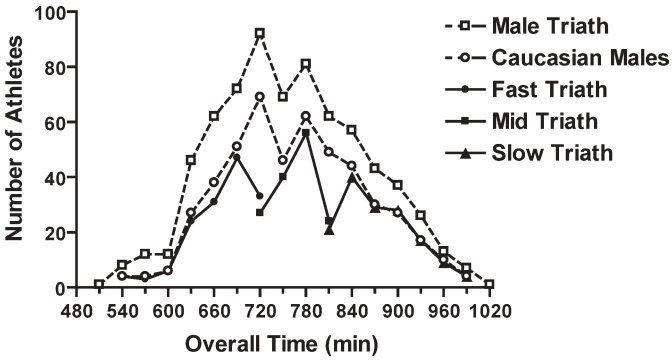

Figure 1. Comparison of the overall race time distribution of the entire field of (open square) male triathletes (Male Triath, $n = 701$) as well as the (open circle) all finishing male Caucasian (Caucasian Males, $n = 488$), (filled circle) the fastest finishing male Caucasian (Fast Triath, $n = 142$), (filled square) the middle of the field finishing male Caucasian (Mid Triath, $n = 141$) and (filled triangle) the slowest finishing male Caucasian (Slow Triath, $n = 142$) athletes who completed either the 2000 and/or 2001 South African Ironman Triathlons. One-hundred and fifteen male athletes completed both events and the 2001 times for these athletes were included in the analysis.

BDKRB2 or *NOS3* genotype effects on the observed differences in age, weight and BMI between the groups (data not shown).

Individual *BDKRB2* and *NOS3* genotype and allele frequencies in the triathlete and control groups

As originally hypothesized, there was a significant difference in the genotype distribution of the −9/+9 *BDKRB2* gene polymorphism when the Fast Triath group (43−9/−9, 29.9%; 66 −9/+9, 45.8% and 35 + 9/+9, 24.3%) was compared with the Con group (39−9−9, 19.3%; 117 − 9/+9, 57.9% and 46+9/+9, 22.8%) ($\chi^2 = 6.4$, $P = 0.042$). The −9/−9 genotype was significantly higher in the Fast Triath than in the Con group (odds ratio of 1.8, 95% confidence interval = 1.1–2.9, $P = 0.032$) (Table 2). As expected, no significant differences were found in the *BDKRB2* genotype distributions when the slower triathlete groups were compared with the control group ($\chi^2 = 5.0$, $P = 0.081$). There was, however, a significant difference in the genotype distribution of the −9/+9 *BDKRB2* gene polymorphism when the three Triath groups (114−9/−9, 27.0%; 200−9/+9, 47.4% and 108+9/+9, 25.6%) were added together and compared with the Con group ($\chi^2 = 6.7$, $P = 0.035$). The −9/−9 genotype was significantly higher in the combined triathlete groups than in the Con group (odds ratio of 1.5, 95% confidence interval = 1.0–2.3, $P = 0.046$). There was a tendency for a linear trend of the −9/−9 genotype distribution among the four groups ($\chi^2 = 3.5$, $P = 0.061$). There were no significant differences in the frequencies of the allele distributions between any of the triathlete and control groups (data not shown). Similar *BDKRB2* genotype and allele frequency distributions were obtained when only the South-African-born individuals were analysed (data not shown).

