Linkage between a muscle-specific CK gene marker and $\dot{V}O_{2max}$ in the HERITAGE Family Study

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ABSTRACT

RIVERA, M. A., L. PÉRUSSE, J.-A. SIMONEAU, J. GAGNON, F. T. DIONNE, A. S. LEON, J. S. SKINNER, J. H. WILMORE, M. PROVINCE, D. C. RAO, and C. BOUCHARD. Linkage between a muscle-specific CK gene marker and $\dot{V}O_{2max}$ in the HERITAGE Family Study. *Med. Sci. Sports Exerc.*, Vol. 31, No. 5, pp. 698–701, 1999. **Purpose:** We have reported a significant association between $\dot{V}O_{2max}$ in the sedentary state and its response (Δ $\dot{V}O_{2max}$) to an endurance training program with a muscle-specific creatine kinase (CKMM) gene polymorphism. The purpose of this study was to test the hypothesis of genetic linkage between the same CKMM marker and $\dot{V}O_{2max}$ in the sedentary state as well as Δ $\dot{V}O_{2max}$. **Methods:** Sib-pair linkage analysis was performed on 277 full sib-pairs from 98 Caucasian nuclear families of the HERITAGE Family Study. $\dot{V}O_{2max}$ was measured during cycle ergometry tests before and after 20 wk of endurance training. The CKMM polymorphism was detected by the polymerase chain reaction and digestion with the *NcoI* restriction enzyme. **Results:** Frequencies for the rare (1170 base pairs) and common (985 + 185 base pairs) alleles were 0.32 and 0.68, respectively. No significant linkage ($t = -0.02$, $P = 0.49$) was detected between the CKMM marker and the age and sex adjusted $\dot{V}O_{2max}$ ($mL \cdot kg^{-1} \cdot min^{-1}$) in the sedentary state. However, after adjustment of Δ $\dot{V}O_{2max}$ for the effects of age, sex, initial $\dot{V}O_{2max}$, and body mass, evidence for linkage between the CKMM locus and Δ $\dot{V}O_{2max}$ was suggestive ($P = 0.04$). **Conclusion:** The present results provide further support for the notion that the CKMM gene, or some gene in close linkage disequilibrium with it, may contribute to individual differences in the $\dot{V}O_{2max}$ response to endurance training. **Key Words:** DNA, POLYMERASE CHAIN REACTION, GENETIC VARIATION, EXERCISE, RESTRICTION ENZYME, CHROMOSOME 19

Several reports provide support for the hypothesis that muscle-specific creatine kinase (CKMM) is a legitimate candidate gene to investigate in relation to maximal oxygen uptake ($\dot{V}O_{2max}$) and its response (Δ $\dot{V}O_{2max}$) to endurance training. An early study indicated that a CKMM protein charge variant was weakly associated with the ability to perform during a 90-min endurance test (1). An improved resistance to fatigue was observed in CKMM gene knockout mice (10). A recent report from our laboratory has shown a significant association between the CKMM genotype and the Δ $\dot{V}O_{2max}$ in both parents and adult offspring of the HERITAGE Family Study (8). In the offspring, the homozygotes for a CKMM *NcoI* rare allele had a significantly lower Δ $\dot{V}O_{2max}$ than heterozygotes or homozygotes for the common allele. Furthermore, the frequency of homozygotes for the rare CKMM *NcoI* allele was at least three times that of the other genotypes among the lowest decile of the Δ $\dot{V}O_{2max}$ (low responders to training). This genotype was not observed among the high responders to training (upper decile of Δ $\dot{V}O_{2max}$). In the same study (8), the $\dot{V}O_{2max}$ of the parents in the sedentary state was significantly associated with the CKMM genotype.

Given these data it would seem useful to examine the cosegregation within families between the CKMM *NcoI* marker and $\dot{V}O_{2max}$ as well as Δ $\dot{V}O_{2max}$. The cosegregation within families approach is a further strategy that allows us to examine the likelihood that the CKMM *NcoI* marker is genetically linked to a locus affecting $\dot{V}O_{2max}$ and its Δ to endurance training. Since we have already demonstrated a statistically significant association (association is a property of the alleles) between the CKMM *NcoI* rare allele and a low Δ $\dot{V}O_{2max}$ in biologically unrelated adults and in a sample of unrelated adult offspring (8), we thought that it was of interest to examine the likelihood of genetic linkage

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for $\dot{V}O_{2max}$ in the sedentary state as well as the responsiveness to endurance training (Δ $\dot{V}O_{2max}$) by examining the allelic concordance and discordance in sib-pairs at the CKMM *NcoI* marker and their relations to either of these phenotypes. In contrast to association studies, evidence for linkage implies that the gene or some other gene in the vicinity of the locus is genetically linked to the phenotype of interest. Therefore, the purpose of this study was to test whether there was genetic linkage between the CKMM *NcoI* (3) restriction site polymorphism (RSP) and $\dot{V}O_{2max}$ in the sedentary state as well as Δ $\dot{V}O_{2max}$ in response to the HERITAGE Family Study endurance training program.

