Published in final edited form as:

NMR Biomed. 2021 January ; 34(1): e4402. doi:10.1002/nbm.4402.

Molecular correlates of MRS-based 31phosphocreatine muscle resynthesis rate in healthy adults

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Abstract

Dynamic phosphorus MRS (³¹P-MRS) is a method used for in vivo studies of skeletal muscle energetics including measurements of phosphocreatine (PCr) resynthesis rate during recovery of submaximal exercise. However, the molecular events associated with the PCr resynthesis rate are still under debate. We assessed vastus lateralis PCr resynthesis rate from ³¹P-MRS spectra collected from healthy adults as part of the CALERIE II study (caloric restriction) and assessed associations between PCr resynthesis and muscle mitochondrial signature transcripts and proteins (NAMPT, NQO1, PGC-1 α , and SIRT1). Regression analysis indicated that higher concentration of nicotinamide phosphoribosyltransferase (NAMPT) protein, a mitochondrial capacity marker, was associated with faster PCr resynthesis. However, PCr resynthesis was not associated with greater physical fitness (VO₂ peak) or messenger ribonucleic acid levels of mitochondrial function markers such as NQO1, PGC-1 α , and SIRT1, suggesting that the impact of these molecular signatures on PCr resynthesis may be minimal in the context of an acute exercise bout. Together, these findings suggest that ³¹P-MRS based PCr resynthesis may represent a valid non-invasive surrogate marker of mitochondrial NAMPT in human skeletal muscle.

Keywords

³¹P-MRS; mitochondria; muscle; nicotinamide phosphoribosyltransferase

INTRODUCTION

Phosphorus MRS (³¹P-MRS) provides *in vivo* non-invasive measurements of skeletal muscle function in humans.¹ Parametric ³¹P-MRS measurements reflecting the recovery time constant of phosphocreatine (PCr), also referred to as the *PCr resynthesis rate*, have been of interest in exercise science because they reflect mitochondrial capacity or maximum adenosine triphosphate (ATP) synthesis via coupled creatine kinase reactions in skeletal muscle. These parametric measurements have shown physiological relevance

CONFLICTS OF INTEREST

The authors have none to declare.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

in assessments of the skeletal muscle mitochondrial capacity and/or function in athletes, sedentary individuals, and individuals with Type 2 diabetes² under normoxic, hypoxic, or exercise conditions.^{3–7} Specifically, PCr resynthesis rate has been shown to decrease from endurance-trained athletes to lean sedentary individuals to obese sedentary subjects to individuals with Type 2 diabetes mellitus.² In addition, exercise training can shorten the PCr resynthesis rate of sedentary individuals⁸ and enhancements in PCr resynthesis rate are associated with enhancements of insulin sensitivity.^{9,10} Together these findings have made skeletal muscle ³¹P-MRS based PCr resynthesis rate a promising biomarker for studies of skeletal muscle biochemistry.

However, the biological mechanisms underlying ³¹P-MRS based PCr resynthesis rate during recovery of submaximal muscle contraction are not fully understood. During exercise, PCr undergoes breakdown to maintain ATP concentration constant in the face of ATP hydrolysis, and the rate of replenishment is driven by several putative physiological and molecular factors. Muscle oxygen availability is one such factor: in exercise trained individuals, fractional inspired oxygen during plantar flexion modulates PCr resynthesis rate under normoxic and hypoxic conditions,^{7,11,12} although in untrained individuals only fairly severe hypoxia influences PCr resynthesis rate.⁶ PCr resynthesis rate is influenced by molecular factors as well. For example, greater ATP_{max} (maximum ATP resynthesis rate (mM/L) for measuring maximum phosphorylation capacity: a ³¹P-MRS readout closely related to PCr resynthesis rate) has been shown to be associated with greater nicotinamide phosphoribosyltransferase (NAMPT) protein level in skeletal muscle in a combined sample of athletes and individuals with Type 2 diabetes or obesity before and after a 3-week exercise intervention.¹³ However, it is not clear whether this correlation still holds within any sub-group of this combined sample, or whether it holds in sedentary individuals who have not undergone exercise training. Recently, relationships between PCr resynthesis rate and molecular signatures of mitochondrial function in human skeletal muscle (NAD(P)H quinone dehydrogenase 1 (NQO1), peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α), and sirtuin 1 (SIRT1)) have been postulated, but not confirmed.^{14,15}

We examined relationships between PCr resynthesis rate and protein or messenger ribonucleic acid (mRNA) levels of multiple mitochondrial genes (NAMPT, NQO1, PGC-1α, and SIRT1) in individuals enrolled in a clinical trial. We hypothesized that, because NAMPT protein is a key component of human skeletal muscle, which facilitates the fast recovery of PCr when it is depleted, greater NAMPT protein levels would be associated with faster PCr resynthesis rate.

