In vivo P31 NMR demonstrates reduced ATP synthesis rate in skeletal muscle in a murine cancer cachexia model

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Introduction– Development of cachexia is the most common manifestation of advanced malignant disease that occurs in the majority of cancer patients before death and, according to Warren, is responsible for the death of 22% of cancer patients (1). Implantation of a fast-growing tumor to mice (Lewis lung carcinoma) results in a clear cachectic state characterized by profound muscle wasting (2). *In vivo* NMR spectroscopy allows measurements of physiological biomarkers in intact systems (3,4), and has recently shown mitochondrial dysfunction in burns (5), which is also characterized by severe muscle wasting. Here, we present results showing (a) reduced rate of ATP synthesis using *in vivo* saturation-transfer ^{31}P NMR spectroscopy, and (b) aberrant gene expression in a clinically relevant cancer cachexia model.

Materials and Methods– NMR spectra of hind limb were acquired 14 days after intramuscular (hind leg) inoculum of 4 x 10^5 Lewis lung carcinoma cells obtained from exponential tumors. All NMR experiments were performed in a horizontal bore magnet (proton frequency 400 MHz, 21 cm diameter, Magnex Scientific) using a Bruker Avance console. A 90° pulse was optimized for detection of phosphorus spectra (repetition time 2 s, 400 averages, 4K data points). Saturation 90°-selective pulse trains (duration 36.534 ms, bandwidth 75 Hz) followed by crushing gradients were used to saturate the γ-ATP peak. The same saturation pulse train was also applied downfield of the inorganic phosphate (Pi) resonance, symmetrically to the γ-ATP resonance. T_1 relaxation times of Pi and phosphocreatine (PCr) were measured using an inversion recovery pulse sequence in the presence of γ-ATP saturation. An adiabatic pulse (400 scans, sweep with 10 KHz, 4K data) was used to invert Pi and PCr, with an inversion time between 152 ms and 7651 ms. Biopsies were harvested from the left gastrocnemius muscle. RNA was extracted, purified, and quantified and genomic analysis was performed following standard Affymetrix protocols(Affymetrix, CA, USA).

Results– Representative ^{31}P NMR spectra from control and cancer-bearing mice are shown in Fig. 1. ATP synthesis rate was significantly reduced in cancer-bearing mice (Table 1). Fig. 2 shows that uncoupling protein 3 (UCP3), and forkhead box O 3 alpha (Fox03alpha) expressions were significantly upregulated (P=0.00003 and P=0.0002 respectively) and this corroborated in part previous findings (6). Meanwhile, peroxisome proliferator activated receptor-gamma coactivator-1beta (PGC-1beta) was significantly downregulated (P=0.006). PGC-1alpha expression levels did not change (P=0.46).

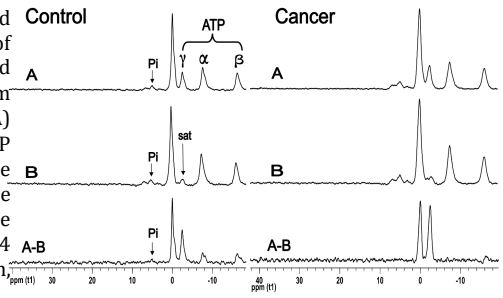

Figure 1. NMR spectra of *in vivo* 31P NMR saturation-transfer performed on the hind limb skeletal muscle of awake mice. Representative summed 31P-NMR spectra acquired from normal and burned mice before (A) and after (B) saturation of the γATP resonance, with the difference spectrum between the two (A-B). The arrow indicates the position of the saturation (sat) by rf irradiation (-2.4 ppm). P_i, inorganic phosphate; ppm, chemical shift in parts per million.

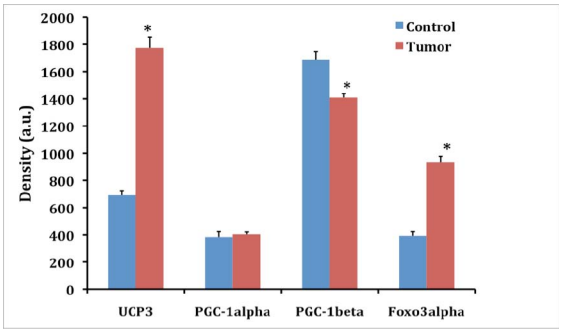

Figure 2. Gene expression levels (mRNA) in cancer cachexia. Asterisks indicates statistical significance (t-test).

Table 1. Results of *in vivo*^{31}P-NMR saturation transfer experiments performed on the hindlimb skeletal muscle of mice. ATP synthesis rate (reaction $P_i \rightarrow \gamma ATP$).

	Healthy Controls (n = 10)	Cancer (n = 6)	⊗(%)	P-value
$\Delta M/M_0$	0.484 ± 0.036	0.304 ± 0.051	–37.2	0.011
T_{1obs} (s)	1.59 ± 0.14	1.50 ± 0.14	-5.6	NS
κ_f (s^{-1})	0.304 ± 0.026	0.203 ± 0.038	–33.2	0.038
ATP (μmol/g)	1.19 ± 0.14	0.87 ± 0.19	–26.9	0.054
P_i (μmol/g)	0.280 ± 0.062	0.222 ± 0.079	-20.7	NS
ATP synthesis rate (μmol/g/s)	0.085 ± 0.013	0.045 ± 0.013	–47.1	0.029

Values are means ± SE; $\Delta M/M_0$ is the fractional change in P_i magnetization as a result of saturation transfer; T_{1obs} is the observed spin lattice relaxation time of P_i during γATP saturation in seconds; κ_f is the rate constant for the reaction $P_i \rightarrow \gamma ATP$, calculated as $(1/T_{1obs}) \times (\Delta M/M_0)$. ATP synthesis is calculated as $[P_i] \times \kappa_f$. A bioluminescence assay kit was used to assess ATP concentration. Unpaired Student's t-test was used for the comparisons.

Discussion– NMR-measured unidirectional ATP synthesis flux primarily reflecting rate through the F1F0-ATP synthase enzyme, with the coupled glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase reactions being negligible. Protons extruded from the mitochondrial matrix during electron transport drive ATP synthesis during re-entry through the F1-ATPase. Our *in vivo* NMR results showed significantly reduced rate of ATP synthesis rate and were cross-validated by results from analysis of genomics in skeletal muscle tissue showing alterations in key metabolic gene expressions (i.e., UCP3 and PGC-1beta) which cause aberrant mitochondrial biogenesis and uncoupling leading to cachexia. FoxO3 activation alone causes dramatic cachexia *in vivo* (7). Our findings suggest that cachexia and muscle wasting in cancer are due to reduced ATP synthesis rate that may be regulated upstream by key regulatory genes. These findings implicate mitochondrial dysfunction in distal skeletal muscle in cancer cachexia. Our NMR approach advances the development of novel *in vivo* non-destructive research approaches in murine models of cancer cachexia, suggests biomarkers for investigation of biomedical paradigms, and thus may contribute to novel therapeutic development.

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