Metabolic flux analysis of hepatic mitochondrial oxidation of hyperpolarized [1-^{13}C] and [2-^{13}C] pyruvate *in vivo*

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Introduction: ^{13}C MRS studies in the liver are challenging due to abundant intracellular lipid resonances, impairing detection of the ^{13}C glutamate labeling commonly used to measure tricarboxylic acid (TCA) cycle fluxes [1]. The carboxyl resonances typically detected by hyperpolarized ^{13}C MRS (180-200 ppm region) do not interfere with lipid resonances and allows studying real-time *in vivo* metabolism in a sensitive manner. In this study, we assessed hepatic metabolism in vivo in real time using combined and separate injections of hyperpolarized (HP) [2-^{13}C]pyruvate and [1-^{13}C]pyruvate to independently detect the contributions to mitochondrial metabolism related to pyruvate carboxylase (PC) and pyruvate dehydrogenase (PDH) activities. The enzymatic fluxes were quantified by metabolic modeling.

Materials and Methods: ***Polarization:*** Neat ^{13}C-labelled pyruvic acid prepared with trityl radical (Albeda) was polarized in a 7 T custom-built DNP polarizer with microwave irradiation (196.8 GHz, 55mW) for 1.5hr at 1.00 ± 0.05 K. The frozen sample was rapidly dissolved with 5.5 ml preheated deuterated phosphate buffer (~pH 7.5). The solution was automatically transferred into a separator/infusion pump placed inside the animal bore. ***Animal:*** Male Sprague Dawley rats (n=3, 219 ±69.8g, fed) were anesthetized with isoflurane. A femoral vein and artery were catheterized for substrate injection and blood pressure measurements, respectively. ***MR acquisition*:** Hyperpolarized ^{13}C-pyruvate was injected through the femoral vein. *In vivo* ^{13}C MRS measurements were respiratory gated and cardiac triggered with a repetition time of 3 s in a 9.4T/31cm horizontal bore magnet (Varian/Magnex). 30° BIR4 adiabatic RF excitation pulses with proton decoupling were applied with a custom-built quadrature ^{1}H/single loop ^{13}C surface coil placed on the liver of the rat. Liver tissue extracts were obtained by freeze-clamping. ***Data analysis:*** Metabolite peak areas were quantified by fitting using Bayesian Analysis. Spectra were summed for ratiometric analysis using relative ratios with pyuvate, and single spectra were analyzed to obtain time courses of peak areas. Error bars indicate ± SEM. ***Model:*** The model is described by eight isotopic mass balance equations for aspartate (ASP, C1 and C4), malate (MAL, C1 and C4), oxaloacetate (OAA, C1 and C4), fumarate (FUM, C1/C4) and bicarbonate (BIC) (Fig. 1). Repeated RF excitations were taken into account and the relaxation time (T_1) of metabolites was assumed to be 20 s. Pyruvate recycling was not included in this model.

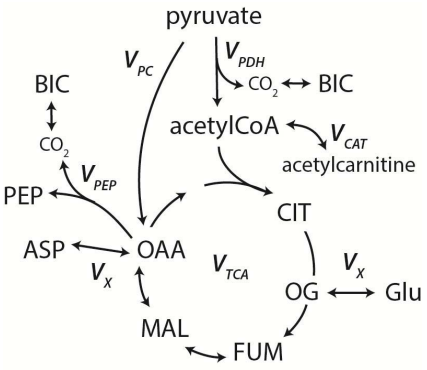

Fig. 1. Metabolic model applied to estimate kinetic parameters.

Table 1. Kinetic parameters

V_{TCA} [µmol/g/min wet weight]	0.81 ± 0.36
V_X [µmol/g/min wet weight]	1.27 ± 0.29
V_{PEPCK} [µmol/g/min wet weight]	2.40 ± 0.37
V_X/V_{TCA}	1.57

Results and Discussion: Following the injection of [1-^{13}C]pyruvate, ^{13}C labeling of aspartate (C1 and C4), malate (C1 and C4), ^{13}C-bicarbonate, alanine (C1) and lactate (C1) were consistently observed (Fig. 2). In several experiments, the ^{13}C labeling in C1 and C4 of fumarate was also detected. Interestingly, the injection of [2-^{13}C]pyruvate did not result in the detection of metabolites downstream of PDH, such as acetylcarnitine (C1), citrate (C5) and glutamate (C5). The co-injection of both substrates, with identical concentration and polarization levels, showed results similar to the separate injections. Both observations suggest, as was reported previously that pyruvate carboxylase (PC) activity predominates in the liver [2]. The ^{13}C labeling was described with the mathematical model (Fig. 3), estimating fluxes of V_{TCA}, V_{PEPCK}, V_X and the metabolic rate V_{PC}/[pyr] and V_{PDH}/[pyr]. The estimation of V_{PEPCK} assumes a fast equilibration of CO_2 with bicarbonate, and thus includes the activity of carbonic anhydrase. The estimated metabolic fluxes of V_X , V_{PEPCK} and V_{TCA} (Table 1) are slightly higher than previous reported values [1, 3]. V_{TCA} was estimated to be 0.81 ± 0.36 µmol/g/min wet weight, V_X 1.27 ± 0.29 and the relative ratio to V_{TCA} was 1.31.

Conclusion: HP [1-^{13}C] and [2-^{13}C] pyruvate studies showed that pyruvate carboxylation predominates over pyruvate oxidation in the liver of fed rats. HP ^{13}C MRS enables the direct estimation of hepatic metabolic fluxes using ^{13}C labeling of TCA cycle intermediates within a single 1-min experiment, enabling comparative studies of different metabolic states.

References: [1] D. Befroy *et al.*, Nat Med 20:98-102 (2014)**.** [2] M. Merritt *et al. ,* PNAS 47: 19084–19089 (2011) [3] B. Jucker *et al .,* J Biol Chem 273:12187-12194 (1998)

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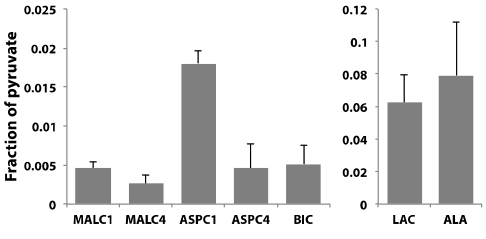

Fig. 4. Metabolite ratios measured in summed spectra relative to that of the injected substrate pyruvate.

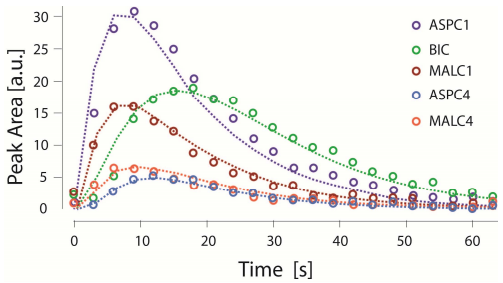

Fig. 3. Time courses of peak areas (spheres) of the ^{13}C labeled metabolites observed in the liver following HP [1-^{13}C]pyruvate injection. Dashed line indicates the fit to the metabolic model.

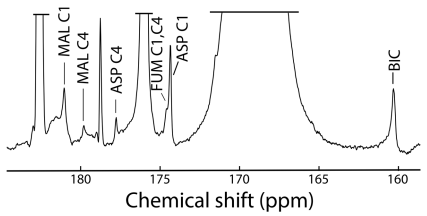

Fig. 2. ^{13}C MRS spectrum following [1-^{13}C]pyruvate metabolism.