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***In vivo* ³¹P MRS detection of an alkaline inorganic phosphate pool with short T1 in human resting skeletal muscle**

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Abstract

Non-invasive determination of mitochondrial content is an important objective in clinical and sports medicine. ³¹P MRS approaches to obtain information on this parameter at low field strength typically require in-magnet exercise. Direct observation of the intra-mitochondrial inorganic phosphate (Pi) pool in resting muscle would constitute an alternative, simpler method. In this study, we exploited the higher spectral resolution and signal-to-noise at 7T to investigate the MR visibility of this metabolite pool. ³¹P *in vivo* MR spectra of the resting soleus (SOL) muscle were obtained with ¹H MR image-guided surface coil localization (six volunteers) and of the SOL and tibialis anterior (TA) muscle using 2D CSI (five volunteers). A resonance at a frequency 0.38 ppm downfield from the cytosolic Pi resonance (Pi₁; pH 7.0 ± 0.04) was reproducibly detected in the SOL muscle in all subjects and conditionally attributed to the intra-mitochondrial Pi pool (Pi₂; pH 7.3 ± 0.07). In the SOL muscle, the Pi₂/Pi₁ ratio was 1.6 times higher compared to the TA muscle in the same individual. Localized 3D CSI results showed that the Pi₂ peak was present in voxels well away from blood vessels. Determination of the T1 of the two Pi pools in a single individual using adiabatic excitation of the spectral region around 5 ppm yielded estimates of 4.3 ± 0.4 s vs 1.4 ± 0.5 s for Pi₁ and Pi₂, respectively. Together, these results suggest that the intra-mitochondrial Pi pool in resting human skeletal muscle may be visible with ³¹P MRS at high field.

Keywords

magnetic resonance spectroscopy; tissue pH; skeletal muscle; ³¹P MRS; fiber type; inorganic phosphate; mitochondria

INTRODUCTION

Ever since the first demonstration that ³¹P MR spectroscopy can be used to measure cellular energy and proton balance in human organs *in vivo* (1,2), assessment of mitochondrial content and function in skeletal muscle has been an important objective in clinical and sports medicine (3). The idea has been that differences in capacity for aerobic ATP synthesis between muscles with high vs low mitochondrial content should give rise to detectable metabolic contrast during exercise using *in vivo* ³¹P MRS techniques.

For example, the rate of recovery of the phosphocreatine (PCr) signal after exercise has been used extensively to assess differences in mitochondrial content and/or function in health and disease (e.g. (3–6)). However, the recovery of this signal is determined not only by the rate of mitochondrial activity, but also by other factors including muscle perfusion and muscle acidification and as such, the precise intensity of the preceding exercise (7). Therefore, interpretation of such kinetic measurements in a clinical setting is not straightforward. Another drawback of these types of experiments is that they require exercise in the magnet, which requires specialized MR-compatible hardware and patient cooperation. Even though elegant approaches have been designed to limit patient exertion (8), exclusion of certain patient groups including young children cannot be prevented. As such, a non-invasive method to assess mitochondrial content under resting conditions would constitute a significant advantage over approaches requiring exercise.

Well over two decades ago, the first evidence that such a direct measure of mitochondrial content may be feasible was provided by the observation in cardiac muscle of a second inorganic phosphate (Pi) pool that was proposed to originate from the mitochondrial matrix (9,10). If so, the amplitude of this peak could provide a non-invasive index for mitochondrial content of the tissue. Detection of a second Pi resonance has, to our knowledge, never been observed in resting human skeletal muscle. This may be due to the limited signal-to-noise ratio (SNR) and spatial resolution, combined with a limited spectral resolution at low field strength since the chemical shift difference between the two Pi signals at physiological temperature is expected to be only 0.4 ppm (9–11) and the partial volume of mitochondria in skeletal muscle is expected to be between 5 and 10%.

In the present study we performed localized ^{31}P MRS of the soleus (SOL) and tibialis anterior (TA) muscles at a field strength of 7T. These muscles were selected as they differ significantly in oxidative capacity (12), while the higher spectral resolution and SNR at 7T offers unique capability to obtain high quality ^{31}P MRS data. A resonance 0.38 ppm downfield from the cytosolic Pi resonance was reproducibly detected in ^{31}P MR spectra obtained from resting muscle from healthy human subjects, with relaxation time data and signal localization providing strong indications that the signal could originate from the mitochondrial Pi pool.

EXPERIMENTAL

Data were collected at 7 tesla on a Philips Achieva MRI scanner (Philips Healthcare, Best, The Netherlands). Subjects were placed feet first in the magnet in a supine position. MR data were acquired from the left lower leg; sandbags were placed around the ankle and knee to prevent involuntary movement during or in-between scans. The study adhered to the local Medical Ethics Committee guidelines.

