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Increased Skeletal Muscle Phosphocreatine Recovery after Sub-maximal Exercise is Associated with Increased Carotid Intima-Media Thickness

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Abstract

Background—The association between skeletal muscle mitochondrial function and CVD risk in healthy subjects is unknown.

Methods—Forty subjects were evaluated for CVD risk with lipid profile, oral glucose tolerance test and measurement of carotid intima-media thickness (cIMT). Skeletal muscle mitochondrial function was determined by phosphocreatine recovery after sub-maximal exercise with ³¹Phosphorous-MRS and represented as τ PCr.

Results— τ PCr was positively associated with age ($r=+0.41$; $P=0.009$) and cIMT ($r=+0.50$; $P=0.001$) on univariate analyses. In multivariate regression analysis controlling for age, the association between τ PCr and cIMT remained significant ($\beta=0.003$; $P=0.03$). This association remained significant after controlling for traditional risk factors for CVD including age, gender, tobacco use, BMI, blood pressure, cholesterol and fasting glucose in a combined model ($\beta=0.003$; $P=0.04$; R^2 for overall model: 0.53; $P=0.008$ for overall model).

Conclusions—These data suggest a novel association between skeletal muscle τ PCr and increased cIMT, independent of age or traditional CVD risk factors.

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Keywords

MRS; spectroscopy; mitochondria; cardiovascular risk

Introduction

Inherited mitochondrial disorders are associated with a variety of cardiovascular diseases (CVD) including conduction defects, cardiomyopathy, septal and ventricular hypertrophy and hypokinesia [1]. Conversely, atherosclerosis is associated with local mitochondrial dysfunction and mitochondrial DNA damage within the cardiovascular system [2]. However, the association of mitochondrial dysfunction with CVD in otherwise healthy subjects is not yet known. As a first step to assessing the relationship between mitochondrial function and CVD risk in otherwise healthy men and women, we measured mitochondrial function in skeletal muscle *in vivo* and determined its association with CVD risk markers. In this study, we determined skeletal muscle mitochondrial function using ^{31}P Phosphorous-magnetic resonance spectroscopy (^{31}P -MRS) to assess phosphocreatine (PCr) recovery rate after sub-maximal exercise among healthy men and women, and related PCr recovery with carotid intima-media thickness (cIMT). PCr recovery after increasing demand by sub-maximal exercise is primarily an oxidative process [3]. Therefore, the rate of PCr recovery is thought to reflect *in vivo* mitochondrial oxidative function [4] with prolonged PCr recovery indicating decreased mitochondrial oxidative function. We hypothesized that prolonged PCr recovery, as represented as increased τPCr , would be associated with unfavorable CVD risk markers including cIMT.

Material and Methods

Study Subjects

Forty healthy men and women between the ages of 18 and 55 without known CVD or symptoms of CVD were recruited from the Boston community between July 2008 and August 2009. Subjects were recruited without regard to weight. Data from these subjects were included in a larger study evaluating the association between mitochondrial function and age [5]. However, the relationship between mitochondrial function and CVD risk factors has yet to be reported. Subjects were otherwise healthy without known endocrine dysfunction or chronic illness. Written informed consent was obtained from each subject before testing in accordance with the Subcommittee on Human Studies at the Massachusetts General Hospital (MGH).

^{31}P Phosphorous Magnetic Resonance Spectroscopy Protocol

Mitochondrial function was determined using ^{31}P -MRS to assess PCr re-synthesis following sub-maximal exercise as previously reported [5]. Briefly, subjects were placed in a 60-cm bore, Siemens 3.0T Tim Trio System with an operating frequency of 49.879 MHz for ^{31}P Phosphorous-spectroscopy. Baseline resting spectra were obtained after which subjects were instructed to perform sub-maximal exercise for 3 minutes at a constant load [40% Maximal Voluntary Contraction (MVC)] by performing bilateral quadriceps contractions at 0.5 Hz followed by a 5 minute recovery period. An 8-cm diameter radiofrequency surface coil, tuned for ^{31}P Phosphorous, was fastened proximal to the superior aspect of the patella over the anteromedial aspect of the right thigh, keeping the coil's axis perpendicular to the z-axis of the magnet. A hard pulse of 200 μsec was used for excitation of the ^{31}P Phosphorous signal. The amplitude of the RF pulse was optimized to obtain a maximal ^{31}P Phosphorous signal in single shot acquisitions. Spectral analysis was performed using integrated area-under-the-curve. Mitochondrial function was determined by fitting the time course of PCr

area-under-the-curve during recovery with an exponential curve ($\beta_3 + \beta_2 (1 - e^{-(t/\tau)})$) to generate τ PCr, which describes mitochondrial phosphorylation potential. The initial rate of PCr recovery (V_{iPCr}), represented as $((60/\tau PCr) \times PCr \text{ depletion})$ is another kinetic parameter used to describe PCr recovery and is also associated with mitochondrial oxidative capacity. V_{iPCr} , however, is thought to be insensitive to end-of-exercise metabolic conditions and is favored by some investigators [6].

