Annals of Applied Sport Science, vol. 4, no. 2, pp. 01-08, Summer 2016
DOI: 10.18869/acadpub.aassjournal.4.2.1

www.aassjournal.com
ISSN (Online)**: 2322 – 4479**
ISSN (Print)**: 2476–4981**

Original Article

www.AESAsport.com
Received: 06/03/2016
Accepted: 26/06/2016

Single Nucleotide Polymorphisms within Calcineurin-Encoding Genes are Associated with Response to Aerobic Training in Han Chinese Males

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ABSTRACT

Calcineurin, which functions in calcium signaling, is expressed in skeletal and cardiac muscle and has been linked to sensitivity to muscle strength training. It is also proposed to contribute to individual aerobic endurance. To investigate the relationship between calcineurin-encoding genes and aerobic endurance traits, 126 young-adult Han Chinese males were enrolled in an aerobic exercise training study. Participants were genotyped for polymorphisms within the 5 genes (PPP3CA, PPP3CB, PPP3CC, PPP3R1 and PPP3R2) encoding calcineurin using restriction fragment length polymorphism polymerase chain reaction (PCR-RFLP) or matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). Participants underwent 18 weeks of aerobic exercise training (running). Before and after the training period, maximal oxygen uptake (VO2max) and 12 km/h running economy were measured. Statistical analyses were performed using chi-square test and analysis of variance. The baseline value of VO2max was significantly associated with rs3804423 and rs2850965 loci in the PPP3CA gene ($P<0.05$). The training responsiveness of VO2max was significantly associated with the rs3804358 locus in PPP3CA and the rs4671887 locus in PPP3R1 ($P<0.05$). The training responsiveness of running economy was significantly associated with rs3739723 in PPP3R2 ($P<0.05$). These findings indicate that polymorphisms within the calcineurin-encoding genes may partially explain individual differences in response to aerobic training.

KEY WORDS: ***Calcineurin, Running Economy, VO_{2max}, Polymorphism.***

INTRODUCTION

The serine/threonine phosphatase calcineurin (CN) is widely distributed in eukaryotes, is directly regulated by calcium ions in the process of cellular signal transduction, and plays a role in dephosphorylation (1). During exercise training, calcium ions flow activates CN to cause calcium ions fluctuations and thereby plays a role as an active receptor in muscle contraction (2, 3).

Calcium ions can transmit neuromuscular signals and thereby trigger related gene expression, and CN can activate the expression of many genes, including calcium ions uptake genes and energy metabolism genes (4). The CN-mediated signal transduction pathway is one of the most important pathways for inducing calcium ions; it causes calcium ion signals to interact jointly with the regulatory mechanisms of other messengers and regulate the functions of

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cells (5). As such, it is found in cells of various tissues, including brain, skeletal muscle, cardiac, and cardiovascular endothelium cells, as well as T lymphocytes (6).

According to experiments by Nordsborg *et al.* (2010), exercise-induced increases in Na (+), K (+) pump alpha1 and beta1 mRNA expression in trained subjects are more pronounced after high- than after moderate- and low-intensity exercise, and Ca^{2+} regulates alpha1 mRNA expression in oxidative muscles via Ca $^{(2+)}$ / calmodulin-dependent protein kinase (CaMK) and calcineurin signalling pathways (7). In an investigation on the causal link between the limitation of myofibre transitions and modulation of calcineurin activity, an exercise of both high frequency and amplitude (swimming) or low frequency and amplitude (running) can induce an initial activation followed by the inhibition of calcineurin (8). These studies indicate a role for CN in response to exercise. Given its interaction with calcium, presence in muscle tissues, and fluctuations during exercise, CN is proposed to correlate with individual differences in response to aerobic training.