Single muscle scans

Surface coil localized ^{31}P MR spectra (TR = 5s, bandwidth 5 kHz, 2048 data points; 128 averages) were acquired from resting SOL muscle in six healthy male volunteers (age range 24–38 years). Subjects were not involved in any specific physical training program but were reasonably physically active (exercise one-to-three times a week, self-reported). ^1H and ^{31}P data were obtained with a custom-built transmit and receive double tuned loop coil with a diameter of 5 cm and custom-built interface boxes with transmit-receive switches to enable ^{31}P and ^1H signal excitation and reception. The coil was carefully placed to receive signal primarily from the medial SOL muscle of the lower leg by manually palpating the most ventral rim of the tibia bone and placing the center of the coil directly above this area. Excitation of the ^{31}P spins was accomplished using a pulse-acquire sequence with a hard pulse of 200 μs and 800 W of RF peak power, aimed to achieve a nominal pulse angle of 30

degrees. After placement of the subject in the scanner, the location of the coil was verified by transverse gradient echo images acquired with the double tuned coil. Subsequently, a B_0 map was acquired of several slices in the region of interest for the image based shimming algorithm (13). Shimming was performed by manually drawing a region of interest in the medial SOL muscle.

In one of the volunteers, 3D CSI (FOV, $150 \times 150 \times 400$; matrix size, $10 \times 10 \times 8$; hamming weighted acquisition and post processing with four averages at the center lines of k-space (14), and adiabatic excitation as described below) was performed to obtain a localized spectrum of the SOL muscle to exclude major contributions from the blood vessels in the subcutaneous fat and/or bone marrow adjacent to the coil. The TR of this experiment was optimized at 1.2 times the T1 value of the Pi_2 signal (i.e. 1680 ms).

Finally, in another subject, the T1 relaxation time of Pi_1 and Pi_2 was measured by experiments with increasing TR in the medial vastus muscle. To ensure full excitation of both signals, an adiabatic 90° half passage pulse of 3.3 ms duration with the transmitter frequency set to 5.0 ppm downfield from the PCr peak was applied. Six different spectra were obtained, with increasing repetition times from 500 ms and 1024 averages to 30 s and 16 averages (measurement time > 60 min). As this experiment is very long, T1 relaxation effects in the SOL muscle were studied qualitatively in three volunteers by acquiring ^{31}P MR spectra with a shorter TR (TR = 250, 2048 averages, bandwidth 5 kHz, 1024 complex data points).

^{31}P MRS of soleus and tibialis anterior muscle

In a separate group of subjects ($n = 5$, 4 males, 1 female, age range 22–46 years),

2D CSI (FOV, 160×160 ; matrix size, 8×8 ; TR = 1680 ms; hamming weighted acquisition and post processing with 32 averages at the center lines of k-space (14), and adiabatic excitation with a half passage pulse) was applied. For this experiment, a custom-built combined 1H and ^{31}P coil setup was used, with square coils for ^{31}P (10 cm linear dimension) and 1H (12 cm linear dimension) in transmit/receive mode (15). Two 2D CSI datasets were acquired: one with the coil placed at the posterior side of the leg for SOL muscle, and one with the coil at the anterior side for the TA muscle. The 2D data set was oriented perpendicular to the orientation of the muscle of interest. Shimming was performed as described above, by manually drawing a region of interest in either the TA or the SOL muscle.

Data processing

^{31}P MR spectra were processed and analyzed in the time domain using the jMRUI software package (16). Peak areas were obtained by fitting Lorentzian line shapes to the resonance positions of two distinct signals for inorganic phosphate (Pi_1 and Pi_2), glycerol phosphoethanolamine (GPE), glycerol phosphocholine (GPC), PCr, a doublet for the γ - and α - signals of ATP, NADPH and a triplet for the β peak of ATP. The line width of the Pi_2 peak was constrained to the line width and phase of the (main) Pi_1 peak. The amplitudes of the doublets and triplet of ATP were constrained to their known relative amplitudes (i.e. γ -ATP₁ = γ -ATP₂; α -ATP₁ = α -ATP₂ and β -ATP₁ = β -ATP₃ = $0.5 * \beta$ -ATP₂). Tissue pH was calculated from the shift in resonance position (S) of the two Pi peaks compared to the reference position of PCr according to: $pH = 6.75 + \log((3.27-S)/(S-5.69))$ (17).