METHODS

Subjects. The HERITAGE Family Study is a multicenter clinical trial conducted by five institutions in the United States and Canada. The overall objective of the study is to investigate the role of the genotype in cardiovascular, metabolic, and hormonal responses to aerobic exercise training and to changes in cardiovascular disease and diabetes risk factors. The aims, design, and measurement protocol of the study have been described before (2). The sample for the present study consists of 495 subjects from 98 Caucasian nuclear families. These families were composed of both parents and at least two offspring (mean of 3.2 offspring per family) aged between 17 and 65 yr. Both before and after training, $\dot{V}O_{2max}$ determinations were available in 475 subjects. Two hundred and seventy-seven (277) pairs of sibs were available for the linkage analysis in the sedentary state phenotype and 260 such pairs for Δ $\dot{V}O_{2max}$ in response to endurance training. The study protocol had been previously approved by each of the Institutional Review Boards of the HERITAGE Family Study research consortium. Written informed consent was obtained from each participant.

HERITAGE endurance exercise training program. Briefly, subjects exercised an average of three times per week for 20 wk following a standardized protocol that required the use of a cycle ergometer (Universal Aerobicycle IV, Cedar Rapids, IA) in the sitting position. The cycle ergometer was connected to a computer system (Universal Mednet, Cedar Rapids, IA) that adjusted the power output of the ergometers to maintain constant training heart rates. During the initial 2 wk, subjects trained at a heart rate associated with 55% of each subject's $\dot{V}O_{2max}$ for 30 min per session. This was gradually increased to 50 min by the end of the 14th week at the heart rate associated with 75% of $\dot{V}O_{2max}$. These levels of intensity and duration were maintained through the remaining 6 wk.

Maximum oxygen uptake ($\dot{V}O_{2max}$) measurements. Both before and after the training program, two maximal exercise tests were performed on separate days on a cycle ergometer (SensorMedics Ergo-Metrics 800S, Yorba Linda, CA) connected to a metabolic measurement cart (SensorMedics 2900). The exercise tests were conducted at almost the same time of day with at least 48 h between tests. In the first test, initial power output was 50 W for 3 min, followed by increases of 25 W each 2 min until volitional exhaustion. In special circumstances (older, smaller, or less fit individuals), the test was started at 40 W, with increases of 10–20 W each 2 min thereafter. During the first two stages of the second maximal exercise test, subjects exercised for 10–12 min at an absolute (50 W) and at a relative power output equivalent to 60% of their $\dot{V}O_{2max}$. They then exercised for 3 min at a relative power output that was 80% of their $\dot{V}O_{2max}$, after which resistance was increased to the highest power output attained in the first maximal test. If the subjects were able to pedal after 2 min, power output was increased each 2 min thereafter until they reached volitional fatigue. Throughout each exercise test, $\dot{V}O_2$, $\dot{V}CO_2$, and $\dot{V}_E$, were recorded as a rolling average of three 20-s intervals using the SensorMedics 2900 metabolic measurement cart. Gas analyzers were calibrated before each maximal test with gases of certified concentrations. Post-test calibration verifications were also conducted after each maximal test. Heart rate was determined from an electrocardiogram and values recorded during the last 15 s of each stage. The criteria for $\dot{V}O_{2max}$ were: respiratory exchange ratio >1.1, heart rate within 10 beats·min^{-1} of the predicted maximal for age, and a plateau in $\dot{V}O_2$ (Δ <100 mL·min^{-1} in the last three consecutive 20-s averages). All subjects achieved a $\dot{V}O_{2max}$ by one of these criteria in at least one of the two tests. After the two maximal tests were completed, both tests were averaged if $\dot{V}O_2$ was within 5% of each other. Otherwise, the test with the higher $\dot{V}O_2$ was chosen.