CN is composed of a regulatory subunit (CnB) and a catalytic subunit (CnA). CnB includes two isomers, CnB1 and CnB2, which are encoded, respectively, by *PPP3R1* (protein phosphatase 3, regulatory subunit B, alpha) on chromosome 2p16-p15 and *PPP3R2* (protein phosphatase 3, regulatory subunit B, beta) on chromosome 9q31. CnA includes three isomers, CnAα, CnAβ, and CnAγ, which are encoded, respectively, by *PPP3CA* (protein phosphatase 3, catalytic subunit, alpha isozyme) on chromosome 4q21-q24, *PPP3CB* (protein phosphatase 3, catalytic subunit, beta isozyme) on chromosome 10q21-q22, and *PPP3CC* (protein phosphatase 3, catalytic subunit, gamma isozyme) on chromosome 8p21.2 (9). Insertion/deletion polymorphisms within the *PPP3R1* promoter region have been associated with left ventricular hypertrophy and the sensitivity to training for muscular strength (10, 11). Previous reports have also indicated that some variants produce CN with different activity levels and are correlated with better exercise endurance in elite athletes (12, 13). However, few studies have investigated the correlation between polymorphisms within the genes encoding CN and response to aerobic training in a general population.

In this study, single nucleotide polymorphisms (SNPs) within the five genes encoding CN, *PPP3CA*, *PPP3CB*, *PPP3CC*, *PPP3R1* and *PPP3R2*, were investigated for a potential role in aerobic training response. Specifically, genotypes were compared against maximal oxygen uptake (VO_2max) and running economy (RE) to explore the correlation between CN variants and individual differences in response to aerobic training.

MATERIALS AND METHODS

Participants. This prospective study recruited 126 male first-year students from the School of Physical Education of Henan Polytechnic University, China. All participants were healthy individuals of Han population born in Henan province. The mean age, height, and weight for these participants were 19.0 ± 1.6 years, 171.2 ± 6.1 cm, and 59.98 ± 6.61 kg, respectively. Potential participants who had a training history or long-term endurance exercise were excluded, which was recounted by themselves. In this study, informed consent was obtained from all participants, ethical approval was obtained by the Henan Polytechnic University, and the study was conducted within the principles set out in the declaration of Helsinki**.**

Aerobic Exercise and Physiological Indexes. All participants underwent 5000-meter steady aerobic running outside on a track, 3 times per week, for a total of 18 weeks, as in a prior report (13). Before 5000-meter aerobic running, 2-week adaptive training was performed with 1000-meter and 3000-meter running at the first and second week. Then, only general flexibility exercises were performed. During training, all participants lived together in an apartment and had unified food. During each aerobic running, each participant wore a Polar Sport Tester (PST; Polar Company) that determine exercise intensity controlled via the heart rate (HR) corresponding to the individual anaerobic threshold (VT) and was monitored (VT_{HR} ± 3) with a Polar Sport Tester (Polar Electro Inc., Lake Success, NY, USA). For the first 10 weeks, the intensity of training was targeted at 95% of individual VT_{HR}; for the last 8

weeks, subjects were asked to run at 105% of their individual $VT_{HR.}$ The running economy (RE, 12 km/h, reported in VO2 in L/min) and maximal aerobic capacity (VO2max, L/min) were also measured by using an open circuit spirometry (Parvomedics, Sandy, UT, USA) during a continuous incremental protocol on a motorized treadmill (Erich Jaeger Treadmill E5, German) (14). The oxygen consumption was measured on JAEGER Oxygen Analyzer (Germany).

Selection and Genotyping of SNPs. The total lengths of *PPP3CA*, *PPP3CB*, *PPP3CC*, *PPP3R1*, and *PPP3R2* were 323.8 kb, 59.14 kb, 100 kb, 73.66 kb, and 3.385 kb, respectively. They were extended by 2 kb toward the upstream direction and 2 kb toward the downstream direction. SNPs of these 5 genes for Han Chinese people in Beijing were downloaded from www.hapmap.org, and 55 tag SNPs were selected using the Tagger program of the Haploview software version 4.0. Of these, 33 were located in *PPP3CA*, 4 were located in *PPP3CB*, 12 were located in *PPP3CC*, 3 were located in *PPP3R1*, and 3 were located in PPP3R2. See Table 1.