In contrast to the original hypothesis, there were no significant differences in the frequencies of the genotype distributions of the *NOS3* gene G894T polymorphism when the three triathletes and control groups were compared (Table 2). In addition, there were no significant differences in the frequencies of the allele distributions between any of the groups (data not shown). Although there was no significant difference in the GG genotype distribution between the Fast Triath and Slow Triath groups ($\chi^2 = 3.8$, $P = 0.053$), there was, however, a significant linear trend for the GG genotype distribution among the Fast Triath (35.0%), Mid Triath (40.4%) and Slow Triath (46.9%) subjects ($\chi^2 = 4.3$, $P = 0.039$) (Table 2). There was also a significant difference in the GG genotype distribution between the Fast Triath (31.8%, $n = 20$) and Slow Triath (50.5, $n = 47$) groups ($\chi^2 = 4.7$, $P = 0.031$) when only the South-African-born athletes were analysed. Similarly, there was a significant linear trend for the GG genotype distribution among the South-African-born Fast Triath, Mid Triath (40.9%, $n = 43$) and Slow Triath subjects ($\chi^2 = 5.5$, $P = 0.019$). In support of this, the overall finishing times (GG genotype: 768 ± 90 min, $n = 177$ versus T allele: 749 ± 93 min, $n = 257$, $P = 0.034$) as well as the swim (GG genotype: 72 ± 15 min, $n = 172$ versus T allele: 69 ± 12 min, $n = 249$, $P = 0.022$) and cycle (GG genotype: 395 ± 38 min, $n = 164$ versus T allele: 785 ± 43 min, $n = 243$, $P = 0.024$) times of the triathletes with a GG *NOS3* genotype were significantly slower than those with a T allele (i.e. GT or TT genotypes). There were, however, no significant differences in the run times of the athletes with a GG genotype (289 ± 48 min, $n = 169$) and those with a T allele (282 ± 47 min, $n = 245$) of the *NOS3* gene ($P = 0.177$). Triathletes with a GG genotype and a T allele were similarly matched for age, height, weight, BMI and percentage South-African-born individuals. Similar findings were obtained when only the South-African-born athletes were analysed (data not shown).

Table 1. General physiological characteristics of the fastest (Fast Triath), middle of the field (Mid Triath) and slowest (Slow Triath) finishing triathlete groups as well as the control (Con) group

	Fast Triath ($n = 148$)	Mid Triath ($n = 147$)	Slow Triath ($n = 148$)	Con ($n = 203$)
Age (years)	33.0 ± 5.9 (148)[a,b]	34.0 ± 7.4 (147)[c]	36.1 ± 9.3 (148)[b,d]	27.7 ± 9.7 (194)[a,c,d]
Height (cm)	180.4 ± 6.2 (135)	180.5 ± 6.0 (125)	180.8 ± 7.6 (140)	181.2 ± 7.6 (195)
Weight (kg)	74.7 ± 7.2 (146)[a,e,f]	78.5 ± 8.1 (146)[c,f]	80.1 ± 10.4 (147)[e]	82.9 ± 11.5 (199)[a,c]
BMI (kg m^{-2})	23.0 ± 1.5 (134) [a,e,g]	24.1 ± 1.9 (124) [g,h]	24.6 ± 2.6 (139)[e]	25.2 ± 3.2 (193)[a,h]
South-African-born (%)	46.1 (143)[a,e,i]	73.6 (144)[i,j]	64.9 (148)[d,e]	86.7 (196)[a,d,j]

Except for the percentage South-African-born, values are expressed as mean ± SD. The number of subjects (n) is in parentheses.
[a]$P < 0.001$ Fast Triath versus Con.
[b]$P = 0.046$ Fast Triath versus Slow Triath.
[c]$P < 0.001$ Mid Triath versus Con.
[d]$P < 0.001$ Slow Triath versus Con.
[e]$P < 0.001$ Fast Triath versus Slow Triath.
[f]$P = 0.003$ Fast Triath versus Mid Triath.
[g]$P < 0.001$ Fast Triath versus Mid Triath.
[h]$P = 0.002$ Mid Triath versus Con.
[i]$P < 0.001$ Fast Triath versus Mid Triath.
[j]$P = 0.002$ Mild Triath versus Con.

Table 2. The *BDKRB2* −9/+9 and the *NOS3* missense Glu298Asp (G894T) polymorphisms genotype frequencies within the fastest (Fast Triath), middle of the field (Mid Triath) and slowest (Slow Triath) consenting Caucasian male finishers of the 2000 and/or 2001 South African Ironman Triathlons as well as the Caucasian male controls (Con)

