Mitochondrial skeletal muscle uncoupling in a murine cancer cachexia model

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Target Audience: Radiologists, Oncologists, Cancer Scientists.

Purpose– About half of all cancer patients, particularly those with cancers in the gastrointestinal tract and lung, present with cachexia in which disease-associated metabolic changes lead to a severe loss of skeletal muscle mass resulting in a body weight reduction of 30% or more [1]. We tested the hypothesis that cancer promotes mitochondrial uncoupling in skeletal muscle. Several clinically relevant animal models resulting in a cachectic state are characterized by profound muscle wasting, but there is no generally agreed model for preclinical testing of cachexia drug therapy. In this study we used the Lewis lung carcinoma [2]. Here, we used in vivo NMR spectroscopy and ex-vivo by Gas Chromatography Mass Spectrometry (GC-MS) technique to asses ATP synthesis rate and TCA cycle flux respectively, and to determine if their ratio, which provides an index of mitochondrial coupling, is affected in the skeletal muscle in cancer cachexia. Our results were complemented by genomic analysis and electron microscopy and suggest that mitochondria could be pharmaceutically targeted to treat cancer cachexia.

Materials and Methods– Mice were randomized into tumor-free control (C) and tumor-bearing (TB). TB-mice received an intramuscular (right hind leg) inoculum of 4×10^5 Lewis lung carcinoma cells obtained from exponential tumors. *^{31}P NMR spectroscopy.* NMR spectra of hind limb were acquired 14 days after intramuscular (hind leg) inoculum. All NMR experiments were performed in an 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. T1 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. *NMR Data analysis*. ^{31}P NMR spectra were analyzed using the MestReNova NMR software package (Mestrelab Research S.L., v. 6.2.1 NMR solutions, Website: www.mestrec.com. The T1obs relaxation time for Pi and PCr was calculated by fitting the function $y = A_1(1 - A2e-(t/T1obs))$ to the inversion recovery data, where y is the z magnetization and t is the inversion time. *Calculation of ATP concentration*: ATP concentration was measured using a Bioluminescence Assay Kit CLS II, Cat# 1699695 (Roche Diagnostics Corporation, Indianapolis, IN 46250-0414, USA). *Calculation of ATP synthesis rate*: For the calculation of the ATP synthesis rate, information from the ^{31}P-NMR spectra and the previously mentioned biochemically measured concentration of ATP was used, as described by Forsen and Hoffman [3]. TCA flux *assessment:* TCA cycle flux was calculated from the time course of ^{13}C enrichment in C-4 and C-2 of glutamate (mass-isotopomers M+1 and M+2 of glutamate) during an infusion of 2-^{13}C acetate. Plasma acetate concentration and ^{13}C enrichment of glutamate in the gastrocnemius muscle were obtained by GC/MS, as described elsewhere [4]. A novel "fragmented mass-isotopomer" approach for dynamic analysis of 13C mass-isotopomers data measured ex-vivo by Gas Chromatography Mass Spectrometry (GC-MS) technique was used to evaluate skeletal muscle TCA flux at different conditions [5]. *Transmission Electron Microscopy (TEM).* For mitochondrial morphology analysis, the muscles were extracted and dissected in small pieces of 1 mm2 approximately and transferred to glass vials filled with 2% parafomaldehyde and 2.5% glutaraldehyde in cacodylate buffer (Fixative buffer).**Results–** The ^{31}P NMR saturation-transfer experiments were performed to determine the rate of mitochondrial ATP synthesis. Figure 1 shows representative ^{31}P NMR spectra acquired from the control group of mice, before (A) and after (B) saturation of the γ-ATP resonance. On irradiation of the γ ATP resonance, the signal intensities of PCr, Pi, αATP, and βATP resonances all decrease, either by magnetization transfer or direct off resonance saturation. Figure 2 shows that ATP synthesis rate and TCA cycle flux were significantly reduced by 49% and 25% respectively, in cancer-bearing mice (P=0.008 and P<0.003). The ratio of ATP synthesis rate to the TCA cycle flux, which provides an index of mitochondrial coupling, was 32% less in cancer-bearing mice (P=0.036). Our results were cross-validated with genomic analysis, showing aberrant expression levels in key regulatory genes and by electron microscopy showing abnormal giant mitochondria. **Discussion–** The principle finding of this study was that TCA cycle flux by mass spectrometry was significantly reduced in cancer-bearing mice. ATP synthesis rate by ^{31}P NMR was also significantly reduced in cancer-bearing mice, and this confirmed our previous published results suggestive of bioenergetic mitochondrial dysfunction [2]. Importantly, the ratio of ATP synthesis rate to the TCA cycle flux, which provides an index of mitochondrial coupling, was significantly less in cancer-bearing mice. The results are consistent with earlier studies in rats in which an increase in UCP3 expression, induced by T3 treatment [6] or fasting [7], was associated with an increase in mitochondrial uncoupling. The novel results with TEM demonstrate that cancer-induced cachexia causes a profound structural disorganization of skeletal muscle as indicated by the observed fiber disruption, band disarrangement, and dilated sarcoplasmic reticulum in the cachectic gastrocnemius muscle from TB mice. The area of cachectic gastrocnemius intermyofibrillar mitochondria is characterized by the presence of giant mitochondria, which could be attributed to their inability to fuse with each other or with normal mitochondria [8] indicating thus a causal effect of structural mitochondria dysfunction on cancer-induced muscle wasting. **Conclusion-** The novel results presented here provide evidence of mitochondrial uncoupling in cancer cachexia and suggest that cancer-induced cachexia causes a profound functional and structural disorganization of murine skeletal muscle. This work fills a knowledge gap in the integrated and mechanistic view of cancer cachexia, which shares similarities with the muscle wasting that occurs in many chronic diseases and aging.