Table 1 The calcineurin-encoding genes for SNP analysis in this study

Gene	Position	dbSNP ID	Locus	Alleles	Allele frequency*	Allele frequency#	H-W equilibrium	
							χ^2	*P*
PPP3CA								
1	10240141	rs13123962	Intron 1	C/T	C:0.37	C:0.38	0.40	0.48
2	102405174	rs2850972	Intron 1	C/T	C:0.37	C:0.32	0.51	0.42
3	102180970	rs2251238	Intron 10	C/T	C:0.17	C:0.18	1.34	0.21
4	102430245	rs2850355	Intron 1	C/T	T:0.47	T:0.48	0.71	0.28
5	102397986	rs2850965	Intron 1	G/T	G:0.11	G:0.12	0.13	0.85
6	102303778	rs4478191	Intron 2	C/T	T:0.33	T:0.26	1.72	0.15
7	102259114	rs12639641	Intron 2	C/T	C:0.39	C:0.31	5.31	0.01
8	102322069	rs10050301	Intron 2	A/G	A:0.38	A:0.36	0.11	0.87
9	102299759	rs2695215	Intron 2	A/C	A:0.24	A:0.32	2.61	0.10
10	102459770	rs2850371	Intron 1	A/G	G:0.46	G:0.47	2.98	0.09
11	102284612	rs10516472	Intron 2	A/G	G:0.10	G:0.06	0.61	0.39
12	102198890	rs1405686	Intron 10	C/T	T:0.13	T:0.17	1.21	0.21
13	102365194	rs2851007	Intron 1	A/G	A:0.26	A:0.35	2.76	0.08
14	102332047	rs2659509	Intron 2	A/C	C:0.38	C:0.28	0.59	0.40
15	102253020	rs2732512	Intron 2	C/T	C:0.23	C:0.20	0.61	0.42
16	102303638	rs2695220	Intron 2	A/G	G:0.14	0.17	1.43	0.22
17	102241124	rs3804358	Intron 3	C/G	C:0.13	C:0.12	0.45	0.49
18	102260259	rs6820738	Intron 2	G/T	G:0.32	G:0.37	0.40	0.51
19	102260644	rs6532920	Intron 2	A/G	A:0.49	A:0.45	0.09	0.72
20	102317089	rs17030887	Intron 2	C/T	C:0.23	C:0.24	0.09	0.76
21	102303638	rs2659544	Intron 2	C/G	G:0.14	G:0.18	2.43	0.11
22	102298759	rs2695214	Intron 2	A/G	C:0.39	C:0.43	0.10	0.71
23	102303638	rs2659510	Intron 7	A/C	G:0.24	G:0.35	1.89	0.90
24	102322700	rs17030900	Intron 2	C/G	G:0.20	G:0.23	0.34	0.51
25	102320175	rs2850983	Intron 2	A/G	G:0.16	G:0.18	4.71	0.02
26	102454392	rs2044041	Intron 1	A/T	A:0.11	A:0.15	0.76	0.32
27	102439907	rs3789753	Intron 1	A/G	A:0.10	A:0.15	0.78	0.31
28	102480291	rs17031191	Intron 1	C/T	T:0.11	T:0.15	0.01	0.91
29	102406885	rs3804423	Intron 1	A/G	A:0.11	A:0.10	0.007	0.98
30	102312830	rs2659501	Intron 2	C/T	T:0.42	T:0.51	1.22	0.21
31	102247953	rs931870	Intron 3	C/G	C:0.30	C:0.29	0.27	0.59
32	102327937	rs2850988	Intron 2	A/G	A:0.30	A:0.36	0.03	0.89
33	102221146	rs3804357	Intron 7	A/C	A:0.23	0.26	0.12	0.71
34	102442409	rs1031034	intron 1	G/T	T:0.47	T:0.45	0.09	0.73