2 | EXPERIMENTAL

2.1 | Study participants

The present study included 10 men and 18 women, ages 20–50 years, with body mass index (BMI) from 22 to 27.9 kg/m² at the screening visit, all part of the CALERIE II study (clinical trials registration [NCT00427193](#), [NCT02695511](#)) for investigating mitochondrial dysfunction with aging in metabolically healthy humans. The study was approved by the institutional review board (PBRC – 26039) at Pennington Biomedical Research Center

and conducted in accordance with the *Declaration of Helsinki*. All participants provided written informed consent prior to the study. This retrospective analysis involved 28 healthy participants measured at baseline for maximum oxygen intake (VO_2 peak) during a cardiopulmonary fitness test, PCr resynthesis rate by ^{31}P -MRS, and mRNA or proteins from muscle biopsies.

2.2 | Cardiopulmonary fitness

Assessment of cardiopulmonary fitness was performed on a programmable treadmill (Trackmaster® TmX425C, CareFusion, Newton, KS). Participants were fitted with a flexible respiratory mask and gases were continuously measured with an integrated metabolic measurement system (Parvo Medics TrueOne® 2400, Sandy, UT). The test began with a walk at a zero-inclined level for 2 min, and gradually increased at a rate of 1 m/min until the participant became exhausted. Weighted oxygen intake as VO_2 peak was measured in $\text{mL}/\text{min}/\text{kg}$ during the last two minutes of exercise.

2.3 | MRS data acquisition and quantification

All magnetic resonance experiments were performed on a GE 3 T Signa HDxt scanner (GE Healthcare, Milwaukee, WI, USA). A single loop ^{31}P -tuned surface coil, 6 cm in diameter, was used to acquire ^{31}P -MRS data using a simple pulse and acquire sequence with non-selective excitation. Prior to the ^{31}P -MRS measurements, a single shot fast spin echo (SSFSE) imaging sequence was acquired via the scanner's body coil to determine the anatomical location of the *vastus lateralis* muscle and ^{31}P surface coil (using a vitamin E pill as a phantom at the center of the ^{31}P surface coil for localization). Magnetic field shimming was performed on muscle by monitoring the 127.73 MHz signal from the muscle water and fat protons. The position of the *vastus lateralis* muscle was well within the limit of depth of penetration of the surface coil. A (28 mM) ^{31}P -labeled creatine phosphate phantom placed on the top of the surface coil was used for ^{31}P RF calibration through transmit gain adjustment by nullifying/minimizing the signal for a reference 180° hard pulse. Two different ^{31}P -MRS datasets were acquired: baseline and exercise. In a baseline dataset, a spectrum was acquired (repetition time (T_R) 15 s, 16 time averages per spectra, 5 kHz spectral bandwidth,¹⁶ 4096 points, nonselective RF pulse with nominal 90° flip angle; see Figure S1). The purpose of acquisition of the baseline spectrum is to obtain a high-fidelity estimate of the baseline PCr concentration prior to exercise. This high-fidelity estimate allowed us to determine whether the PCr concentration had fully recovered to the baseline value after exercise. Subsequently for the exercise dataset, a series of 75 partially saturated spectra were acquired ($T_R = 1.5$ s, four dummy scans, four time averages per spectrum, 5 kHz spectral bandwidth,¹⁶ 4096 points, non-selective RF excitation with nominal 90° flip angle). During exercise ^{31}P -MRS dataset acquisition, the participant was instructed to begin isometric leg-kicking exercise¹³ against tight and rigid Velcro straps, during which sequential MRS spectra were collected. The relative height of the PCr peak was monitored in real time, and the participant was instructed to stop kicking when the PCr peak height was reduced to $40 \pm 10\%$ of baseline. This range has shown to provide adequate PCr depletion while maintaining physiological pH. However, we validated the pH levels by calculating the chemical shift differences between the Pi and PCr peaks in the time series data. This ^{31}P -MRS pH measurement makes use of the fact that basic and acidic species of

Pi are in rapid exchange at physiological pH.¹⁷ The post-processing of spectra was executed with jMRUI Version 5.2¹⁸ and quantitation of metabolite peaks was performed in the time domain with the AMARES (Advanced Method for Accurate, Robust, and Efficient Spectral fitting) algorithm.¹⁹ Confirmed PCr resonance peak data was transferred into MATLAB (MathWorks, Natick, MA) and fitted with a mono-exponential equation,

$$P(t) = P(0) + D(1 - e^{(-t/\tau)}), \quad (1)$$

using a non-linear least squares method. $P(t)$, the PCr peak at each moment in time t , is expressed as a function of the recovery time parameter (τ) and scaling parameters ($P(0)$ and D). The parameter $P(0)$ represents the PCr level at the end of exercise (ie the beginning of PCr recovery), whereas D is the difference between the PCr levels at the end of exercise and at the end of recovery. $P(0)$, D , and τ are free parameters whose values are estimated from each ³¹P-MRS scan.