	Fast Triath (max $n = 148$)	Mid Triath (max $n = 147$)	Slow Triath (max $n = 148$)	Con (max $n = 203$)
BDKRB2 gene −9/+9 polymorphism				
N	144	133	145	202
−9/−9 genotype	43 (29.9)	30 (22.6)	41 (28.3)	39 (19.3)
−9/+9 genotype	66 (45.8)	66 (49.6)	68 (46.9)	117 (57.9)
+9/+9 genotype	35 (24.3)	37 (27.8)	36 (24.8)	46 (22.8)
NOS3 gene missense Glu298Asp (G894T) polymorphism				
N	143	146	145	163
GG genotype	50 (35.0)	59 (40.4)	68 (46.9)	64 (39.3)
GT genotype	73 (51.1)	66 (45.2)	59 (40.7)	72 (44.2)
TT genotype	20 (14.0)	21 (14.4)	18 (12.4)	27 (16.6)

Values are expressed as the number of subjects with the percentage in parentheses. The maximum number of subjects (max *n*) in each group and the actual number of samples (*N*) genotyped are also shown. For the *BDKRB2* genotype distributions: (i) Fast Triath versus Con, $\chi^2 = 6.4$, $P = 0.042$; (ii) Slow Triath versus Con, $\chi^2 = 5.0$, $P = 0.081$. The −9/−9 genotype distribution for the four groups, χ^2 for linear trend = 3.5, $P = 0.061$. For the *NOS3* genotype distributions: (i) Fast Triath versus Con, $\chi^2 = 1.5$, $P = 0.480$; (ii) Slow Triath versus Con, $\chi^2 = 2.2$, $P = 0.338$. GG genotype distribution for the Fast Triath, Mid Triath and Slow Triath groups, χ^2 for linear trend = 4.3, $P = 0.039$.

The *NOS3* genotype distributions of the groups were in Hardy–Weinberg equilibrium (HWE). Except for the Con group ($P = 0.036$), the *BDKRB2* genotype distributions of the Fast Triath and Slow Triath groups were in HWE.

Combined *BDKRB2* and *NOS3* genotype frequencies

There were no significant differences in the combined *BDKRB2* −9/+9 and *NOS3* G894T polymorphisms genotype distribution of the three triathletes and the control groups ($\chi^2 = 27.2$, $P = 0.294$; Table 3). Similar combined genotype frequency distributions were obtained when only the South-African-born individuals were analysed (data not shown). Closer inspection of Table 3 showed that there was, however, a significant linear trend for the combined −9/+9 and GG genotype distribution ($\chi^2 = 6.0$, $P = 0.015$) as well as the +9 allele (either a +9/+9 or a −9/+9 genotype) and GG genotype distribution among the three triathlete groups when analysed irrespective of country of birth ($\chi^2 = 9.3$, $P = 0.002$) (Table 3) or when only the South-African-born athletes were analysed ($\chi^2 = 7.0$, $P = 0.008$) (data not shown). As expected, there was a tendency for the combined *BDKRB2* −9/−9 and *NOS3* G allele to be over-represented in the Fast Triath subjects ($n = 40$, 28.6%) compared with the Con subjects ($n = 28$, 17.3%) (odds ratio 1.9, 95% confidence interval = 1.1–3.3, $P = 0.028$). All the other possible genotype combinations appeared to be evenly distributed between the groups.

The observed *NOS3* GG genotype effects on overall race time could be attributed to the combined effects of this *NOS3* genotype with the *BDKRB2* +9 allele (Tables 2 and 3).

Table 3. Combined *BDKRB2* −9/+9 and *NOS3* missense Glu298Asp (G894T) polymorphisms genotype frequencies within the fastest (Fast Triath), middle of the field (Mid Triath) and slowest (Slow Triath) finishing triathlete groups as well as the control (Con) group

B2BKR2 genotype	*NOS3* genotype	Fast Triath ($n = 140$)	Mid Triath ($n = 132$)	Slow Triath ($n = 142$)	Con ($n = 162$)
−9/−9	GG	18 (12.9)[a]	14 (10.6)	12 (8.5)	13 (8.0)[a]
−9/−9	GT	22 (15.7)[a]	13 (9.9)	23 (16.2)	15 (9.3)[a]
−9/−9	TT	2 (1.4)	3 (2.3)	6 (4.2)	5 (3.1)
−9/+9	GG	21 (15.0)[b,c]	26 (19.7)[b,c]	38 (26.8)[b,c]	36 (22.2)
−9/+9	GT	33 (23.6)	30 (22.7)	25 (17.6)	38 (23.5)
−9/+9	TT	10 (7.1)	10 (7.6)	3 (2.1)	16 (9.9)
+9/+9	GG	9 (6.4)[c]	14 (10.6)[c]	16 (11.3)[c]	15 (9.3)
+9/+9	GT	18 (12.9)	16 (12.1)	11 (7.8)	18 (11.1)
+9/+9	TT	7 (5.0)	6 (4.6)	8 (5.6)	6 (3.7)