PPP3CB								
35	74904832	rs3763679	Intron 3	C/T	T:0.29	T:0.28	1.14	0.30
36	74867057	rs12644	exon 14, 3′-UTR	A/G	A:0.46	A:0.50	0.38	0.52
37	74891291	rs12245450	Intron 9	A/C	C:0.30	C:0.32	0.76	0.39
PPP3CC								
38	22442424	rs2461483	intron 10	C/T	T:0.49	T:0.37	1.76	0.12
39	22376751	rs10108011	intron 1	A/G	G:0.24	G:0.27	0.35	0.51
40	22361446	rs7821470	intron 1	C/T	C:0.20	C:0.19	0.43	0.45
41	22359385	rs10096012	intron 1	A/G	G:0.14	G:0.13	0.16	0.69
42	22369931	rs1879793	intron 1	C/T	C:0.30	C:0.32	3.61	0.06
43	22446882	rs2449341	intron 12	A/C	C:0.34	C:0.35	0.82	0.36
44	22426198	rs2461489	intron 5	C/T	C:0.49	C:0.44	1.41	0.26
45	22430019	rs1075534	intron 6	A/G	A:0.36	A:0.39	1.76	0.13
46	22375143	rs7837713	intron 1	C/T	C:0.13	C:0.12	0.18	0.68
47	22405456	rs2461494	intron 5	C/T	C:0.18	C:0.24	2.13	0.11
48	22454359	rs7430	3′-UTR	C/G	G:0.40	G:0.33	0.15	0.70
49	22380522	rs13271367	intron 1	A/G	A:0.48	A:0.47	11.2	<0.01
PPP3R1								
50	68330051	rs13004394	intron 1	C/T	C:0.12	C:0.17	1.41	0.22
51	68329099	rs4671887	intron 1	A/C	C:0.36	C:0.33	1.03	0.31
52	68331452	rs2029091	intron 1	C/T	C:0.47	C:0.49	0.12	0.71
PPP3R2								
53	103397893	rs1323433	3′ near gene	A/G	A:0.33	A:0.25	0.18	0.69
54	103394171	rs3739723	exon 1, 3′-UTR	A/T	A:0.28	A:0.22	0.04	0.82
55	103395276	rs1407877	exon 1, 3′-UTR	A/G	A:0.20	A:0.22	0.11	0.75

*: Allele frequency for Chinese Han Population downloaded from Hapmap website (www.hapmap.org), #: Allele frequency for the participants involved in the present study.

Wizard Genomic DNA Extraction Kit (Promega) was used to extract DNA from fresh blood sample (500μL). rs12644 was analyzed using the PCR-RFLP method, while other loci were analyzed using MALDI-TOF-MS (Matrix-Assisted Laser Desorption/ Ionization Time of Flight Mass Spectrometry (10). Assay Designer (Sequenom) was used to design single-base extension primers and PCR primers. DNA samples were diluted to a concentration of 5 ng/μL. Then, 1 μL was mixed with 0.625 μL of PCR buffer, 0.325 μL of 25 mM $MgCl_2$, 0.1 μL of HotStar Taq, 1 μL of 2.5 mM dNTP, 1 μL of PCR primer and 0.95 μL of water, which was performed according to recommendations from manufacturers (DBI®Bioscience). Reactions were conducted in the PCR Thermal Cycler Dice (TaKaRa Biotech, TP600) using the following conditions: Initial denaturation at 94°C for 15 min; and 45 cycles of denaturation at 94°C for 20 s, primer annealing at 56°C for 30 s and elongation at 72°C for 1 min, and extension at 72°C for 3 min. After PCR amplification, the system for next reaction included 0.17 μL of SAP buffer, 0.3 unit of alkaline phosphatase, and 1.53 μL of water. The solution was placed at 37°C for 40 min, then at 85°C for 5 min to cause enzyme inactivation. The reaction system for the single-base primer extension included 0.2 μL of 10x iPLEX buffer, 0.041 μL of iPLEX enzyme, 0.2 μL of termination mix, 0.804 μL of 10 μM extension primer, and 0.755 μL of water. The reaction was performed under the following conditions: 30 s at 94°C; and 5 s at 94°C, 5 s at 52°C, 5 s at 80°C, and 3 min at 72°C, for 45 cycles. This reaction product was treated with 6 mg of cation-exchange resin (Sequenom) and 25 μL of water for suspension. MassARRAY Nanodispenser (Sequenom) was used to apply

the genotyped samples to the spectroCHIP. MALDI-TOF MS (ABI 4800 plus, Applied Biosystems, Foster City, CA, USA) was used for analysis. The results were read by the MassARRAY RT software system (Sequenom), and the genotyping assay was completed using the MassARRAY Typer software.

Statistical Analysis. Double data entry was performed using EpiData version 3.1 to create a data bank, and logic checks were performed. SAS 9.2 (SAS Institute) was used for data analysis using chi-squared and analysis of variance (ANOVA) tests. The two-factor repeated measures ANOVA was used for the correlation between polymorphisms and initial values of endurance phenotypes and the sensitivity to training. Corrected P-values were used for multiple comparisons. The effect sizes of polymorphisms correlated with the VO2max and RE are expressed as Cohen's d (95% CI). P<0.05 was considered to indicate a difference was statistically significant.

