Improving the Vitamin D Status of Vitamin D Deficient Adults Is Associated With Improved Mitochondrial Oxidative Function in Skeletal Muscle

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Objective: Suboptimal mitochondrial function has been implicated in several disorders in which fatigue is a prominent feature. Vitamin D deficiency is a well-recognized cause of fatigue and myopathy. The aim of this study was to examine the effects of cholecalciferol therapy on skeletal mitochondrial oxidative function in symptomatic, vitamin D-deficient individuals.

Design: This longitudinal study assessed mitochondrial oxidative phosphorylation in the gastrosoleus compartment using phosphorus-31 magnetic resonance spectroscopy measurements of phosphocreatine recovery kinetics in 12 symptomatic, severely vitamin D-deficient subjects before and after treatment with cholecalciferol. All subjects had serum assays before and after cholecalciferol therapy to document serum 25-hydroxyvitamin D (25OHD) and bone profiles. Fifteen healthy controls also underwent ^{31}P-magnetic resonance spectroscopy and serum 25OHD assessment.

Results: The phosphocreatine recovery half-time ($\tau_{1/2}$PCr) was significantly reduced after cholecalciferol therapy in the subjects indicating an improvement in maximal oxidative phosphorylation (34.44 $\pm$ 8.18 sec to 27.84 $\pm$ 9.54 sec, $P < .001$). This was associated with an improvement in mean serum 25OHD levels (8.8 $\pm$ 4.2 nmol/L to 113.8 $\pm$ 51.5 nmol/L, $P < .001$). There was no difference in phosphate metabolites at rest. A linear regression model showed that decreasing serum 25OHD levels was associated with increasing $\tau_{1/2}$PCr ($r = -0.41$, $P = .009$). All patients reported an improvement in fatigue after cholecalciferol therapy.

Conclusions: Cholecalciferol therapy augments muscle mitochondrial maximal oxidative phosphorylation after exercise in symptomatic, vitamin D-deficient individuals. This finding suggests that changes in mitochondrial oxidative phosphorylation in skeletal muscle could at least be partly responsible for the fatigue experienced by these patients. For the first time, we demonstrate a link between vitamin D and the mitochondria in human skeletal muscle. **(*J Clin Endocrinol Metab* 98: E509–E513, 2013)**

Mitochondrial oxidative phosphorylation is the primary source of cellular ATP with suboptimal mitochondrial function implicated in disorders in which fatigue is a feature (1–3). Fatigue and myopathy are well recognized in the context of vitamin D deficiency and muscle symptoms may arise independent of derangements in bone biochemistry (4). Phosphorus-31 magnetic resonance spectroscopy (^{31}P-MRS) is a noninvasive tool used to assess mitochondrial function in vivo by measuring the kinetics of high energy phosphate metabolites involved in

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Abbreviations: CV, coefficient of variation; 25OHD, 25-hydroxyvitamin D; PCr, phosphocreatine; $\tau_{1/2}$PCr, phosphocreatine recovery half-time; Pi, inorganic phosphate; ^{31}P-MRS, phosphorus-31 magnetic resonance spectroscopy.

muscle energy metabolism during and after perturbation by exercise. The aim of this study was to examine the effects of cholecalciferol therapy on skeletal mitochondrial oxidative function in vitamin D deficient subjects using ^{31}P-MRS. We hypothesized that suboptimal mitochondrial function contributes to the myopathy in vitamin D-deficient individuals and that cholecalciferol therapy would be associated with improved mitochondrial oxidative function.

Subjects and Methods

Twelve individuals with severe vitamin D deficiency (<15 nmol/L) were studied. All presented to primary care with fatigue and/or muscle cramps between March and May 2012. Exclusion criteria included a history of thyroid, pituitary, or inherited mitochondrial disease. All 12 subjects had their biochemistry assessed and underwent a standardized exercise protocol with an assessment of ^{31}P-MRS prior to being treated with cholecalciferol 20 000 IU on alternate days for 10–12 weeks. Biochemistry and the exercise protocol with ^{31}P-MRS were then repeated. Fifteen age-matched healthy volunteers were recruited as controls. The study was approved by the local ethics committee and written informed consent was obtained from participants.