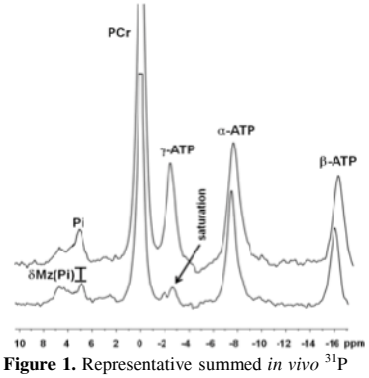

Figure 1. Representative summed *in vivo* ^{31}P NMR spectra acquired from the hind limb skeletal muscle of mice before (upper) and after (lower) saturation of the γ-ATP resonance. The arrow on γ -ATP indicates the position of saturation (sat) by rf irradiation (-

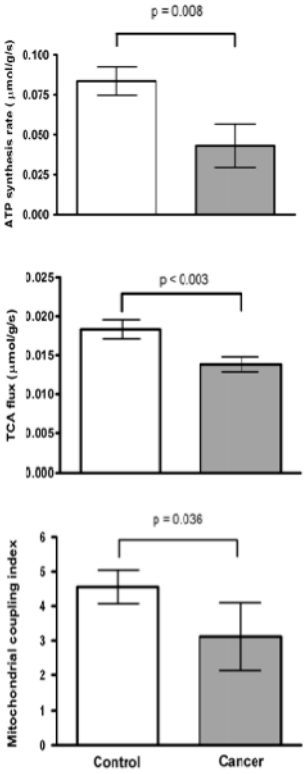

Figure 2. Rates of unidirectional ATP synthesis (upper plot), TCA cycle flux (TCA, middle plot), and the coupling index (lower plot) calculated as the ratio of the rates of ATP synthesis and TCA cycle flux in normal and cancer-bearing mice.

References- 1. Tisdale, M.J., Nat Rev Cancer, 2002. 2(11): p. 862-71. **2.** Constantinou, C., et al., Int J Mol Med, 2011. 27(1): p. 15-24. **3.** Forsen, S. and R. Hoffman. Journal of Chemical Physics 1963. 39(11): p. 2892. **4.** Cline, G.W., et al., J Biol Chem, 2001. 276(23): p. 20240-4. **5.** Shestov, A.A., et al., Neurochemical research, 2012. **6.** Jucker, B.M., et al., Proc Natl Acad Sci U S A, 2000. 97(12): p. 6880-4. **7.** Jucker, B.M., et al., J Biol Chem, 2000. 275(50): p. 39279-86. **8.** Navratil, M., A. Terman, and E.A. Arriaga, Exp Cell Res, 2008. 314(1): p. 164-72.