RESULTS

To determine whether variants in the genes encoding calcineurin are associated with response to aerobic training, genotypes of participants were compared with various exercise measures following an 18-week aerobic training (Table 2). Because the genotype distributions of rs13271367, rs12639641, and rs2850983 did not meet Hardy-Weinberg equilibrium, these loci were not analyzed further. Additionally, because a large amount of data was generated, only statistically significant polymorphic loci are listed in Table 2. Compared to the baseline measures, the participants' VO_2max increased, while their running economy (measured at 12 km/h) decreased. First, genotypes and VO_2max were compared. After correction for multiple comparisons, rs3804423 and rs2850965 in *PPP3CA* were significantly correlated with pre-training value of VO_2max (P<0.05). rs3804358 in *PPP3CA* and rs4671887 in *PPP3R1* were correlated with the sensitivity to training for VO_2max and had marked genotype x training interaction effects; these differences were statistically significant (P<0.05).

Table 2. Single nucleotide polymorphisms associated with VO_2max (L/min)

SNP	VO_2max (pre-training)	VO_2max (post-training)	*P* (genotype)	*P* (training)	interaction (genotype*training)
PPP3CA					
rs2850965			0.001	0.042	0.412
GG(n=4)	3.67±0.36	3.47±0.37			
GT(n=24)	3.65±0.48	3.76±0.49			
TT(n=98)	3.37±0.34	3.43±0.35			
			0.004	0.011	0.091
rs3804423					
AA(n=5)	3.36±0.43	3.55±0.44			
AG(n=22)	3.60±0.48	3.74±0.43			
GG(n=99)	3.39±0.37	3.43±0.39			
			0.217	0.793	0.002
rs3804358					
CC(n=6)	3.25±0.40	3.12±0.12			
CG(n=27)	3.62±0.40	3.52±0.35			
GG(n=93)	3.41±0.39	3.52±0.41			
PPP3R1					
rs4671887			0.514	0.275	0.004
CC(n=21)	3.36±0.0.27	3.34±0.31			
AC(n=49)	3.50±0.40	3.48±0.40			
AA(n=56)	3.43±0.40	3.56±0.44			

Note: Because of the large amount of data, for simplicity Table 2 only lists the loci that reached statistical significance.

Genotypes and performance (response to aerobic training) in running economy were also compared. Again, the data presented in the table (Table 3) include only statistically significant loci. rs3739723 (*PPP3R2*) was correlated with the sensitivity to training for running economy and had a marked genotype x training interaction effect; this difference was statistically significant (P<0.05).

Table 3. Single nucleotide polymorphism associated with running economy (L/min) at 12 km/h

SNP	RE (pre-training)	RE (post-training)	*P* (genotype)	*P* (training)	interaction (genotype* training)
PPP3R2					
rs3739723			0.402	<0.001	0.031
AA(n=7)	2.79±0.08	2.52±0.10			
AT(n=41)	2.65±0.28	2.39±0.24			
TT(n=78)	2.70±0.29	2.50±0.27			

Note: Because of the large amount of data, for simplicity Table 3 only lists the locus that reached statistical significance.
RE: running economy

The effect sizes of SNPs rs2850965, rs3804423, rs3804358, rs4671887, and rs3739723 correlated with VO_2max and running economy are shown in Table 4.

Table 4. Effect size of SNPs significantly associated with VO_2max or running economy [Cohen's d (95% CI)]