Genotype determinations. DNA was extracted from lymphoblastoid cell lines after a standard protocol of digestion by proteinase K and purification with phenol-chloroform. The polymerase chain reaction (PCR) was performed in a DNA thermal cycler (Perkin Elmer Cetus, GeneAmp 9600, Norwalk, CT). Primers for the *NcoI* RSP of the CKMM gene were as follows: 5′ GTG-CGG-TGG-ACA-CAG-CTG-CCG 3′ and 5′ CAG-CTT-GGT-CAA-AGA-CAT-TGA-GG 3′ (4). These provided a product of 1170 base pairs (bp). Total volume of the PCR was 25-μL of a reaction mixture containing 10% DMSO, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 200 μM of each dATP, dCTP, dGTP, and dTTP, 0.3 μM of each forward and backward primers, 0.75 units of Taq polymerase (Perkin Elmer Cetus, Norwalk, CT), and 500 ng of DNA. The amplification protocol was: 1) one cycle of denaturation at 95°C for 5 min; 2) 30 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 45 s; and 3) one final 5 min elongation cycle at 72°C. Preventive contamination measures were taken by the inclusion of PCR reaction mixture without DNA (negative control) in every run of amplification.

RFLP analysis. The PCR product was digested with 10 units of *NcoI*. Restriction digest conditions were those recommended by the enzyme manufacturer (New England Biolabs, Mississauga, Ontario, Canada). The resulting fragments were separated by horizontal electrophoresis on 1.2% agarose gels. Each gel was run for 60 min at 150 mA while refrigerated at 10°C, stained with ethidium bromide, and photographed under UV transmitted lights. The ϕX174 DNA, digested with *HaeIII*, was used as length marker to

TABLE 1. Characteristics of the adult offspring of the HERITAGE Family Study.

Variable	Adult Offspring	
	Men (N = 145)	Women (N = 162)
Age (yr)	25 ± 6	25 ± 6
Body mass (kg)	82 ± 17*	64 ± 13
BMI (kg/m^2)	26 ± 5*	24 ± 5
Baseline $\dot{V}O_{2max}$ (mL·min^{-1})	3305 ± 494*	2056 ± 302
After $\dot{V}O_{2max}$ (mL·min^{-1})	3794 ± 510*	2426 ± 358
Δ $\dot{V}O_{2max}$ (mL·min^{-1})	489 ± 246*	369 ± 189
% Δ $\dot{V}O_{2max}$	15 ± 6*	18 ± 7

Values are Means ± SD.
* Mean for men significantly different from that for women; Both sexes had a significant ($P < 0.05$) increase in Δ $\dot{V}O_{2max}$; BMI = body mass index; Δ $\dot{V}O_{2max}$ (mL·min^{-1}) = After training $\dot{V}O_{2max}$ (mL·min^{-1}) minus Baseline $\dot{V}O_{2max}$ (mL·min^{-1}).

estimate the size of the digested DNA fragments. The allele without the *NcoI* restriction site was designated as allele 1170 bp, while the allele with the polymorphic *NcoI* site was designated as allele 985 + 185 bp.

Statistical analysis. Allele frequencies were estimated using the gene counting method. A Chi-square test was used to examine sex differences in allele and genotype frequencies and to determine whether the observed genotype frequencies were in Hardy-Weinberg equilibrium. Correlation coefficients (Pearson r) were calculated to quantify the degree of linear association between two variables. Pretraining $\dot{V}O_{2max}$ (mL·kg^{-1}·min^{-1}) was adjusted for age and sex, while the Δ $\dot{V}O_{2max}$ (mL·min^{-1}) was adjusted for age, sex, body mass, and pretraining $\dot{V}O_{2max}$. These adjustments were made by linear regression procedures. The pretraining $\dot{V}O_{2max}$ (mL·kg^{-1}·min^{-1}) and Δ $\dot{V}O_{2max}$ (mL·min^{-1}) residuals were used in linkage calculations. These tests, except the linkage calculations, were performed by using the SAS software (SAS Institute, Cary, NC) for personal computer (version 6.08).

Before the linkage analysis, all the families were found to comply with a Mendelian transmission pattern at the CKMM *NcoI* marker. Linkage analysis using the single locus sib-pair method (5,10) was performed to examine the evidence for genetic linkage of each of the two phenotypes ($\dot{V}O_{2max}$ and Δ $\dot{V}O_{2max}$) with the CKMM *NcoI* RSP. The sib-pair linkage method is based on the regression of the squared sib-pair phenotype difference on the estimated proportion of alleles shared identical by descent at the locus. A one-sided *t*-test was used to evaluate whether the regression coefficient was negative. A negative coefficient would indicate linkage. The single locus sib-pair method is a nonparametric "model free" procedure of linkage analysis and assumes that the sib-pairs come from a random mating population and that the population is in gametic equilibrium. The linkage analysis was performed using the SIBPAL program of the Statistical Analysis for Genetic Epidemiology (S.A.G.E.) package, Release 3 (10).