Biochemical Assessment

Biochemical assessments were made using standard methodology.

Carotid Intima-Media Thickness (cIMT)

Measurement of cIMT was performed as previously described [7]. The reported cIMT is the average over the length of the measured segments on the right and left carotid arteries.

Physical Activity, Nutrition and Body Composition Assessment

Physical activity was determined by the average number of steps taken per day over a 7-day period with an accelerometer (NL-800). Calorie and macronutrient intake was assessed by 4-day food records (Nutrition Data System). Lean body mass was determined by DEXA (Hologic, Inc. 4500).

Statistical Analysis

Normally distributed variables were compared using the Student's t-test, and non-normal variables were compared using the non-parametric Wilcoxon Rank Sums test. Nominal variables were compared using the Chi-square test. Univariate regression analysis was performed using the Pearson correlation coefficient. Multivariate regression analysis with standard least squares modeling was performed for cIMT using τ PCr as an independent variable. The relationship between cIMT and τ PCr was adjusted for age, physical activity, lean body mass and measures of dietary intake and insulin sensitivity in separate models as well as traditional CVD risk factors in a combined model. Sensitivity analyses including only obese subjects were also performed. Further analysis on the effects of age and τ PCr on cIMT was performed by ANOVA after stratification into tertiles of age and higher and lower τ PCr. Statistical analysis was performed using JMP (SAS Institute, Inc., Cary, NC). Statistical significance was determined as $P < 0.05$.

Results

Clinical Characteristics of Study Subjects

Demographic data of the study subjects are shown in Table 1A. Study subjects achieved a mean PCr depletion of 18.3 ± 0.8 , end-of-exercise pH of 6.802 ± 0.030 and an end-of-exercise PCr/Pi ratio of 1.26 ± 0.15 suggesting they exerted appropriate effort during the exercise protocol. τ PCr, V_{iPCr} , MVC, PCr depletion, end-of-exercise pH or end-of-exercise PCr/Pi ratio were not related to weight or BMI status.

Relationship of τ PCr to Cardio-metabolic Risk Parameters

τ PCr was strongly associated with age ($r = +0.41$; $P = 0.009$) and cIMT ($r = +0.50$; $P = 0.001$) in univariate analysis (Figure 1A). τ PCr was not associated with physical activity or macronutrient intake. τ PCr was not associated with traditional CVD risk factors including systolic and diastolic blood pressure, LDL cholesterol, HDL cholesterol, triglycerides or fasting glucose, nor with $\log_{10}$ CRP or NEFA.

Multivariate Regression Analysis of the Relationship between τ PCr and Carotid IMT

In separate independent modeling adjusting for age, lean body mass, BMI and physical activity, the association between τ PCr and cIMT remained significant in all models (all $P < 0.05$). Further independent modeling demonstrated the relationship remained significant controlling for measures of dietary intake, including total caloric intake and macronutrient content, and measures of insulin resistance (all $P < 0.05$).

In an expanded model simultaneously adjusting for traditional CVD risk factors including age, gender, tobacco use, BMI, systolic and diastolic blood pressure, LDL and HDL cholesterol and fasting glucose, τ PCr remained significantly associated with cIMT ($\beta = 0.003$; $P = 0.04$; R^2 for overall model: 0.53; $P = 0.008$ for overall model) (Table 1B).

The overall finding demonstrating an association between τ PCr and cIMT controlling for age and traditional risk factors for CVD was recapitulated in analyses limited to obese subjects only (data not shown).