Values are expressed as the number of subjects with the percentage in parentheses. The total number of subjects in each group that were genotyped for both polymorphisms are also shown. The combined genotype distributions $\chi^2 = 27.2$, $P = 0.294$.
[a]Combined −9/−9 genotype and G allele (i.e. −GG or GT genotype) of the Fast Triath versus Con $\chi^2 = 4.9$, $P = 0.027$.
[b]Linear trend among the three triathlete groups ($\chi^2 = 6.0$, $P = 0.015$).
[c]Linear trend among the three triathlete groups with a GG genotype and a +9 allele ($\chi^2 = 9.3$, $P = 0.002$).

The overall race ($P = 0.001$) as well as the swim ($P = 0.008$), bike ($P < 0.001$) and run ($P = 0.025$) split times were significantly slower in the triathletes with the combined *NOS3* GG genotype and *B2BKR2* +9 allele (i.e. −9/+9 or +9/+9 genotype) than the athletes with the other seven combinations of *NOS3* and *B2BKR2* genotypes (Table 4), including those triathletes with a combined *NOS3* G allele (i.e. GG or GT genotype) and a *B2BKR2* −9/−9 genotype (data not shown).

Multivariate analysis for the determination of overall race time

As there were significant differences between the physiological characteristics of the three triathlete groups (Table 1), bivariate analysis was used to describe the relationship between the overall finishing times of the Ironman Triathlons and their different physiological parameters. The athletes' age ($r = 0.172$, $P < 0.001$), weight ($r = 0.242$, $P < 0.001$) and BMI ($r = 0.330$, $P = 0.001$) were all positively correlated with the overall race time.

The variables BMI, combined *NOS3* and *BDKRB2* genotypes (GG genotype and +9 allele versus remaining genotype combinations) and age were included in the model that accounted for 14.6% of the variance in the overall race time for the Ironman Triathlons ($P < 0.00001$, standard error of estimate = 88.2) (Table 5). Although BMI was the most important determinant of overall race time in this model, both age and the combined *NOS3/BDKRB2* genotypes were also significant determinants. Both BMI and age were positively associated with overall race time, whereas the combined *NOS3*/*BDKRB2* genotype was negatively associated. Similarly, these variables accounted for 10.3% of the variance in the bike split time for the event, whereas only BMI and the combined *NOS3*/*BDKRB2* genotype accounted

Table 4. General physiological characteristics and performance of the triathletes with a combined *NOS3* GG genotype and a *BDKRB2* −9/+9 genotype and the remaining eight possible combinations of the missense Glu298Asp (G894T) polymorphism within the *NOS3* gene and −9/+9 polymorphism *BDKRB2* gene

	NOS3 GG genotype and *BDKRB2* +9 allele ($n = 124$)	Remaining *NOS3* and *BDKRB2* genotype combinations ($n = 290$)	*P*-value
Age (years)	35.4 ± 8.2 (124)	33.9 ± 7.6 (290)	0.077
Height (cm)	180.8 ± 7.2 (109)	180.3 ± 6.3 (266)	0.103
Weight (kg)	78.9 ± 9.4 (122)	77.3 ± 8.8 (288)	0.537
BMI (kg m^{-2})	24.2 ± 2.2 (108)	23.8 ± 2.2 (264)	0.201
South-African-born (%)	68.9 (122)	59.3 (285)	0.069
Overall time (min)	784 ± 85 (85)	750 ± 94 (329)	0.001
Swim time (min)	73 ± 15 (120)	69 ± 13 (281)	0.008
Bike time (min)	401 ± 38 (113)	385 ± 43 (275)	<0.001
Run time (min)	294 ± 46 (117)	282 ± 48 (279)	0.025

Except for the percentage South-African-born subjects, values are expressed as mean ± SD. The number of subjects (n) are in parentheses.

