

In vivo substrate oxidation in skeletal muscle of mice

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Introduction Mismatched activities of mitochondrial β -oxidation and tricarboxylic acid cycle (TCA) have been implicated in the development of skeletal muscle insulin resistance [1]. $[2-^{13}\text{C}]\text{acetate}$ has been used to measure the kinetics of TCA, *in vivo*, in skeletal muscle of humans[2], rats[3] and rabbits[4] using dynamic ^{13}C Magnetic Resonance Spectroscopy (MRS). This powerful tool to study aspects of energy metabolism *in vivo* would also be very useful to apply to mice for which a wide variety of specific energy metabolic transgenes have been created. However, performing such experiments in mice poses a number of practical challenges due to their small size. Recently, the feasibility of ^{13}C MRS with $[1,6-^{13}\text{C}]\text{glucose}$ in the mouse brain was demonstrated [5].

The aim of this study was to develop a protocol to examine *in vivo* substrate oxidation in skeletal muscle of mice, in particular to be able to study fatty acid oxidation and subsequent TCA cycle rates in muscle of energy deficient knockout models. Dynamic ^{13}C MRS was applied here to resting hind-limbs of wild-type mice subjected to an intravenous infusion of $[2-^{13}\text{C}]\text{acetate}$ or $[2,4,6,8-^{13}\text{C}]\text{octanoate}$.

Materials and methods: Eight-week old wild-type mice ($n=13$) were fasted 6 h prior to the experiments. They were anaesthetized with 1-2 % isoflurane in a 50:50 % mixture of $\text{O}_2/\text{N}_2\text{O}$ delivered through a face mask and their left-jugular vein was catheterized with a micro-renalthane tube. Animals were positioned prone in the magnet with the right-hind-limb in a 4-turn ^{13}C solenoid coil surrounded by an Alderman-Grant ^1H coil to enable shimming and ^1H decoupling. MRS was performed in a 7T horizontal bore magnet interfaced to a clinical console (Clinscan, Bruker Biospin). The ^{13}C and ^1H coils were tuned to 75.6 MHz and 300.4MHz respectively. Non-localized ^1H decoupled ^{13}C MR spectra were acquired with a 35° flip-angle, $\text{TR} = 300\text{ms}$, $\text{NP}=2048$, $\text{NA}= 2048$, $\text{SW}=20\text{KHz}$. ^1H decoupling was achieved by Waltz-4 centred on glutamate resonances at 2.2 ppm. Baseline spectra were acquired during a saline infusion ($2\mu\text{L}/\text{min}$) over 30 min. Subsequent ^{13}C spectra were acquired either during 150 min of a primed infusion of $[2-^{13}\text{C}]\text{acetate}$ ($200\mu\text{mol}/\text{Kg}/\text{min}$) $n=7$, or during 240 min of a primed infusion of $[2,4,6,8]\text{octanoate}$ ($50\mu\text{mol}/\text{Kg}/\text{min}$), $n=6$. FIDs were Fourier transformed and after baseline subtraction and glutamate resonances of C2 (55.4 ppm) and C4 (34.2ppm), C2 of acetate (24.5 ppm) as well as C2,C4,C6 and C8 of octanoate (36.4 ppm, 29.1ppm, 31.2ppm,14.5 ppm) were Gaussian line-fitted using NUTS-pro software (Acorn NMR, USA). After infusion, plasma was collected and calf-muscles were rapidly excised and freeze-clamped for absolute enrichment measurement and further ^{13}C NMR isotopomer analysis.