Effect of Age and Mitochondrial Function on Carotid IMT

Subjects were stratified by age into tertiles (18–35, 35–45 and 45–55 years old) and further stratified by τ PCr to assess the effects of age and τ PCr on cIMT. As the mean and median value for τ PCr within our sample was 48.3 and 46.1 seconds respectively, the sample was divided into two groups: τ PCr < 45 and ≥ 45 seconds. Subjects within higher age tertiles had higher cIMT as expected, regardless of stratification by τ PCr. Among subjects within the same age tertile, subjects with higher τ PCr had higher cIMT (ANOVA $P = 0.002$; Figure 1B).

Relationship of τ PCr to End-Of-Exercise pH and PCr Depletion

τ PCr was significantly associated with end-of-exercise pH ($P = 0.002$) and PCr depletion ($P = 0.0001$) as previously reported [6]. However, τ PCr remained significantly associated with cIMT in multivariate modeling adjusting for end-of-exercise pH and PCr depletion ($P = 0.001$). Furthermore, V_{iPCr} , a kinetic parameter of PCr recovery thought to be independent of end-of-exercise pH, was also significantly associated with cIMT ($P = 0.01$).

Discussion

This study demonstrates a significant and novel association between skeletal muscle PCr recovery after sub-maximal exercise and cIMT, independent of age, physical activity, lean body mass, measures of dietary intake, insulin resistance and traditional CVD risk markers.

These results significantly extend a prior observation suggesting that mitochondrial function is correlated with the degree of peripheral arterial disease in non-diabetic subjects [8]. Furthermore, treatment of stroke prone hypertensive rats with the mitochondrial anti-oxidant MitoQ₁₀ results in improvements in blood pressure and endothelial function and reduced cardiac hypertrophy [9], suggesting a direct association between mitochondrial function and CVD.

Decreased skeletal muscle mitochondrial function may be related to increased cIMT through several possible mechanisms. Skeletal muscle mitochondrial dysfunction may reflect generalized or cardiac mitochondrial dysfunction, which may be directly causal to the development of CVD. Alternatively, skeletal muscle mitochondrial dysfunction may contribute to insulin resistance and dyslipidemia which in turn contribute to increased cIMT. However, in our study, the relationship between cIMT and τ PCr remained significant after controlling for indices of insulin resistance and dyslipidemia. We further controlled for CRP without altering the results. Lastly, mitochondrial function and CVD risk may be associated

through covariates such as age, physical activity, macro-nutrient composition of diet, weight status and body composition parameters. However, this seems unlikely given that the relationship between τ PCr and cIMT remained significant after controlling for these covariates. Our results therefore suggest a close relationship between skeletal muscle mitochondrial function and cIMT independent of these covariates.

Several different MRS based methods have been used to measure skeletal muscle mitochondrial function *in vivo* including the saturation transfer method utilizing ^{31}P -MRS to measure resting ATP synthesis [10], ^{13}C -MRS to measure substrate oxidation [11], and use of ^{31}P -MRS to measure PCr re-synthesis after sub-maximal exercise as a measure of functional capacity of activated muscle [12]. While these techniques may provide different information regarding mitochondrial function, we elected to use ^{31}P -MRS to measure recovery of PCr after sub-maximal exercise, as this technique was previously validated by comparison with mitochondrial DNA content from skeletal muscle biopsy [13]. Furthermore, a more recent study in rats comparing the use of the saturation transfer method and the PCr recovery method after treatment with the mitochondrial complex I inhibitor diphenyleneiodonium (DPI), demonstrated the PCr recovery method to be a more sensitive indicator of mitochondrial dysfunction. 2 weeks treatment with DPI resulted in a 20% decrease in ADP stimulated mitochondrial oxygen consumption rate in isolated muscles *ex vivo* along with a concomitant 46 % decrease in *in vivo* mitochondrial function as determined by PCr recovery after muscle stimulation. However, no differences were noted in resting ATP synthesis rates as determined by saturation transfer [14].

We report the first observation of a significant relationship between skeletal muscle PCr recovery and cIMT. Although a direct causal relationship can not be established from an observational study, and we can not exclude the possibility that atherosclerotic CVD may result in decreased skeletal muscle PCr recovery through reduced blood flow, the significant relationship between skeletal muscle PCr recovery and cIMT is novel and suggests for the first time the possibility that skeletal muscle dysfunction may be related to and contribute to the development of atherosclerotic disease. It will be important in future studies to confirm these findings and to determine whether mitochondrial function in the myocardium and vasculature are related to atherosclerotic indices. Longitudinal studies will also be needed to assess the timing of the relationship of the development of mitochondrial dysfunction to increased cIMT.