Table 5. Multivariate analysis for the overall race and split times of the Caucasian male triathletes who completed the 2000 and 2001 South African Ironman Triathlons

	β	B	*P*-value
Overall time (min)			
BMI (kg m^{-2})	0.302	13.11	<0.001
NOS3 GG and *BDKRB2* +9 allele	−0.150	−31.48	0.002
Age (years)	0.123	1.53	0.01
Swim time (min)			
Age (years)	0.316	0.54	<0.001
*NOS3*GG and *BDKRB2* +9 allele	−0.101	−2.97	0.043
Bike time (min)			
BMI (kg m^{-2})	0.212	4.11	<0.001
*NOS3*GG and *BDKRB2* +9 allele	−0.150	−14.17	0.004
Age (years)	0.151	0.83	0.004
Run time (min)			
BMI (kg m^{-2})	0.331	7.32	<0.001
*NOS3*GG and *BDKRB2* +9 allele	−0.116	−12.50	0.022

For the overall time: $R = 0.391$, adjusted $R^2 = 0.146$, SEE = 88.2, $P < 0.00001$. For the swim time: $R = 0.368$, adjusted $R^2 = 0.129$, SEE = 12.5, $P < 0.00001$. For the bike time: $R = 0.333$, adjusted $R^2 = 0.103$, SEE = 40.4, $P < 0.00001$. For the run time: $R = 0.368$, adjusted $R^2 = 0.128$, SEE = 45.7, $P < 0.00001$. B, parameter estimate; β, partial correlation coefficient.

for 12.8% of the variance in the run split time. Age and the combined *NOS3/BDKRB2* genotype accounted for 12.9% of the variance in the run split time (Table 5). The combined *NOS3/BDKRB2* genotype was also a significant determinant of the overall race time when only the South-African-born athletes were included in the analysis ($P = 0.007$) (data not shown).

DISCUSSION

The first important finding in this study is that the −9/−9 genotype of the *BDKRB2* gene was over-represented in the entire field of consenting male Caucasian triathletes of the 2000 and 2001 South African Ironman Triathlons. However, when divided into tertiles according to their finishing times, the −9/−9 genotype was only over-represented in the fastest tertile. This was in agreement with our original hypothesis as the fastest tertiles of athletes had superior endurance ability than the athletes in the two slower tertiles. The absence (−9), rather than the presence (+9), of a 9 bp repeat sequence in exon 1 has previously been shown to be associated with increased gene transcription (24) and higher *BDKRB2* mRNA expression (25,34). Increased bradykinin β_2 receptor activity, a key component of the KKS, may therefore be, at least partly, involved in determining endurance performance. The genotype and allele frequencies of the −9/−9 *BDKRB2* gene polymorphism within the groups were similar to the previously reported values (24,35).

This corroborates a recent study in which the −9/+9 polymorphism within the *BDKRB2* gene was shown to be associated with efficiency of muscular contraction (i.e. the energy used per unit of power output during exercise or delta efficiency) and running distance in elite track athletes (22). Muscular contraction efficiency was significantly higher in the group of individuals with a −9/−9 *BDKRB2* genotype. This, together with the evidence that the I allele of the *ACE* gene is associated with endurance performance (3–8) and muscular efficiency (5,14,15), suggests that the KKS is involved in determining the athletic performance. Williams *et al*. (22) have shown that the I allele of the *ACE* gene together with the −9 allele of the *BDKRB2* gene was associated with endurance performance of elite athletes. Although the ACE is also a key component of the RAS (36), genes encoding other components of the RAS such as angiotensinogen and the angiotensin receptors have not been shown to be associated with athletic ability (4,6).