MRS protocol

MRS data were acquired using a 3T Achieva scanner (Philips, Andover, Massachusetts) with a 14-cm diameter phosphorus surface coil for transmission/reception of signal and the built-in body coil for anatomical imaging (5). A purpose-built exercise apparatus was developed for operation with the imaging scanner. This apparatus permitted a controlled plantar flexion (between 0° and 30°) to isolate and exercise the soleus and gastrocnemius muscles with the patient lying supine, using restraining straps to prevent recruitment of the quadriceps. Subjects performed an exercise protocol consisting of 3 minutes rest, 3 minutes of plantar flexion at 0.5 Hz, and 6.5 minutes of rest after the exercise to measure recovery to equilibrium. A fixed load of 35% of maximum voluntary contraction (determined prior to spectroscopy) was used to accurately measure oxidative metabolism in recovery while maintaining a stable pH (6). Phosphorus spectra were collected at 10-second intervals throughout the exercise using a fully adiabatic 1D-ISIS (1 dimensional image-selected in vivo spectroscopy) sequence to localize signal to gastrocnemius and soleus muscles.

Quantification of phosphocreatine (PCr), inorganic phosphate (Pi), and pH was performed using the AMARES time domain fit routine in the jMRUI processing software (version 3.0) (7, 8). Standard methods were used to calculate the parameters of oxidative metabolism and pH handling (6). Exponential fits to the recovery data were made to estimate the half-times for recovery to equilibrium of PCr($\tau_{1/2}$ PCr) and ADP($\tau_{1/2}$ ADP). Muscle pH was assessed using the chemical shift between phosphocreatine and inorganic phosphate peaks (6).

Biochemistry

Serum samples were obtained from participants at both visits. Serum 25-hydroxyvitamin D (25OHD) was measured using the DiaSorin liaison method (DiaSorin, Stillwater, Minnesota). Two quality controls are routinely used in the assay with mean values of 37 and 157 nmol/L and interassay coefficient of variations (CVs) of 12.6% and 4.9%, respectively. The intraassay CV for this method is less than 8%, with a functional sensitivity of 6 nmol/L. Further details have been described previously (9). Serum calcium was analyzed using the Roche Modular P (Indianapolis, Indiana) with an intraassay CV of 0.6% and an interassay CV of 2.2%.

Outcome measures and power calculation

The primary outcome was to compare mitochondrial function ($\tau_{1/2}$PCr, $\tau_{1/2}$ADP) in 12 symptomatic vitamin D-deficient subjects before and after cholecalciferol therapy using ^{31}P-MRS. The sample size was estimated from pilot studies of healthy individuals at our center who underwent repeat ^{31}P-MRS to determine repeatability. Using the Bland-Altman method, we expected to find a maximum of 9% variability in $\tau_{1/2}$PCr from repeated measurements (10). To detect a 10% change in $\tau_{1/2}$PCr with a significance of $P = .05$ at 90% power, we estimated that a minimum of 10 subjects was needed. Statistical analyses were undertaken using Minitab version 16 software package (State College, Pennsylvania). All data are expressed as mean ± SD. After ensuring parametric distribution with the Kolmogorov-Smirnov method ($P > .15$), we used paired t tests to make comparisons before and after cholecalciferol. A linear regression model was built to examine the relationship between 25OHD and $\tau_{1/2}$PCr. Significance was set at $P < .05$.

Results

Subject clinical characteristics

Participants presented to the primary care unit with fatigue and myopathy. The mean age (±SD) of the subjects was 33.7 ± 9.8 years (range 18.1–50.4 years). One patient had a complex medical history including hepatitis C, depression, epilepsy, and anemia and was on thiamine, folic acid, vitamin B, levetiracetam, and amitriptyline. The remaining participants had no other relevant past illness. Two participants were Caucasian, 2 were Afro-Caribbean, and 8 were of Southeast Asian ethnicity. Five females and 7 males participated. There was no change in diet, medication, or exercise pattern between the 2 study visits. Symptoms of fatigue and myopathy were confirmed on the first visit, with all patients reporting an improvement in symptoms at their second visit. The 15 controls were 31.3 ± 7.0 years of age (range 22.3–45.9 years).