The CSI datasets were visualized using 3DiCSI software (18), and voxels were identified in the SOL and TA muscles. Care was taken to exclude the major blood vessels as well as subcutaneous fat. The corresponding free induction decays were exported and fitted using

the jMru software package after filtering using Hankel Lanczos singular values decomposition (HLSVD). Filtering was applied to remove signals of PCr and ATP which were only partially excited as the bandwidth of the adiabatic pulse was limited to the region around 5 ppm. Prior knowledge on Pi_2 versus Pi_1 was applied as described above. T1 values were fitted to the standard progressive saturation equation $S(t) = S_{\infty} (1 - e^{-TR/T1})$, where $S(t)$ is the signal intensity at each time point and S_{∞} is the signal in fully relaxed spectra.

Statistics

Differences between the pH calculated using the Pi_2 and the Pi_1 resonances and between Pi_2/Pi_1 in SOL and TA were compared with a paired t-test.

RESULTS

Single muscle scans

In all volunteers, surface coil localized ^{31}P MR spectra of the SOL muscle region were obtained with line widths of 35 ± 5 Hz of the PCr signal. PCr signals did not show any splitting, indicating that possible effects of poorly shimmed regions on the line shapes were minimal. All signals, i.e. resonances from cytosolic Pi (Pi_1), PCr, GPE, GPC and ATP (19,20), that are typically observable at lower field strengths if proton decoupling is used could be observed at 7T without any decoupling (Fig. 1). Additionally, signals originating from other phosphorylated muscle metabolites were detected at 5.1 and -8.2 ppm. These signals were attributed to a second Pi pool (Pi_2) in a muscle compartment with alkaline pH (pH 7.3) (Fig. 1) and pyridine nucleotides (NADPH) (21), respectively. Specifically, the resonance frequencies of the two distinct pH pools were 4.76 ± 0.06 ppm and 5.14 ± 0.07 ppm, relative to the PCr resonance. This indicated a significant difference in tissue pH for the two compartments (7.29 ± 0.07 for Pi_2 and 6.95 ± 0.04 for Pi_1 , $p < 0.000001$). The line width of the Pi_1 signal was 0.47 ± 0.07 ppm averaged over all volunteers.

The 3D CSI experiment in the SOL muscle confirmed that, despite the lower SNR of this measurement, a distinct signal was visible for Pi_2 also in a localized spectrum of this muscle (Fig. 2). Fitting of this resonance showed that it constituted 13% of the main Pi_1 resonance. The separation between Pi_1 and Pi_2 was 0.39 ppm.

The quantitative T1 measurements using adiabatic excitation and nonlinear curve fitting of an monoexponential function to the data yielded T1 estimates of 1.4 ± 0.5 s for Pi_2 and 4.3 ± 0.4 s for Pi_1 ; sample spectra and the curve-fit are shown in Figure 3. At fully relaxed conditions, $TR = 30$ s, the ratio of Pi_2/Pi_1 was 0.07. In the remaining volunteers, the same qualitative signal behavior was seen with respect to the different T1 relaxation properties, i.e. the ratio of Pi_2/Pi_1 increased from 0.13 ± 0.05 to 0.21 ± 0.2 as TR was decreased from 5 s to 250 ms. This is in agreement with the expected signal gain, using T1 relaxation values of 1.4 s for Pi_2 and 4.3 s for Pi_1 , if we reached an effective flip angle of 23 degrees at the used power settings (i.e. close to the nominal flip angle of 30 degrees).

^{31}P of soleus and tibialis anterior muscle—The 2D CSI experiments on the SOL and TA muscles in the same subject showed a consistent and significant decrease in the Pi_2/Pi_1 ratio in all volunteers in the TA muscle compared to the SOL muscle ($p = 0.035$) (Fig. 4). Assuming identical cytosolic Pi concentrations in resting SOL and TA, this result thus predicts that the mean ratio of $Pi_{2_SOL}/Pi_{2_TA} = 0.11/0.07 = 1.6$.

DISCUSSION

The main finding of the present study was the reproducible detection of a resonance 0.38 ppm downfield from the cytosolic Pi signal in surface coil-localized ^{31}P NMR spectra from the resting SOL muscle in healthy human subjects at 7T. Both a quantitative T1 measurement with adiabatic excitation as well as qualitative experiments with short and long TR values showed that the T1 of the Pi_2 signal was shorter than the T1 of the cytosolic Pi pool. Based on various considerations as outlined below, we tentatively attribute the signal at 5.1 ppm to the Pi pool inside the mitochondrial matrix. To our knowledge, this would constitute the first report of the *in vivo* observation of a second Pi signal in resting mammalian skeletal muscle.