In the KKS, ACE is responsible for degrading kinins, of which bradykinin is one, into inactive peptide fragments (19). As the I allele of the *ACE* gene is associated with lower ACE activity (37), individuals with an I allele would be expected to have higher bradykinin levels than those with a DD genotype. At physiological doses bradykinin, which acts via the bradykinin β_2 receptors, concurrently increases skeletal muscle blood flow and glucose uptake (19). Studies have shown that bradykinin enhances the autophosphorylation capacity of the insulin receptor, IRS-1 (38), which in turn stimulates GLUT4 translocation and glucose uptake via an insulin-dependent pathway. In addition, bradykinin may mediate the increase in skeletal muscle insulin sensitivity that accompanies exercise (18). Bradykinin may also affect skeletal muscle glucose uptake via an insulin-independent pathway. Recently, Shiuchi *et al*. (39) have shown that ACE-inhibitors, resulting in increased bradykinin levels, increase GLUT4 translocation without affecting the autophosphorylation of IRS-1. They suggest that the improved glucose uptake is mediated via bradykinin-induced NO production. Bradykinin release is increased in contracting skeletal muscle and acts via the bradykinin β_2 receptors, located on the plasma membrane of skeletal muscle cells and the vascular

endothelium, to activate ecNOS and the production of the vasodilator NO (19,40,41). In addition, NO activation of guanylate cyclase has been shown to contribute to the adenosine monophosphate activated protein kinase (AMPK)-stimulation of glucose uptake and GLUT4 translocation in heart muscle (42). In some studies of isolated skeletal muscle, bradykinin did not increase glucose uptake (43,44), suggesting that its effects on glucose uptake are endothelium-dependent and mediated via the β_2 receptor.

With this in mind, in this study there was a tendency ($P = 0.028$) for the $-9/-9$ *BDKRB2* genotype combined with an *NOS3* G allele (i.e. GG or GT genotype) to be over-represented in the fastest finishing triathletes. The functional significance of this *NOS3* polymorphism is still unclear; however, the ecNOS Asp_{298} variant of the protein, which is produced by the T allele, appears to be more susceptible to proteolytic cleavage (33). This may result in a lower steady-state ecNOS activity and, subsequently, lower levels of NO production (33). The frequency of the G894T *NOS3* alleles within the groups was similar to previously reported values (45).

Although NO is an important vasodilator in smooth muscle, it also reversibly inhibits mitochondrial cytochrome oxidase, responsible for transferring electrons to molecular oxygen, by competing for available oxygen (46). This decreases oxygen consumption in skeletal muscle and heart mitochondria, and it has been suggested that under physiologic conditions, NO regulates mitochondrial metabolism to optimize the ratio between oxygen consumption and energy production (21,47). If NO production is inhibited or limited, oxygen consumption at any given workload will increase, reducing the efficiency of the working muscle. Increased bradykinin levels associated with the I allele of the *ACE* gene, increased expression of the *BDKRB2* gene in skeletal muscle and/or increased ecNOS activity will lead to an increase in NO production. The resultant decrease in oxygen consumption may increase the efficiency of contracting skeletal muscle and will be beneficial during endurance exercise such as triathlons. In fact, the frequency of the biologically relevant *ACE* (II) and *BDKRB2* ($-9/-9$) combination was significantly higher in the fastest athletes compared with the controls ($P = 0.029$). In addition, the frequency of the I allele and $-9/-9$ combination was also significantly higher in the fastest athletes (23.2%) compared with the controls (13.4%, $P = 0.021$) (M. Collins, unpublished observation). This needs to be investigated further with a larger sample size. Although we have shown differences in double genotype combinations, future studies with a larger sample size will allow the evaluation of the effect of the triple genotype combination that is the *BDKRB2*, *NOS3* and *ACE* combination. At present, the sample size is too small to allow accurate statistical tests to be performed.

Although there was a tendency of the $-9/-9$ *BDKRB2* genotype combined with an *NOS3* G allele to be over-represented in the fastest finishing triathletes compared with the control subjects ($P = 0.028$), any possible biological importance of this observation must nevertheless be interpreted with caution. Athletes with the GG *NOS3* genotype combined with a *BDKBR2* +9 allele were significantly slower in all three disciplines as well as in overall finishing times, than athletes with any other genotype combination, including those with a combined GG and $-9/-9$ genotype. This suggests that either the GG *NOS3* genotype may not be more advantageous with respect to endurance performance as originally suggested or that its effects are masked by the *BDKBR2* $+9/-9$ genotype. The reasons and mechanisms for this are unclear and need to be further investigated. This apparent discrepancy could be explained by the fact that the relative number of athletes with a GG *NOS3* genotype combined with a $-9/+9$ *BDKRB2* genotype was significantly under-represented ($P = 0.002$) in the fastest finishers (12.3%) when compared with the slowest finishers (27.3%).