2.4 | Human muscle tissue biopsy

The muscle biopsy was completed on the leg that was not used to collect ³¹P-MRS data. Participants provided approximately 600 mg of muscle using the Bergstrom technique²⁰ in the *vastus lateralis*. Briefly, the skin surface area was disinfected with 7.5% povidone-iodine prep solution (Aplicare, Forest Hills, NY). A topical anesthesia was induced with a mixture of 2% lidocaine HCl (Hospira, Lake Forest, IL) and 0.5% bupivacaine HCl (AUROMEDICS Pharma, Dayton, NJ) to produce local anesthetic effects at the location of muscle biopsy. A 0.75 cm incision was made into the skeletal muscle fascia, fascia fibers were teased apart with the blunt edge of the scalpel, the Bergstrom needle was inserted to remove muscle tissue, and the wound was closed using bandage. The tissue was further processed for mRNA and protein extractions.

2.5 | Statistical analysis

Results are reported as mean $\pm$ standard deviation. Statistical analysis was performed using STATA software Version 12.0 (StataCorp, College Station, TX). We studied the relationship between τ and a set of molecular mitochondrial signatures (NAMPT protein and mRNA levels of PGC-1 α , NAMPT, SIRT1, and NQO1) as well as VO₂ peak. We hypothesized that VO₂ peak and the mitochondrial signatures would be associated with τ . To perform this analysis, we took an incremental model building approach to understand both the independent and joint effects of mitochondrial signatures on τ . Specifically, we implemented the regression model shown in Equation 2 to relate a dependent variable y to independent variables $x_1, \dots, x_k$:

$$y_i = \alpha + \sum_{j=1}^k \beta_j x_{ij} + e_i, \quad e_i \sim N(0, \sigma^2) \quad (2)$$

where the dependent variable y_i is the i th subject's τ , α is the intercept of the regression line, β_j is the regression coefficient for the j th independent variable, and e_i is the additive error term. In the first stage of analysis, we estimated one such regression model for each mitochondrial signature as well as VO₂ peak; i.e., each model had a single independent

variable x_1 , representing the mitochondrial signature or VO_2 peak. In the second stage of analysis, we estimated one regression model for each possible pair of mitochondrial signatures; i.e., each regression model had an independent variable x_1 representing the first mitochondrial signature of the pair, and another independent variable x_2 representing the second mitochondrial signature of the pair. We did this to determine which significant independent variables from the first stage of analysis were most strongly associated with τ . Finally, to understand which mitochondrial signatures were most strongly associated with τ when all are considered jointly as independent variables, we estimated a final model containing all mitochondrial signatures as joint independent predictors. In each case, we used the method of least squares to fit the model. The statistical analysis includes Bonferroni correction for multiple comparisons, and adjusted R^2 is reported as a goodness of fit measure for each regression model.

ANCOVA analyses were conducted to investigate significant associations between the BMI or the depth of subcutaneous fat (fat layer between the ^{31}P -surface coil and the target tissue) and the response variable (τ). Gender and age of subjects were also tested as significant confounder variables during multiple regression analysis. Details of the ANCOVA analysis can be found in supplemental information.

3 | RESULTS

3.1 | Variability in PCr resynthesis rate among healthy individuals

Representative time courses of submaximal exercise were analyzed for all subjects (see Table S2) and two representative curves representing individuals with fast and slow PCr resynthesis rate are illustrated in Figure 1. PCr resynthesis times calculated from mono-exponential model fits are indicated as relatively short (24.1 s) and long (73.3 s).

