In vivo high-resolution magic angle spinning proton NMR spectroscopy of Drosophila melanogaster flies as a model system to investigate mitochondrial dysfunction in trauma

N. Psychogios[1,2], Y. Apidianakis[3], V. Righi[1,2], H. Szeto[4], R. G. Tompkins[5], L. G. Rahme[3,5], and A. A. Tzika[1,2]

[1]NMR Surgical Laboratory, Department of Surgery, Massachusetts General Hospital and Shriners Burn Institute, Harvard Medical School, Boston, MA, United States, [2]Department of Radiology, Athinoula A. Martinos Center for Biomedical Imaging, Massachusetts General Hospital, Charlestown, MA, United States, [3]Molecular Surgery Laboratory, Massachusetts General Hospital and Shriners Burn Institute, Harvard Medical School, Boston, MA, United States, [4]Department of Pharmacology, Joan and Sanford I. Weill Medical College of Cornell University, New Yor, Joan and Sanford I. Weill Medical College of Cornell University, New York, NY, United States, [5]Department of Surgery, Massachusetts General Hospital and Shriners Burn Institute, Harvard Medical School, Boston, MA, United States

Introduction– Trauma resulting from a wide range of injuries impacts skeletal muscle metabolism and is the fifth overall cause of death of Americans, leading to 100,000 deaths in the US annually. Use of state-of-the art, *in vivo* NMR techniques to elucidate metabolic derangement following skeletal muscle trauma and the effects of a potential therapeutic agent is of public health relevance. Also, use of *Drosophila melanogaster* as a model organism is of interest due to the remarkable parallels between metabolism in *Drosophila* and mammals (1,2). Indeed, study of *Drosophila* metabolism is an emerging field that can potentially elucidate conserved metabolic mechanisms. Furthermore, powerful genetic tools available in *Drosophila* research make the fruit fly a particularly tractable model organism in which to probe metabolic pathways and lead to a better understanding of human metabolic disorders. Here, we examined the feasibility of a novel, *in vivo* high-resolution magic angle spinning proton NMR spectroscopy (HRMAS ^{1}H NMR) approach (Fig. 1) towards the investigation of the metabolic derangements in traumatized flies. We hypothesized that these metabolic derangements can be ameliorated by treatment with a novel mitochondria protective peptide (Szeto-Schiller, SS-31) (3).

Materials and Methods– We used male wild-type (WT) and mutant *chico* (exhibiting reduced insulin signaling) *Drosophila* flies (n=6 per group). Saline and SS-31 were injected 12 hours post-injury. The flies were placed in the spectrometer and special care was taken to avoid injury during moving in and out of the rotor. Prior to fly insertion in the spectrometer, flies were immobilized by placing them on ice for less than 1 min and were kept at 4°C while in the spectrometer. ^{1}H HRMAS experiments were performed on a Bruker Bio-Spin Avance NMR spectrometer (600.13 MHz) using a 4mm triple resonance (^{1}H, ^{13}C, ^{2}H) HRMAS probe (Bruker). The temperature was kept at (or adjusted to) 4°C by a BTO-2000 unit in combination with a MAS pneumatic unit (Bruker). The flies were placed into 4mm zirconium oxide (Zirconia, Bruker) rotors with spherical inserts. Eight μl D_2O (deuterium lock reference) containing 50 mM TSP (trimethylsilyl propionic-2,2,3,3-d_4 acid, M_w=172, δ=0ppm, external chemical shift reference) were added to the rotor with the sample. To avoid contact between flies and D_2O, a small piece of parafilm was inserted on top of the insert. The MAS rotation frequency was stabilized at 2.0 ± 0.001 kHz by a MAS speed controller. One-dimensional ^{1}H HRMAS NMR single-fly spectra were acquired on all samples using a rotor synchronized Carr-Purcell-Meiboom-Gill (CPMG) spin echo pulse sequence, [90°-(τ-180°-τ)n], which works as a T2 filter to remove the spectral broadening. The inter-pulse delay (τ = 500μs) was synchronized to the MAS rotation frequency. The number of transients was 256 with 32,768 (32k) data points. The 1D fully-relaxed spectra were also obtained using a single pulse sequence with water pre-saturation signal. A line-broadening apodization function of 3.0 Hz was applied to all HRMAS ^{1}H FIDs prior to Fourier transformation. Spectra were referenced with respect to TSP, manually phased, and a Whittaker baseline estimator was applied to subtract the broad components of the baseline prior to peak area calculations using MestReC software. Peak heights were scaled with respect to TSP.