RESULTS

The physical characteristics of the sample are presented in Table 1. $\dot{V}O_{2max}$ (mL·kg^{-1}·min^{-1}) in the sedentary state was significantly ($P < 0.05$) correlated with age (r = −0.25) in male adult offspring. Δ $\dot{V}O_{2max}$ (mL·min^{-1}) was correlated ($P < 0.05$) with body mass in both male (r = 0.15) and female (r = 0.18) adult offspring. The allele frequencies (1170 bp allele = 0.32 and 985 + 185 bp allele = 0.68) were not significantly different between the male and female offspring ($P > 0.05$). The observed genotypic distributions did not differ from those predicted by the Hardy-Weinberg equilibrium.

No significant linkage ($t = -0.02$, $P = 0.49$, sib-pairs = 277) was detected between the CKMM marker and $\dot{V}O_{2max}$ (mL·kg^{-1}·min^{-1}) in the sedentary state. However, the analysis yielded weakly suggestive evidence ($t = -1.7$, $P = 0.04$, sib-pairs = 260) for genetic linkage between the CKMM locus and the covariate (age, sex, body mass, and pretraining $\dot{V}O_{2max}$) adjusted Δ $\dot{V}O_{2max}$ (mL·min^{-1}).

DISCUSSION

The present study tested the hypothesis of genetic linkage between $\dot{V}O_{2max}$ in the sedentary state and Δ $\dot{V}O_{2max}$ with the CKMM *NcoI* marker in sib-pairs from the HERITAGE Family Study. We failed to detect linkage with $\dot{V}O_{2max}$ in the sedentary state since the observed squared sib-pair differences for this trait did not decrease with an increase in the proportion of alleles shared identically by descent by the sib-pairs at the CKMM *NcoI* locus. The observation of a suggestive linkage between Δ $\dot{V}O_{2max}$ and the CKMM *NcoI* marker in the present study implies that siblings sharing two alleles identical by descent (IBD) at the CKMM *NcoI* locus have a more similar Δ $\dot{V}O_{2max}$ than sib-pairs sharing fewer alleles IBD. One may regard the present finding as providing additional support, although not strong, for the notion that the CKMM gene or a gene in close proximity may influence individual variation in the $\dot{V}O_{2max}$ response to endurance training of sedentary subjects.

The present study adds to our previous finding of a significant association between the CKMM genotype and the Δ $\dot{V}O_{2max}$ to endurance training in the HERITAGE Family Study (8). In fact, since linkage analysis is less powerful than association studies (7), it would not be surprising to find stronger evidence for association than for linkage at a true causal gene, even if the marker was more polymorphic. Moreover, the earlier observations that a CKMM protein charge variant was weakly associated with endurance performance (1) and the report that knocking out the CKMM gene in mice led to a slightly improved resistance to fatigue (11) strengthen the notion that CKMM is an important gene for the $\dot{V}O_{2max}$ responsiveness to endurance training in sedentary subjects. Even though we cannot rule out the possibility that another gene in linkage disequilibrium with the CKMM gene (region q13.2-q13.3 of chromosome 19 (6)) could be responsible for the suggestive linkage with the responsiveness to endurance training, the evidence accumulated thus far strongly suggests that the CKMM gene is responsible for these genetic effects.

The fact that there was no evidence for linkage with $\dot{V}O_{2max}$ in the sedentary state cannot be readily explained. One possibility is that the contribution of the locus is not very large and that its effects are maximized and thus made more detectable when people are exposed to regular exercise. On the other hand, we were not able to associate this particular CKMM marker with the status of elite endurance athlete (9). Besides the fact that the NcoI marker may not be the true cause of the associations reported in our other studies (8), it is also possible that at the elite level there are other genes playing more important roles, thus reducing even further the contribution of the CKMM locus. It would be useful next to identify the true CKMM mutation, indexed by the *NcoI* polymorphism, responsible for the variation in $\dot{V}O_{2max}$ trainability. In conclusion, the present findings provide further support for the notion that the CKMM gene plays a role in the responsiveness of $\dot{V}O_{2max}$ to endurance training.

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