Results & Discussion: Oxidation of $[2-^{13}\text{C}]\text{acetate}$, and of its precursor $[2,4,6,8-^{13}\text{C}]\text{octanoate}$, in skeletal muscle of mice cause ^{13}C enrichment in glutamate C4 from a first turn of the TCA cycle. A subsequent turn labels glutamate C2 and C3. After the start of the $[2-^{13}\text{C}]\text{acetate}$ infusion, a resonance for acetate C2 (24.1ppm) was immediately visible in the first ^{13}C MR spectrum (Fig.1). Glutamate was sufficiently ^{13}C enriched in both infusions to allow the detection of the carbons C2 and C4. During $[2-^{13}\text{C}]\text{acetate}$ infusion, the glutamate C4 resonance was detected at the onset of the infusion ($\sim 10\text{min}$) whereas carbon C2 became visible later in time ($> 30\text{min}$). Mono-exponential fitting of glutamate carbon resonances revealed that the time of half-maximal ^{13}C enrichment (t_{50}) of glutamate C4, an index of TCA cycle flux, was $7.2\pm 1.8\text{min}$ and $19.9\pm 3.9\text{min}$ for glutamate C2 ($p<0.02$). Relative contribution of anaplerotic pathways to the TCA cycle flux was calculated from the ratio glutamate C2/glutamate C4, which in our experimental conditions, was 0.40 ± 0.10 , within the range of values reported previously [4,6]. Unlike $[2-^{13}\text{C}]\text{acetate}$, the conversion of $[2,4,6,8-^{13}\text{C}]\text{octanoate}$ into glutamate C4 was much slower with a t_{50} of $30.7\pm 4.2\text{min}$ ($p<0.002$) whereas t_{50} for glutamate C2 was $38.3\pm 11.8\text{min}$, not significantly different from that one of glutamate C4. The relative contribution of anaplerosis to TCA cycle during $[2,4,6,8-^{13}\text{C}]\text{octanoate}$ infusion was 0.42 ± 0.20 , not significantly different from the one observed in the $[2-^{13}\text{C}]\text{acetate}$ infusion. During the $[2,4,6,8-^{13}\text{C}]\text{octanoate}$ infusion, a sequential lipid/octanoate accumulation occurs (see Fig.1) This suggests that: first, fatty acid oxidation and not TCA cycle is rate-limiting for ^{13}C glutamate enrichment; second, during a constant lipid overload, only a limited amount of lipids are oxidized and reach the TCA cycle. Further analysis of the different multiplets of glutamate, as well as absolute enrichments of the pool will be performed in order to estimate absolute fluxes of TCA cycle and anaplerosis.

Conclusion: This study shows the feasibility of dynamic ^{13}C measurements *in vivo* in skeletal muscle of mice, with a relative good resolution, to infer on specific metabolic pathways, namely at the level of muscular substrate oxidation. This paves the way to apply this technique in investigations of the dynamics of energy metabolism in skeletal muscle of knockout models.

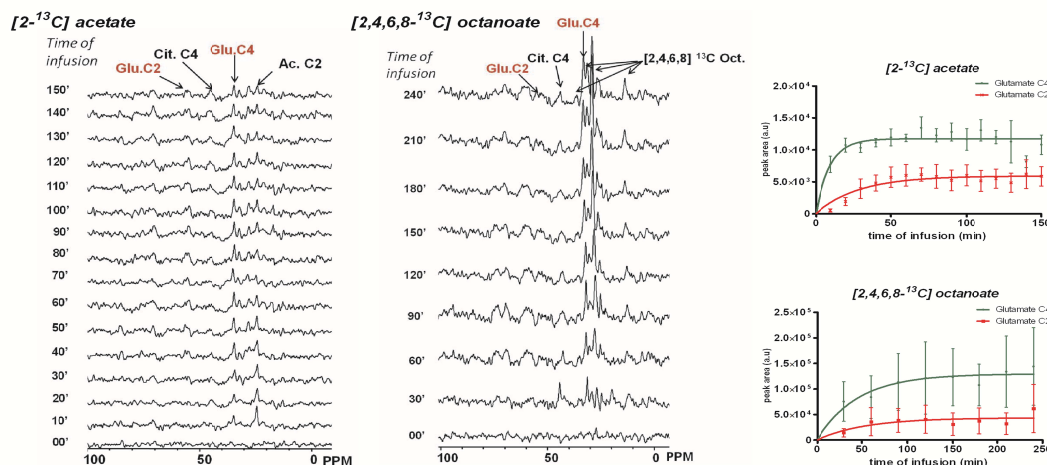


Figure 1 Stack-plots of *in vivo* ^1H decoupled ^{13}C MR spectra acquired from the hind-limb of a mouse during 150 min of $[2-^{13}\text{C}]\text{acetate}$ or 240 min of $[2,4,6,8]\text{octanoate}$. Resonances of glutamate carbons (Glu. C2, C4,) acetate (Ac.C2), citrate (Cit.C4) and $[2,4,6,8]\text{octanoate}$ could be identified in the ^{13}C spectra. Mono-exponential fittings of absolute areas of ^{13}C glutamate C4 and C2 during: (above) 150 min of $[2-^{13}\text{C}]\text{acetate}$ infusion in 7 mice; and (below) during 240 min of $[2,4,6,8-^{13}\text{C}]\text{octanoate}$ infusion in 6 mice.

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