Other metabolite pools within the sampled tissue may potentially also contribute to a signal at this resonance position. For instance, signals from the blood in the muscle could influence the results via contributions of Pi from the plasma (22,23) because the pH from the blood is alkaline (pH 7.4) similar to the pH in the mitochondrial compartment (24). As we used surface coil localization in a major part of our experiments, blood vessels within the subcutaneous fat very close to the RF coil could have contributed significantly to our results. The data from the 3D CSI experiment, however, confirmed the hypothesis that the Pi signal at 5.1 ppm mainly originates from inside the muscle tissue. The voxel was completely located inside the muscle and remote from any large blood vessels in the subcutaneous fat. The major leg vessels, which are localized somewhat deeper than the voxel, are unlikely to contribute significantly to the signal as the sensitivity of the coil this deep in the posterior compartment is very low. Additionally, a hamming filter was used to reduce the side-lobes of the point spread function inherent to the CSI experiment. A distinct Pi_2 signal was discernable in these spectra, which constituted about 13% of the main Pi_1 signal. In the muscle tissue itself, a simple calculation suggests that any contribution of Pi from the blood to the resonance at 5.1 ppm was only minor (less than 15%). The free Pi concentration in blood is on the order of 1 mM (25) and the partial volume of blood in resting skeletal muscle is less than 5%. Consequently, assuming that (i) the T1 value of Pi in the blood is similar to the T1 of the Pi_2 signal, (ii) the concentration of Pi_2 is similar to Pi_1 (i.e. 5 mM (17)) and (iii) the partial volume of the mitochondria in the SOL is ~7%, the signal contribution of plasma Pi is maximally 15% of the peak at 5.1 ppm.

A second signal that could contribute to the observed resonance at 5.1 ppm is 2-DPG from the erythrocytes in the blood. Concentrations of this compound have been reported between 5 and 10 mM (22). However, the resonance position of the doublet of 2-DPG has been reported at 5.5 ppm (23) while the combined peak of extracellular Pi and 2-DPG has been observed at 5.33 ppm (24). These chemical shifts are 0.4 and 0.2 ppm downfield, respectively, from the chemical shift of the Pi_2 resonance that we observed. Given the spectral resolution and line widths obtained in this study, any significant signal from 2-DPG should have been detected as a separate peak.

The Pi_2 signal could potentially also have originated from Pi in the extracellular space if the MR visibility of the Pi pool inside the mitochondria is limited at body temperature (26–28). Previously, based on this assumption, an alkaline Pi signal has been assigned to be extracellular Pi in ^{31}P MR spectra obtained in human brain (29,30). However, the contribution of extracellular space to the signal obtained in muscle is significantly less than in brain, due to structural differences between the two tissues. Past ^{31}P MRS studies in isolated perfused rat hearts at 37°C first reported a resonance 0.4 ppm downfield of the cytosolic Pi peak (9,10). The authors concluded, based on three observations, that this resonance originated from the mitochondrial matrix. First, the chemical shift of the Pi resonance was in close agreement with the consensus that the intramitochondrial pH is 0.4

pH units more alkaline than the cytosolic pH at a physiological temperature (11). Second, the T_1 of the putative intramitochondrial Pi pool was shorter than the T_1 of the cytosolic Pi pool (9). Third, infusion of valomycin, an inhibitor of the mitochondrial respiratory chain, resulted in quenching of the signal (9,10). A number of independent *in vitro* studies have up to now reported corroborating evidence that the intramitochondrial Pi pool is ^{31}P MRS visible (11,26).

In the present study we likewise found a shorter T_1 for the Pi signal tentatively attributed to the intramitochondrial pool compared to the cytosolic Pi pool (Fig. 3), although the T_1 value we obtained for Pi_1 was somewhat lower compared to a recent study at 7T (31).

Additionally, the 2D CSI experiment in SOL and TA muscle showed a reduced Pi_2/Pi_1 in the TA muscle compared to the SOL muscle. Previous studies have shown both a reduced oxidative capacity (12) as well as reduced SDH activity (32) in the TA muscle compared to the SOL muscle in humans. It is possible that cytosolic Pi levels differ between the two muscles, as differences in Pi levels have been observed between fast twitch and slow twitch muscles in humans (33) and animals (34,35). However, as human biopsy data showed a similar fiber type composition of TA and SOL muscles (32), we assume that cytosolic Pi concentrations do not differ between the TA and SOL muscles. If the amplitude of the Pi_2 resonance scales with mitochondrial density, these results would indicate that the mitochondrial density of human SOL muscle is 1.6-fold higher than for human TA muscle. This number matches the finding by Forbes and coworkers of a 1.5 fold faster (22 vs 32 s) apparent time constant for PCr recovery in SOL vs TA muscle in human subjects (12). The agreement between this well established MR-readout of *in vivo* mitochondrial capacity and the results of our CSI measurements of the Pi_2/Pi_1 ratio in these calf muscles further supports our hypothesis that resonance Pi_2 originates from the mitochondrial compartment in muscle.