As country-matched controls for the international athletes were not available, the percentage South-African-born subjects in the control group was significantly higher than those in the triathlete groups. To overcome this problem, all data were additionally analysed using only the South-African-born subjects. There was no evidence from the data that population stratification affected our results. The athletes born outside of South Africa did complete the event in a faster time but this would be expected as the more serious athletes are more likely to travel to an international event. Recreational or slower athletes are less likely to travel and usually limit their participation to local events.

Another limitation of this study was that the control group was not in HWE ($P = 0.036$) for the $-9/+9$ polymorphism within the *BDKRB2* gene. HWE states that genotype frequencies are constant from one generation to the next, assuming there is no selection pressure acting on a particular allele, no mutation, no migration and no population stratification. In addition, deviations from HWE observed in association studies may result from genotyping errors, selection bias or the use of very small sample sizes such that the allele frequencies observed in the sample are not a true representation of the population from which the sample is drawn. In our study, even though all the controls were South-African-born, their ancestral lineage is not known and so population stratification may in fact be the explanation for the deviation from HWE observed. Alternatively, as the control group in this study did not contain individuals who had trained or participated in an ultra-endurance event and consisted predominantly of subjects who participated recreationally in power and sprint sporting codes, it is possible that the control subjects did not represent the general South African Caucasian population.

In summary, the results of this study suggest that variants of the *BDKRB2* and *NOS3* genes which contribute to increased KKS activity are associated with the endurance performance of Ironman triathletes. It is, however, highly unlikely that performance is solely associated with genes encoding for proteins in a single biological pathway, such as the KKS. Owing to the involvement of many biochemical and physiological pathways during exercise (1), it is more likely that several genes, each encoding for proteins involved in different biological systems and having a small but significant influence on the systems, are probably involved in the endurance phenotype (2). In conclusion, the $-9/+9$ polymorphism within the *BDKRB2* gene, together with the *NOS3* missense Glu298Asp polymorphism, is associated with the observed performance in the 2000 and 2001 South African Ironman Triathlons.

MATERIALS AND METHODS

Subjects

Participants were recruited from the 701 male triathletes who completed either the 2000 (272 finishers) and/or the 2001 (544 finishers) South African Ironman Triathlons, of which 115 completed both events as previously described (48). The triathlons consisted of a 3.8 km swim, a 180 km cycle and a 42.2 km run. Prior to the events, each competitor was sent a detailed explanation of the study and invited to participate. Triathletes who agreed to participate in the study were interviewed at race registration to ensure full understanding of the study, completed an informed consent form and personal particulars questionnaire. Four-hundred and forty three of the consenting male Caucasian triathletes with varying athletic ability were included in this study. The triathletes were divided into tertiles according to their overall race finishing times to produce three groups including the fastest finishers (Fast Triath, $n = 148$), middle of the field finishers (Mid Triath, $n = 147$) and the slowest finishers (Slow Triath, $n = 148$). In addition, 203 apparently healthy Caucasian control subjects (Con) that had not participated in or trained for an ultra-endurance event were recruited from the greater Cape Town Metropolitan area. The athletes were divided into the three finishing times groups because the hypothesis for this study was that the advantageous genotypes of the *BDKBR2* ($-9/-9$) and *NOS3* (GG) genes would occur at a higher frequency in the fastest triathletes (Fast Triath) compared with both the slower triathletes (Mid Triath and Slow Triath) and control (Con) subjects. The slower triathlete groups were included in this study to exclude the possibility that the advantageous genotypes were generally over-represented in this population of endurance athletes. It was further hypothesized that the slower triathlete groups would either have similar genotype frequencies to the control group or an intermediate genotype distribution. As shown in Table 2, not all the samples in each group were genotyped for both polymorphisms. Approval for this study was obtained from the Research and Ethics Committee of the Faculty of Health Sciences, University of Cape Town.