All patients were severely vitamin D deficient at their first assessment. All were noted to have a significant improvement in 25OHD status after cholecalciferol therapy prior to the repeat MRS muscle study ($P < .001$). There was no significant difference in serum calcium, phosphate, or alkaline phosphatase levels before and after cholecalciferol (Table 1). The mean serum 25OHD of the controls was 44.2 nmol/L.

Table 1. Biochemistry, ^{31}P-MRS Measurements Undertaken on Skeletal Muscle and Questionnaires in Vitamin D-Deficient Subjects Before and After Cholecalciferol Therapy and in Healthy Controls

Mean ± SD	Baseline (Vitamin D Deficient)	Follow-Up (After Vitamin D Supplementation)	*P* Value	Healthy Controls
Biochemistry				
Serum 25OHD, nmol/L	8.83 ± 4.28	113.8 ± 51.5	<.001	44.2 ± 29.4
Serum calcium, mmol/L	2.29 ± 0.1	2.35 ± 0.1	.24	NA
Serum phosphate, mmol/L	0.99 ± 0.1	1.12 ± 0.2	.19	NA
Alkaline phosphatase, U/L	86.7 ± 15.5	78.5 ± 9.5	.16	NA
Rest				
Pi, mM	2.63 ± 0.48	2.78 ± 0.47	.38	2.94 ± 0.59
PCr, mM	31.77 ± 3.20	32.09 ± 4.19	.77	32.39 ± 1.28
Pi/PCr	0.083 ± 0.02	0.087 ± 0.02	.35	0.090 ± 0.02
pH, U	7.04 ± 0.03	7.05 ± 0.03	.28	7.04 ± 0.03
End of exercise				
PCr drop, fractional	0.25 ± 0.14	0.24 ± 0.10	.81	0.21 ± 0.08
Nadir pH, U	6.99 ± 0.08	7.00 ± 0.05	.34	6.91 ± 0.28
Maximum proton efflux, mM/min	3.63 ± 4.37	3.70 ± 1.83	.95	2.59 ± 1.69
Recovery				
$\tau_{1/2}$PCr, sec	34.44 ± 8.18	27.84 ± 9.54	<.001	35.66 ± 14.62
$\tau_{1/2}$ADP, sec	26.84 ± 6.58	21.93 ± 6.81	.003	27.49 ± 9.12

Abbreviation: NA, not available. Data are expressed as mean ± SD. Paired *t* tests were undertaken for comparisons before and after cholecalciferol therapy.

Postexercise PCr and ADP recovery, indices of mitochondrial oxidative function ($\tau_{1/2}$PCr, $\tau_{1/2}$ADP) improved after cholecalciferol therapy ($\tau_{1/2}$PCr: before calciferol 34.44 ± 8.18 seconds, after calciferol 27.84 ± 9.54 seconds; $P < .001$). The resting metabolites including Pi, PCr, and pH did not differ significantly. There was also no difference in PCr depletion (fractional PCr drop) or differences in nadir pH and maximum proton efflux at the end of exercise (Table 1). The $\tau_{1/2}$PCr of healthy controls was 35.66 ± 14.62 seconds, with Figure 1 illustrating $\tau_{1/2}$PCr values in controls compared with vitamin D-deficient subjects before and after vitamin D therapy. A pooled regression analysis showed lower serum 25OHD levels correlated with longer $\tau_{1/2}$PCr ($r = -0.41$, $P = .009$).

Discussion

The maximal mitochondrial oxidative phosphorylation rate was enhanced ($\tau_{1/2}$PCr and $\tau_{1/2}$ADP recovery times were reduced) after cholecalciferol therapy in severely vitamin D-deficient, symptomatic individuals. Correcting vitamin D deficiency was associated with an improvement in symptoms of myopathy and fatigue in all participants. There was no evidence of abnormal resting metabolic parameters often found in conditions with altered membrane permeability when compared with healthy controls (6). To the best of our knowledge, this is the first study in humans to demonstrate an improvement in parameters of mitochondrial function as vitamin D-deficient individuals became vitamin D replete.

A recent meta-analysis concluded that vitamin D supplementation did not result in an improvement in muscle strength in vitamin D-replete adults, although there was evidence to suggest an increase in muscle strength in adults with 25OHD of 25 nmol/L or less (11). A randomized controlled trial investigated muscle strength and resting phosphorus metabolites in vitamin D-deficient patients and found improvements in muscle strength but no changes in resting phosphorus metabolites in the vitamin D-treated group (12). However, the authors did not acquire dynamic phosphorus data while exercising and hence did not compute oxidative phosphorylation capacity ($\tau_{1/2}$PCr and $\tau_{1/2}$ADP). The patient groups were neither symptomatic nor severely vitamin D deficient.