Overall, based on the chemical shift value, the T_1 characteristics of the Pi_2 resonance at 5.1 ppm, the small contribution of extracellular space to the signal in skeletal muscle, and the difference in the Pi_2/Pi_1 ratio level between the SOL and TA muscles, we conclude that it is highly likely that the signal we observe at 5.1 ppm originates from the mitochondrial matrix. At this point, it is impossible to provide sure evidence of the mitochondrial origin of the signal. The drug-based experimental approaches previously used by Garlick and coworkers in perfused rat hearts to further test this hypothesis (9,10) are not feasible in human subjects. Therefore, alternative validation strategies need to be performed to further gain insight in the origin of the signal. These include studies in selected human populations with known changes in mitochondrial content (e.g. endurance-trained athletes).

In vivo detection of ^{31}P NMR signal from the intramitochondrial Pi pool will likely have most impact on the clinical investigation of mitochondrial function in human muscle disease. Specifically, it would enable a non-invasive read-out of any changes in mitochondrial density in response to training or disease by longitudinal measurements of Pi_2 in ^{31}P NMR spectra from resting muscle without any need for prolonged exercise in the magnet (33,36). Additionally, it should benefit recent efforts to obtain validated computational models of cellular metabolic networks including mitochondria (37–40). Specifically, a number of alternative computational models of the mitochondrion have recently been proposed in the literature (38–40). ^{31}P MRS-based information on the *in vivo* absolute intramitochondrial Pi concentration and transmembrane pH gradient would provide invaluable data for accurate parameterization and validation, respectively, of these models.

A quantitative estimate of the concentration of the Pi_2 metabolite pool in SOL muscle could not yet be obtained from all subjects in our experiments, due to the B1 inhomogeneity of the surface coil used. However, a simple calculation using the single CSI data set indicates that

the Pi_2 signal constitutes 6% of the Pi_1 signal after partial saturation correction. Assuming 7% mitochondrial tissue volume, this would result in a Pi_2 concentration of 4.2 mM, which is in reasonable agreement with literature values.

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Abbreviations used

ATP	adenosine triphosphate
CSI	chemical shift imaging
2-DPG	diphosphoglycerate
GPC	glycerol phosphocholine
GPE	phosphoethanolamine
NADPH	pyridine nucleotides
PCr	phosphocreatine
Pi	inorganic phosphate
SNR	signal-to-noise ratio

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Figure 1.

A typical ^{31}P MR spectrum of the resting SOL muscle of a healthy volunteer, with on the right the region between 2.5 and 5.5 ppm enlarged. Signals of an extra Pi pool and phosphoethanolamine (GPE) are visible. Peak assignments: two signals for inorganic phosphate (Pi_1 and Pi_2), glycerol phosphocholine (GPC), glycerol phosphoethanolamine (GPE) phosphocreatine (PCr), three signals for ATP and pyridine nucleotides (NADPH).

Figure 2.

(left) CSI spectra from the resting lower leg overlaid on a proton image in the background (gradient echo with a TR of 128 ms, $0.5 \times 0.5 \times 5 \text{ mm}^3$ resolution). CSI data were collected with a FOV of $150 \times 150 \times 400$ and a matrix size of $10 \times 10 \times 8$. The voxel in green is the selected voxel in the SOL muscle. (right) the selected voxel enlarged, zero-filled and 26 Hz exponential filter were applied. A distinct signal at 5.1 ppm is visible, 0.4 ppm downfield of the main Pi signal.

Figure 3.

Estimation of the T1 value of the two Pi peaks using a saturation-recovery experiment, with an adiabatic RF excitation pulse centered at 5.1 ppm. On the left, signal intensities of Pi_1 (black circles) and Pi_2 (grey circles) are shown with increasing TR. Values are scaled to fully relaxed conditions. Nonlinear curve fitting of an exponential function to these data yielded a shorter T1 for Pi_2 compared to Pi_1 . On the right, zoomed sections of MR spectra in the region around 5 ppm of this experiment for TRs of 0.5, 2 and 10s are shown. Note that the spectra are normalized to the signal intensity of the main Pi peak (Pi_1).

Figure 4. Histogram of the Pi_2/Pi_1 ratio in different muscles in five different subjects. This ratio was significantly higher in SOL muscle (black) compared to TA (grey).