Conclusion

In summary, our study demonstrates a strong and novel association between skeletal muscle PCr recovery and cIMT independent of age, physical activity, lean body mass, dietary intake, insulin resistance, or traditional CVD risk markers. This study suggests a novel factor which may contribute to atherosclerotic disease.

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Figure 1A.

Figure 1B.

Figure 1.

Figure 1A. τ PCr is positively associated with carotid IMT. $R=+0.50$; $P=0.001$. Normal weight subjects are depicted by open circles while obese subjects are depicted by closed circles.

Figure 1B. Carotid IMT is associated with τ PCr independent of age. Carotid IMT is depicted in the y-axis in our cohort stratified by age on the x-axis and τ PCr on the z-axis. $P=0.002$ by ANOVA.

Table 1A

Characteristics of study subjects. The data are presented as mean $\pm$ SEM.

N	40
Age (years):	37.9 $\pm$ 1.7
Demographic:	
Gender: [male (%)]	19 (48 %)
Weight category: [obese (%)]	29 (73 %)
Race: [Caucasian (%)]	21 (53 %)
Tobacco (pack years):	4.0 $\pm$ 1.2
Anthropometric:	
Weight (kg):	66.0 $\pm$ 1.7
BMI(kg/m ²):	32.0 $\pm$ 1.1
Waist circumference (cm):	104.0 $\pm$ 2.9
Vital Signs:	
Systolic BP (mm Hg):	119 $\pm$ 2
Diastolic BP (mm Hg):	74 $\pm$ 2
Biochemical:	
Total Cholesterol (mmol/l):	4.57 $\pm$ 0.15
Triglycerides (mmol/l):	1.15 $\pm$ 0.13
HDL Chol (mmol/l):	1.25 $\pm$ 0.05
LDL Chol (mmol/l):	2.87 $\pm$ 0.13
NEFA (mM)	0.55 $\pm$ 0.03
Fasting glucose (mmol/l):	4.9 $\pm$ 0.2
2-hour glucose on OGTT (mmol/l):	6.3 $\pm$ 0.3
Fasting insulin (mU/l)	7.82 $\pm$ 0.98
HOMA:	1.91 $\pm$ 0.41
HbA1c (%):	5.6 $\pm$ 0.1
Log ₁₀ CRP	1.07 $\pm$ 0.11
³¹ P-MRS:	
End-of-Exercise pH:	6.802 $\pm$ 0.030
End-of-Exercise PCr/Pi ratio:	1.26 $\pm$ 0.15
PCr depletion post exercise:	18.3 $\pm$ 0.8
MVC (kg):	48.0 $\pm$ 3.8
τ PCr (sec):	48.3 $\pm$ 2.7
Vi _{PCr} (mM/min):	24.7 $\pm$ 1.5

- Intracellular pH (pH) was calculated by comparing the chemical shift in ppm (δ) of the median area Pi peak relative to that of PCr, using the calibration curve: $\text{pHi} = 6.85 + \log_{10}(3.56 - \delta)/(\delta - 5.64)$.
- Maximum voluntary contraction (MVC) for the quadriceps muscles was measured using standard weights with the subjects in a bilateral leg-extension exercise apparatus within the bore of the MRI scanner before commencement of the exercise protocol. MVC was determined as the maximum amount of weight that could be lifted once.

- All subjects except two had an end-of-exercise pH of <7 and all subjects except one had an end-of-exercise PCr/Pi ratio <4 to indicate sufficient exercise effort. A sensitivity analysis excluding these three subjects was performed without significantly affecting the results. Therefore the results are presented with the three subjects' data included.

Table 1B

Multivariate regression analysis for carotid IMT including τ PCr and traditional cardiovascular risk factors simultaneously in a combined model among all subjects (n=40).

Parameters	β Estimate	Standard Error	<i>P</i>
τ PCr:	0.003	0.001	0.04
Age:	0.005	0.002	0.03
Gender: (male or not)	0.0006	0.02	0.98
Tobacco use:	-0.002	0.003	0.61
BMI:	-0.0002	0.004	0.96
Systolic BP:	0.0007	0.002	0.75
Diastolic BP:	0.002	0.002	0.37
LDL Cholesterol:	0.0005	0.0007	0.48
HDL Cholesterol:	0.002	0.002	0.44
Fasting blood glucose:	0.001	0.0009	0.17

R^2 for the overall model is 0.53 ($P=0.008$).