Although our data did not show overt abnormalities of mitochondrial oxidative function in the vitamin D-deficient subjects when compared with healthy controls, cholecalciferol therapy resulted in significant enhancement of mitochondrial maximal oxidative phosphorylation. The wide range of healthy control data reflects the wide biological variability in mitochondrial function. A linear regression model confirmed the relationship between vitamin D and mitochondrial function. As vitamin D levels fall, mitochondrial recovery kinetics increase. $\tau_{1/2}$PCr is a widely accepted measure of maximal mitochondrial capacity. It is a function that reflects a composite of mechanisms including mitochondrial number, oxidative enzyme content, mitochondrial components, and vascular supply of substrates and oxygen (13). Perturbations in any or all of the above mechanisms may lead to suboptimal mitochondrial oxidative function. Lower $\tau_{1/2}$PCr

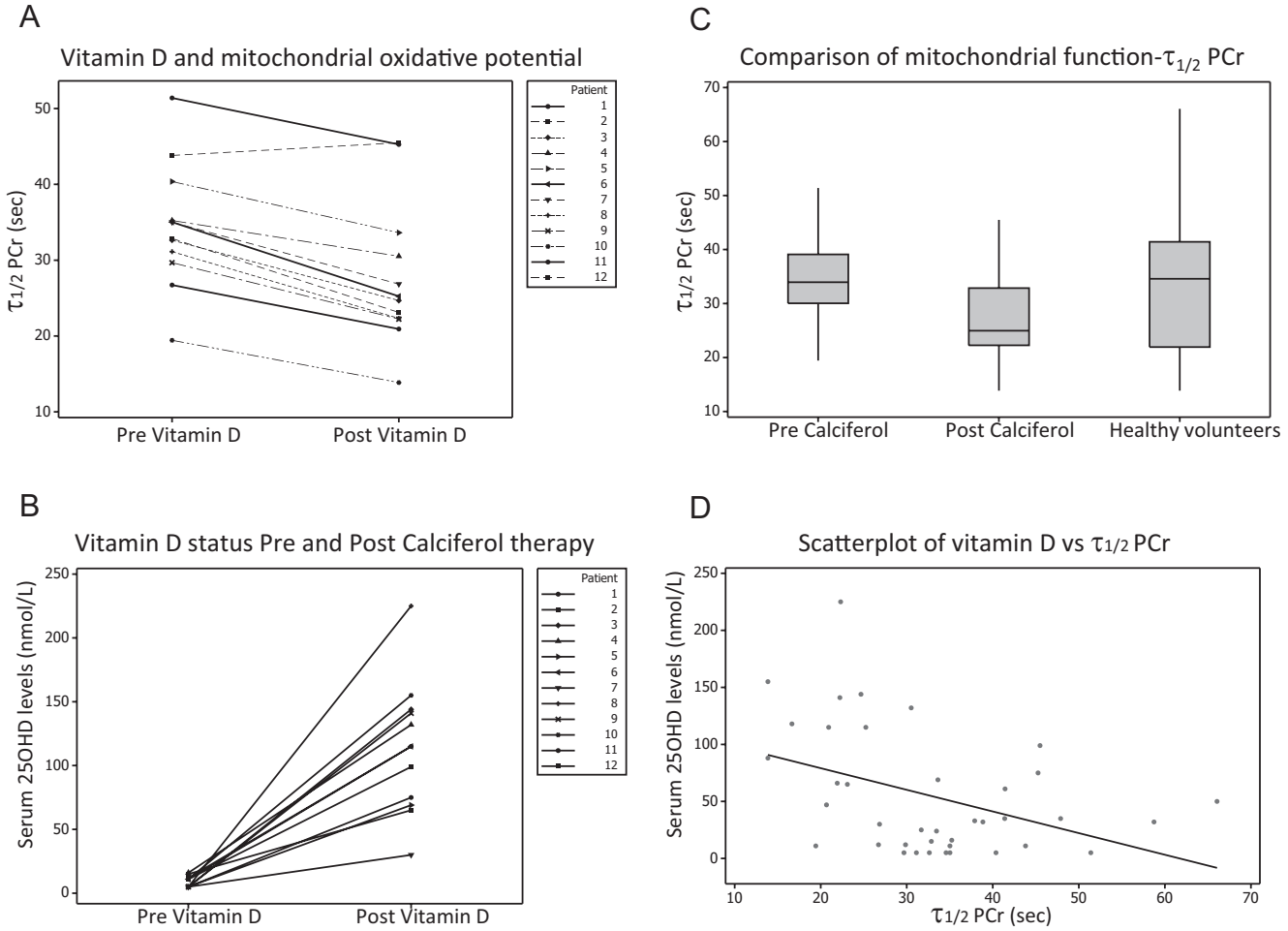

Figure 1. A–D, Comparison of $\tau_{1/2}$PCr kinetics before and after cholecalciferol therapy (A); serum 25OHD levels at presentation with vitamin D deficiency and then after cholecalciferol therapy (B); comparison of mitochondrial function ($\tau_{1/2}$PCr) before and after vitamin D therapy and in healthy volunteers (C); a linear regression model examining the relationship between serum 25OHD and $\tau_{1/2}$PCr. This model includes data from both before and after vitamin D treatment subjects and healthy controls ($r = -0.41$, r^2 adjusted = 14.67%, $P = .009$) (D).

represents enhanced maximal mitochondrial oxidative phosphorylation. Long-term endurance training and ageing have been reported to positively and negatively modulate mitochondrial function, respectively (14, 15).

Mitochondrial oxidative phosphorylation measurement using ^{31}P-MRS demonstrates low intraindividual variability with biological variability accounting for the vast majority of measurement variability (16). An advantage of our study design lies in its ability to control for confounding variables. Earlier pilot work in our unit demonstrating an intraindividual CV of 9% in healthy controls is pertinent when interpreting the study findings. It is also important to highlight the fact that an 8-week study of increased physical activity in 10 healthy controls at our center did not alter mitochondrial function as measured by $\tau_{1/2}$PCr (17). Moreover, our test-retest reproducibility is consistent with another center that has cross-validated ^{31}P-MRS by comparing oxidative capacity with high-resolution mitochondrial respirometry isolated from muscle biopsy tissue (18).