	rs2850965	rs3804423	rs3804358	rs4671887	rs3739723
pre-training					
aa/ab	0.01(-0.89,0.96)	0.51(-0.41,1.48)	0.82(-0.08,1.90)	0.37(-0.19,0.87)	0.42(-0.19,0.94)
aa/bb	0.76(0.29,1.23)	0.09(-0.35,0.53)	0.32(-0.11,0.79)	0.20(-0.34,0.68)	0.32(-0.11,079)
ab/bb	0.75(0.30,1.21)	0.62(0.22,1.13)	0.56(0.16,0.97)	0.18(-0.23,0.61)	0.08(-0.35,0.50)
post-training					
aa/ab	0.59(-0.35,1.57)	0.56(-0.43,1.56)	1.20(0.19,2.04)	0.37(-0.20,0.94)	0.42(-0.21,1.13)
aa/bb	0.12(-0.31,0.57)	0.30(-0.13,0.75)	0.87(0.40,1.29)	0.53(0.03,1.10)	0.08(-0.40,0.57)
ab/bb	0.91(0.41,1.32)	0.86(0.42,1.35)	0.07(-0.31,0.48)	0.21(-0.22,0.65)	0.48(0.04,0.91)

Note: Value is Cohen's d (95% CI) effect size. d <0.2, 0.2 - 0.5, and > 0.8 are, respectively, small, medium, and large effects. "a" refers to the low frequency of alleles, "b" refers to the high frequency of alleles.

DISCUSSION

To build on previous studies that indicated that variants in the genes encoding CN are associated with sensitivity to strength training (9-13), this study assessed possible associations between SNPs in the genes encoding CN and markers of response to aerobic training, VO_2max and running economy. Four significant SNPs were identified as associating with initial values of VO_2max or the sensitivity to training: rs2850965, rs3804423, and rs3804358 in *PPP3CA* and rs3739723 in *PPP3R1*. A fifth SNP, rs3739723 in *PPP3R2*, was correlated with the sensitivity to training for running economy. The latter finding is similar to the findings of Bray *et al.* (15).

The VO_2max value depends on both central and peripheral factors. The central mechanism is limited by cardiac structure, function, and cardiac cell maturation. Cardiac hypertrophy also depends on the CN signaling pathway (16). In CnAβ-deficient mice, CN activities are lower, their hearts are smaller, and they have reduced capacity to respond to myocardial hypertrophy caused by external stimuli (17). Similarly, if mutations are introduced in CnB to block its phosphatase activity activated by calcium ions, then mouse vascular development is abnormal; this suggests that the Ca^{2+}-CN signaling pathway

functions early in vascular development and the surrounding tissues (18); in other words, CN can influence the cardiac structure and function. Thus, polymorphisms within the *PPP3CA* gene may affect the skeletal muscle. rs3739723 in the *PPP3R2* gene was found in this study to be correlated with the sensitivity to training for running economy, which is a determining factor for aerobic capacity under submaximal load (19).

The peripheral mechanism of VO_2max regulation mainly includes oxidase activity, muscle fiber types, and mitochondrial density. CN may participate in several of these activities. CnA, encoded by *PPP3CA*, regulates CN expression in the skeletal muscle (20). Additionally, CN up-regulates slow muscle troponins in mouse myoblasts and rat soleus muscles (21). In trangenic mice with highly expressed CN, there is an increase in the expression of myoglobins and slow muscle troponins and in GLUT4 expression in skeletal muscle (22). Thus, CN is proposed to regulate the expression of GLUT4. Further, CN has an important effect upon mitochondrial biogenesis (23, 24). Finally, a loss of skeletal muscle oxidative capacity was observed after CN inhibitors were orally given to organ transplant patients (25). Therefore, CN plays important regulatory roles in muscle function. Combined with our results, these studies indicate that CN may be important to determining exercise capacity.

CONCLUSION

In summary, the rs2850965, rs3804423, and rs3804358 polymorphisms within the *PPP3CA* gene as well as the rs4671887 polymorphism in the *PPP3R1* gene were correlated with the initial values of VO_2max or the sensitivity to training, and the rs3739723 polymorphism in the *PPP3R2* gene was correlated with the sensitivity to training for running economy. Therefore, variants of CN-encoding genes may partly explain the individual differences in response to aerobic training.

APPLICABLE REMARKS

- The SNPs, rs2850965, rs3804423, and rs3804358 in *PPP3CA* and rs3739723 in *PPP3R1*, were identified as associating with initial values of VO_2max or the sensitivity to training.
- The SNP, rs3739723 in *PPP3R2*, was correlated with the sensitivity to training for running economy.

ACKNOWLEDGMENTS

This work was supported by Science and Technology Department of Henan Province (Grant No.142700410395).

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