Detection of hyperpolarized 13C labeled ketone bodies in vivo

Brett William Clive Kennedy[1,2], Mikko Iivari Kettunen[1,2], De-En Hu[1,2], Sarah Elizabeth Bohndiek[1,2], and Kevin Michael Brindle[1,2]

[1]*Department of Biochemistry, University of Cambridge, Cambridge, England, United Kingdom,* [2]*Cambridge Research Institute, Cancer Research UK, Cambridge, England, United Kingdom*

Background

The ketone bodies, acetoacetate and D-β-3-hydroxybutyrate, are important metabolites during periods of fasting, accounting for nearly 70% of the oxygen consumption in the brain[1]. Clinically, loss of lean muscle mass and body fat due to tumor burden in the presence of adequate nutrition (cachexia) is commonly seen with cancer. A significant proportion of mortality is often due to complications arising from this aberrant metabolism[2]. In cachexia, blood glucose is reduced and unlikely to remain the sole metabolic fuel sustaining tumor growth; it has been shown previously, in xenograft tumor models, that D-β-3-hydroxybutyrate and acetoacetate are used in direct proportion to their rate of supply[3]. The current study sought to probe ketone body metabolism *in vivo* using a variety of hyperpolarized ^{13}C labeled ketone body substrates and precursors.

Methods

[U-$^{13}C_4$] sodium D-β-3-hydroxybutyrate (NaHB) and [1,3-$^{13}C_2$] ethyl acetoacetate (EAcAc) were purchased from Cambridge Isotope Laboratories, MA. [1,3-$^{13}C_2$] lithium acetoacetate (AcAc) was prepared by saponification of the ethyl ester with lithium hydroxide and recrystallization from methanol/ether[4]; [3-$^{2}H_1$ 1,3-$^{13}C_2$] DL-β-3-hydroxybutyric acid (HBFA) was prepared by borodeuteride reduction of the ethyl ester, followed by saponification in methanol/sodium hydroxide and ether extraction of the free acid[5]. Substrates were polarized either as saturated aqueous solutions, or as the neat acid, in the presence of 15mM OX 63 radical (GE Healthcare, Amersham UK), with the exception of [1,3-$^{13}C_2$] ethyl acetoacetate, which was polarized as the neat ester in the presence of 30% DMSO and 15mM AH11501 (GE Healthcare, Amersham UK). Each preparation contained 1.2mM gadolinium chelate (Dotarem) and samples were polarized in an alpha-prototype polarizer (Oxford Instruments, UK) at their optimal microwave frequency (~93.965GHz, 100mW) for approximately 90 minutes before dissolution. Experiments *in vivo* were performed in a vertical 8.9cm bore magnet at 9.4T (Oxford Instruments, UK) with a Varian INVOA Unity Plus spectrometer. A 24-mm surface coil was placed either directly over either the heart/liver region (EAcAc, AcAc, HBFA) of a non-tumor bearing mouse, or over the tumor of an EL-4 tumor bearing C57BL/6 adult female mouse (AcAc, NaHB, HBFA). Following dissolution, 0.2mL of hyperpolarized fluid was injected intravenously through a tail-vein catheter (final concentration of each substrate, EAcAc: 80mM, AcAc: 50mM, NaHB: 40mM HBFA: 60mM). ^{13}C spectra were acquired without slice selection for up to one-minute following injection. Data were collected with a nominal flip angle of 10° as follows – EAcAc: 8kHz, 1536 points, T_R = 0.2s, T_E = 0.5ms; NaHB: 8kHz, 1536 points, T_R = 1s, T_E = 0.8ms; AcAc: 8kHz, 432 points, T_R = 1s, T_E = 1ms; HBFA: 18kHz, 3456 points, T_R = 1s, T_E = 0.1ms. Acquisition began approximately fifteen seconds after injection of the hyperpolarized substrate, with the summation of approximately the first fifteen seconds of data after acquisition presented below (Figure 1).