3.2 | NAMPT is associated with variations in PCr resynthesis rates of human skeletal muscle

Parameters of linear regression were obtained in a systematic, incremental approach to regression from baseline group using “one,” “two,” or “multiple” independent variables (Tables 1 and S1). In the first stage of analysis considering only a single (“one”) independent variable, negative correlation was found between the dependent and the independent variables. NAMPT protein and mRNA levels were significantly associated with faster PCr resynthesis time at baseline ($p < 0.0083$) (Table 1 and Figure 2). In addition, VO_2 and mRNA levels of PGC-1 α , SIRT1, and NQO1 were not significantly associated with PCr resynthesis time (Table 1 and Figure 2). Subsequently, in another regression model involving the combined analysis of a pair of independent variables, NAMPT protein was found to be more strongly associated with the outcome variable than NAMPT mRNA levels (Table S1). Finally, NAMPT protein turned out to be the only significant predictor when all of the mitochondrial signatures were considered jointly in one multiple regression model. ANCOVA analyses in Table S3 suggest that BMI and the depth of subcutaneous fat in the exercised thigh were not significantly associated with the PCr recovery time constant. Moreover, neither gender nor age were significant predictors of the PCr recovery time constant. The inclusion of these variables in multiple regression models did not substantially

modify any of the associations between our independent variables of primary interest and the PCr recovery time constant. The details have been discussed in the Supporting Information.

4 | DISCUSSION

We identified a significant association between ^{31}P -MRS based measurements of PCr resynthesis rate and NAMPT protein levels in the *vastus lateralis*. These findings advance our knowledge of physiological mechanisms related to PCr resynthesis rate measurements made in ^{31}P -MRS studies of the skeletal muscle.

The association between NAMPT protein levels and PCr resynthesis rate is biologically relevant, given the role it plays in energy metabolism. In mammals, NAMPT is a rate-limiting enzyme that directly catalyzes the condensation of nicotinamide (NAM) with α -D-5-phosphoribosyl-1-pyrophosphate to synthesize nicotinamide mononucleotide (NMN).^{21,22} NMN is a pivotal precursor in the biosynthesis of nicotinamide dinucleotide (NAD^+). However, mammals lack the capacity to directly convert NAM into NMN. Therefore, it is biologically important to salvage and recycle NAD^+ . Synthesized NAD^+ becomes a key cofactor in oxidative phosphorylation within the inner mitochondrial membrane. Oxidative phosphorylation leads to ATP production,²³ which in turn influences the coupled reactions of creatine kinase that are crucial for contraction and relaxation of the myocyte²⁴ and therefore critically determines PCr resynthesis rate.

Two human studies have supported the importance of NAMPT as well. In a combined group of lean and obese metabolically healthy adults and individuals with Type 2 diabetes, exercise training increased NAMPT protein in the skeletal muscle in parallel with increased post-training maximum ATP synthesis and $\text{VO}_{2\text{max}}$.¹³ In addition, a 12-week exercise-induced reduction of plasma NAMPT (possibly due to increased muscle sequestration of NAMPT) was associated with a corresponding improvement in an oral glucose tolerance test.²⁵ We are however presenting the first confirmation that elevated NAMPT protein levels may be directly associated with faster PCr resynthesis rate in healthy adults who did not undergo exercise training.

We did not observe significant associations between PCr resynthesis rate and additional molecular markers associated with mitochondrial function, including SIRT1, PGC-1 α , and NQO1 mRNA levels, which might reflect the fact that mRNA levels do not always predict the corresponding protein levels. Although NQO1 is utilized along with NAD^+ in reductase reactions,^{26–28} and SIRT1 utilizes NAD^+ as a cofactor for protein deacetylase reactions,^{29,30} mitochondrial NAD^+ is mostly unaffected even when there is significant SIRT1 activity.^{31,32} This compartmentalized usage of NAD^+ protects the mitochondrial NAD^+ pool, which is critical to the oxidative production of ATP. Although important for skeletal muscle functioning, the specific usage of NAD^+ in other compartments outside the mitochondrion by PGC-1 α , SIRT1, and NQO1 may be one reason these factors have minimal association with PCr resynthesis rate.

Our study is not without limitations. Our study only included metabolically healthy, moderately fit individuals, and it is therefore not sure whether our findings extend to

additional groups such as older adults, children, endurance-trained athletes, or individuals with Type 2 diabetes, some of whom may have differing levels of NAMPT protein¹³ as well as different PCr resynthesis rates.² Additionally, our measurements were specific to a single skeletal muscle, the vastus lateralis. Future work should determine the generalizability of our finding to other settings.

The skeletal muscle NAMPT protein content is known to be positively correlated with mitochondrial mass and function during exercise intervention.¹³ In addition, the activities of skeletal muscle mitochondrial enzymes (citrate synthase, beta-hydroxyacyl-coenzyme A dehydrogenase, and cytochrome C oxidase) remain unchanged in response to exercise.³³ Since the muscle fiber typology substantially influences the time to recover from exercise,³⁴ the relationships among NAMPT content and the muscle fiber typology or distribution are also important to address but are beyond the scope of the current paper.