The precise basis for the muscle symptoms observed in vitamin D deficiency is unclear. Hormone-responsive elements on the mitochondria genome could up-regulate cellular energy production through genomic and nongenomic mechanisms (19). Vitamin D receptors mediate the action of 1,25 dihydroxyvitamin D and are expressed in several tissues. Mitochondria in skeletal muscle fibers can take up cytoplasmic Ca^{2+} released from the sarcoplasmic reticulum during twitch and tetanic responses (20). Such Ca^{2+} uptake may stimulate mitochondrial ATP production, although it may not be essential (21). Experiments on vitamin D-deficient chick muscles demonstrated alterations in oxidative phosphorylation and an inability of muscle mitochondria to retain Ca^{2+} (22). An established link between cytosolic and mitochondrial Ca^{2+} concentrations, identification of transport mechanisms, and the proximity of mitochondria to Ca^{2+} release sites supports the notion that Ca^{2+} can be an important signaling molecule in the energy metabolism interplay of the cytosol with the mitochondria (23). Vitamin D may

therefore play a significant role in Ca^{2+} uptake by the mitochondria which in turn are involved in the orchestration of cellular metabolic homeostasis.

There are limitations to our study: the number of subjects studied was relatively small, the study was not placebo controlled, and neither patient nor investigators were blinded during treatment. We did not use comprehensive fatigue questionnaires because of their low specificity to vitamin D deficiency, their intrusive nature, and issues of translation. We do not know whether vitamin D supplementation to asymptomatic individuals will result in changes in mitochondrial oxidative phosphorylation, and there is much to learn about the relationship between vitamin D status and mitochondrial function. However, our data offer clear proof of principle that ^{31}P-MRS is a valuable platform for further studies in this area.

In conclusion, these data show that cholecalciferol therapy in symptomatic, vitamin D-deficient individuals results in improved mitochondrial oxidative function as measured by ^{31}P-MRS.

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