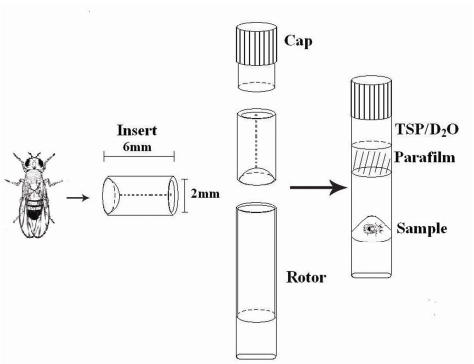

Figure 1. *Experimental approach of in vivo HRMAS ^{1}H NMR for the investigation of live Drosophila at 14.1 T. External standard trimethylsilyl- propionic-2,2,3,3-d4 acid (TSP).*

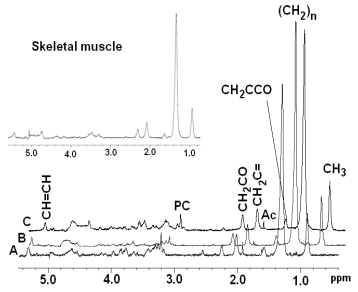

Figure 2. *In vivo 1D HRMAS ^{1}H CPMG spectra of: **A)** old WT not injured, **B)** old WT injured, and **C**) chico mutants. Lipid components: C**H**$_3$ (0.89 ppm), (C**H**$_2$)n (1.33 ppm), C**H**$_2$C-CO (1.58ppm), Acetate (Ac, 1.92 ppm), C**H**$_2$C=C (2.02 ppm), C**H**$_2$C=O (2.24 ppm), β-alanine (β-Ala, 2.55 ppm), phosphocholine (PC, 3.22 ppm), and phosphoethanolamine (PE, 3.22 ppm), glycerol (4.10, 4.30 ppm 1,3-CH; 5.22 ppm 2-CH$_2$), C**H**=C**H** (5.33 ppm). The spectrum in the inset is from the thorax of dissected flies and thus represent primarily skeletal muscle; note their similarity to spectra for whole flies.*

Results– Representative *in vivo* 1D HRMAS ^{1}H CPMG spectra of *Drosophila* flies are shown in Fig. 2. Note that the metabolic profile of aged WT flies (B) was similar to the one of *chico* non-injured mutant flies (C), which have a phenotype of insulin resistance, and exhibit a substantial increase in both $(CH_2)_n$ lipids at 1.33 ppm and CH_2C-CO lipids at 1.58 ppm, attributed to intra- (IMCL) and extramyocellular (EMCL) respectively, as well as increases in other lipids. Quantitative analysis showed a significant increase in (C**H**$_2$)n lipids (an insulin resistance biomarker) at 1.33 ppm in old WT injured flies (Table 1). Also C**H**=C**H** lipids at 5.33 ppm, an apoptosis biomarker, were significantly increased in old WT injured flies. Both (C**H**$_2$)n and C**H**=C**H** lipids were normalized following injection of SS-31 (Table 1).

Discussion– Indeed, elevated IMCL levels are associated with insulin resistance, a metabolic dysfunction in skeletal muscle trauma (4). Also ceramide (5.33 ppm) accumulation decreases insulin stimulated GLUT4 translocation to the plasma membrane and, consequently, decreases glucose transport (5), resulting in insulin resistance. Our findings agree with our prior report in murine burn trauma (6), and provide evidence for the hypothesis that trauma in aging is linked to insulin signaling (7). This link may explain the mitochondrial dysfunction that accompanies insulin resistance that occurs in trauma and aging, in mammals since a mitochondrial protective peptide normalized insulin resistance and apoptosis biomarkers. Our approach advances the development of novel *in vivo* non-destructive research approaches in *Drosophila*, suggests biomarkers for investigation of biomedical paradigms, and contributes to novel therapeutic development.

Table 1. Quantity (μmol/g) of selected lipid components in live *Drosophila* from ^{1}H HRMAS NMR

Lipid components		**CH_3**	**$(CH_2)_n$**	**CH_2CCO**	**$CH_2C=$**	**CH_2CO**	**CH=CH**
Chemical shift (δ, ppm)		**0.89 ppm**	**1.33 ppm**	**1.58 ppm**	**2.02 ppm**	**2.24 ppm**	**5.33 ppm**
WT	not injured	0.18 ± 0.01	1.41 ± 0.08	0.06 ± 0.003	0.13 ± 0.01	0.07 ± 0.01	0.08 ± 0.01
	injured	0.27 ± 0.03	2.10 ± 0.25	0.16 ± 0.08	0.24 ± 0.06	0.13 ± 0.03	0.13 ± 0.02
	% change, P-value	50.0, **0.022 (*)**	48.94, **0.024 (*)**	166.67, 0.26	84.62, 0.085	85.71, 0.071	62.50, **0.015 (*)**
WT 12 h	injured+saline	0.26 ± 0.003	1.94 ± 0.05	0.13 ± 0.01	0.22 ± 0.004	0.10 ± 0.01	0.13 ± 0.004
	injured+SS31	0.17 ± 0.002	1.29 ± 0.02	0.06 ± 0.002	0.12 ± 0.002	0.06 ± 0.001	0.08 ± 0.002
	% change, P-value	-34.62, **< 0.001 (*)**	-33.51, **< 0.001 (*)**	-53.85, **0.0013 (*)**	-44.45, **< 0.001 (*)**	-40.00, **0.00012 (*)**	-38.46, **< 0.001 (*)**

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