DNA extraction and genotyping

Approximately 4.5 ml of venous blood was collected from each subject into an ethylenediaminetetraacetic acid (EDTA) vacutainer tube by venupuncture of a forearm vein. These samples were stored at 4°C until DNA extraction was performed as described by Lahiri and Nurnberger (49). The extracted DNA was stored at 4°C until subsequent polymerase chain reaction (PCR) analysis.

The subjects were genotyped for the $-9/+9$ polymorphism within exon 1 of the *BDKRB2* gene using a nested PCR assay. At least 100 ng of genomic DNA was initially amplified using the following forward (5′-GCCCTTGAAAGATGAGCTG-3′) and reverse (5′-AACTCCCCACGACCACAG-3′) primers to produce 266 and/or 275 bp fragments as described by Braun *et al*. (35). Two microlitres of the primary PCR reaction was re-amplified to produce 100 and/or 91 bp fragments using the following forward (5′-TCTGGCTTCTGGGCTCCG AG-3′) and reverse (5′-AGCGGCATGGGCACTTCAGT-3′) primers as described by Williams *et al*. (22). Both the primary and secondary PCR reactions were carried out in a total volume of 50 μl containing DNA template, 20 pmol each of the forward and reverse primers, 20 mM Tris–HCl pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$, 125 μM each of dNTP (dATP, dCTP, dTTP and dGTP) and 5 U of *Taq* DNA polymerase, using a PCR express thermal cycler (Hybaid Ltd., Middlesex, UK). The amplification was performed with an initial denaturing step at 94°C for 5 min, followed by 35 cycles of denaturing at 94°C for 1 min, annealing for 1 min at 54°C during the primary PCR reaction and 60°C during the secondary PCR reaction and extension at 72°C for 1 min, with a final extension step at 72°C for 5 min. The secondary PCR products were resolved on 7.5% polyacrylamide gels and visualized under UV light after ethidium bromide staining.

The subjects were also genotyped for the missense Glu298Asp (G894T) polymorphism in exon 7 of the *NOS3* gene using the previously described standard protocol (27,50). Briefly, the steps were as follows. Forward and reverse primer sequences were the following: 5′-AAGGCAGG AGACAGTGGATGGA-3′ and 5′-CCCATGCAATCCCTT TGGTGCTCA-3′. A 248 bp amplicon was obtained after PCR which was then digested with *Dpn*II for 3 h at 37°C. The restriction enzyme did not cleave the wild-type G allele, whereas the mutant T allele was cleaved into two fragments of 158 and 90 bp. These were separated on a 3% agarose gel. Heterozygotes displayed all three fragments upon digestion.

Statistical analysis

Data were analysed with the STATISTICA version 7 (StatSoft, Inc., Tulsa, OK, USA) and GraphPad InStat version 2.05a (GraphPad Software, San Diego, CA, USA) statistical programmes. Differences in genotype and allele frequencies as well as in the percentage of South-African-born individuals, between the triathlete and control groups were determined by either the Pearson χ^2, Yates corrected χ^2 or Fisher exact tests. The odds ratios, 95% confidence intervals and χ^2 test of linear trend were determined using the GraphPad InStat software. Any significant differences between the characteristics of the triathlete and control groups and sub-groups were determined by a one-way analysis of variance (ANOVA). When the overall F-value was significant, a Tukey's honest significance *post hoc* test was used to determine the specific differences. Data that were not normally distributed was log-transformed. Bivariate correlations were used to determine the relationship between overall race and split times and physiological parameters. Physiological parameters and genotype data were used in multivariate analysis, by the use of forward stepwise regression, to determine the model which best predicted overall race time during the 2000 and 2001 South African Ironman Triathlons. Statistical significance was accepted when $P < 0.05$ and $P < 0.025$ when combined gene–gene interactions or effects were analysed. The required sample size was determined using QUANTO 0.5 as previously described (8). HWE was established using the Genepop web version 3.4 program (http://wbiomed.curtin.edu.au/genepop/).

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Conflict of Interest statement. The authors declare no conflict of interest.

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