In summary, we found that, in healthy and moderately fit adults' human skeletal muscle, NAMPT protein is associated with PCr resynthesis rate. This finding supports the importance of mitochondrial NAD⁺ and helps clarify the biological correlates of the ³¹P-MRS based PCr resynthesis rate signal. Specifically, the relationship between NAMPT content and the mitochondrial oxidative phosphorylation supports the notion that NAMPT availability may be a key regulator of mitochondrial oxidative phosphorylation capacity. NAMPT protein content and NADP(H) level are correlated,³⁵ and thus measuring NAD⁺ concentrations should be a measurement target in future studies, using different measurement techniques than those described here.³⁶

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

Financial support for this research provided by National Institute of Health and Pennington Biomedical Research Foundation is gratefully acknowledged. We very much appreciate the support and contribution of Lauren M. Sparks, Connie Murla, and clinical staff of the Pennington Biomedical Clinical Cores. Also, we appreciate the diligence of our research participants throughout the duration of the study.

FUNDING INFORMATION

The research project described here was supported by National Institutes of Health Grants R01 AG030226 and U01 AG020478, and funded in part by P30 DK072476 and U54 GM104940. Additional support was provided by the Pennington Biomedical Research Foundation.

Abbreviations:

ATP	adenosine triphosphate
BMI	body mass index
mRNA	messenger ribonucleic acid
NAD	nicotinamide dinucleotide

NAM	nicotinamide
NAMPT	nicotinamide phosphoribosyltransferase
NMN	nicotinamide mononucleotide
NQO1	NAD(P)H quinone dehydrogenase 1
PCr	phosphocreatine
PGC-1α	peroxisome proliferator-activated receptor gamma coactivator-1 alpha
SIRT1	sirtuin 1
VO₂	rate of oxygen intake (peak/maximum)
³¹P-MRS	phosphorus MRS

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FIGURE 1.

Time series of PCr along with model fit of human vastus lateralis muscle (A) showing a faster PCr resynthesis rate (ie short $\tau = 24.12$ s) and (B) showing a slower PCr resynthesis rate (ie long $\tau = 73.3$ s) during the exercise bout. The corresponding ^{31}P -MRS spectra are shown beside these curves

FIGURE 2.

Scatter plot of time constant, τ (s), versus VO_2 peak (mL/min/kg) from participant's fitness test at baseline (A) and versus relative NAMPT proteins (NAMPT arbitrary units/ glyceraldehyde-3-phosphate dehydrogenase arbitrary units) (B), versus NAMPT mRNA (C), versus NQO1 mRNA (D), versus PGC1 α mRNA (E), and versus SIRT1 mRNA (F) of participants' muscle biopsies at baseline. A linear fit is indicated with a line including the 95% confidence interval (CI)

TABLE 1

Parameters of the linear regression model (Equation 2) using data from the baseline study visit are presented for VO₂ peak, NAMPT protein, and mRNA levels of NAMPT, NQO1, PGC-1 α , and SIRT1. The time constant τ is the PCr recovery time estimated from ³¹P-MRS measurements using Equation 1. Adjusted R^2 is a goodness of fit measure for the linear regression model. The linear regression outputs are the intercept alpha (α), regression coefficient beta (β), and standard error (E). The sign of β represents positive or negative correlation between the dependent and independent variables. The magnitude of coefficient β signifies the mean change in the dependent variable for a unit change in the value of the independent variable while keeping other independent variables constant. The p -value for each independent variable is the probability of the null hypothesis that the independent variable has no relationship with the dependent variable. $p < 0.0083$ is considered statistically significant after applying Bonferroni correction for multiple comparisons. “No of obs.” represents how many data items were used in each regression analysis. This number is less than total number of participants in the study due to issues in collecting the measurements, such as inadequate muscle tissue available for assays.

Dependent variable	Independent variable	β	α	E	$p > t $	Adjusted R^2	No of obs.
τ	VO ₂ peak	-6.47	58.19	3.13	0.049	0.1364	28
τ	NAMPT protein	-369.88	54.28	124.77	0.007	0.253	23
τ	NAMPT mRNA	-29.88	70.36	10.33	0.008	0.235	24
τ	NQO1 mRNA	-82.96	47.68	132.10	0.536	0.0176	23
τ	PGC1 α mRNA	-1.70	59.44	0.94	0.083	0.0867	25
τ	SIRT1 mRNA	-5.85	48.01	5.49	0.298	0.0056	24

Significant variables ($p < 0.0083$) in bold face type.