Results

EAcAc, AcAc and HBFA can be polarized to a high degree and exhibit long T_1 values *in vivo*. NaHB, with its substantially lower polarization and T_1 could be observed *in vivo*, but without detection of further metabolism. Metabolites formed during the course of the experiment are labeled with solid black arrows; expected but unobserved metabolites are marked with broken blue arrows. EAcAc (**A**) is converted rapidly into AcAc with an apparent rate constant of 0.043s^{-1}; this likely takes place in the blood due to non-specific esterase activity. AcAc (**B**) and NaHB (**C**) did not appear to be metabolized further, whereas labeled acetoacetate (1-2% of the HBFA peak intensity) was observed after administration of hyperpolarized HBFA (**D**). Modulation of metabolism was attempted by co-injection of each hyperpolarized tracer with equimolar amounts of its partner metabolite, but this did not yield any change in the observed metabolism. Some animals were also fasted for 24 hours prior to the experiment with a view to increasing endogenous ketone body levels; again, no significant changes were detected in the metabolism of these hyperpolarized tracers.

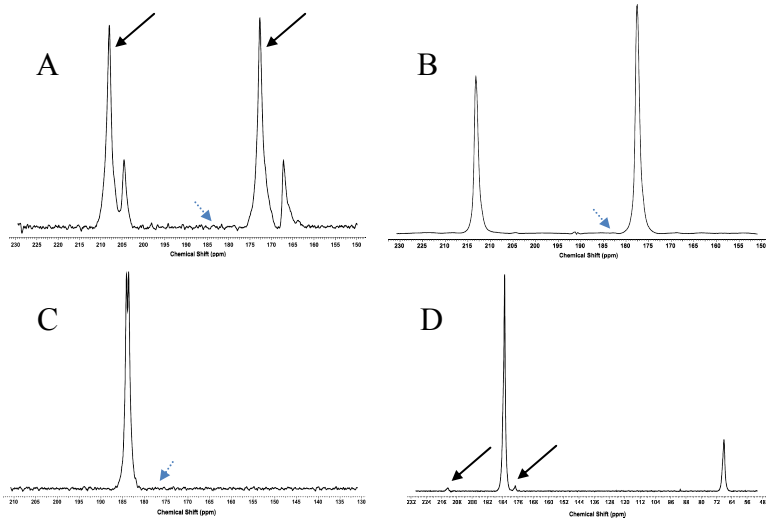

Figure 1
Above: spectra showing the summation of approximately the first 15s of data acquired *in vivo*. **A**, **B** and **D** show spectra when the surface coil was placed over the heart/liver region; spectrum **C** shows the result when the coil was place over an EL-4 tumor.
Right: table showing the relative polarization of each substrate and their respective *in vitro* T_1 values.

Substrate	Polarization (%)	$^{13}C_1$ T_1 (s)	$^{13}C_2$ T_1 (s)
[1,3-$^{13}C_2$] ethyl acetoacetate	20	27	28
[1,3-$^{13}C_2$] lithium acetoacetate	22	41	33
[U-$^{13}C_4$] sodium D-β-3-hydroxybutyrate	7	16	5
[3-$^{2}H_1$ 1,3-$^{13}C_2$] DL-β-3-hydroxybutyric acid	30-40	37	24

Discussion

D-β-3-hydroxybutyrate dehydrogenase, the enzyme that catalyzes the interconversion of D- β-3-hydroxybutyrate and acetoacetate is a mitochondrial, membrane-bound enzyme[6]. The reaction is thought to be near-to-equilibrium and therefore the proportion of these two metabolites has been used as an indicator of the intramitochondrial NAD$^+$/NADH ratio[7]. Since these substrates must cross both the plasma and mitochondrial membranes before metabolism can occur it was thought that this might limit observable metabolism in the hyperpolarized ^{13}C experiment and therefore we also used membrane permeable ethyl acetoacetate[8]. However, while the ester was rapidly metabolized into acetoacetate, this was most likely due to esterase activity in blood as no further metabolism was observed. Furthermore, synthesis of the doubly-labeled hydroxybutyric acid, with the deuterium at the C(D)OH position, resulted in observable generation of labeled acetoacetate. This was most likely due to the long *in vivo* T_1 values and high polarizations achieved. Whilst we could not modulate the observed rate of acetoacetate production in these particular experiments, we nevertheless anticipate that this new substrate may yield information on mitochondrial oxidation state *in vivo*.

Acknowledgements: This study was funded by Cancer Research UK and supported by GE Healthcare.

[1]Owen OE, Biochem. Mol. Biol. Educ. 2005 33:246-51. [2]Tisdale MJ, Nat. Rev. Cancer 2002 2:862-871. [3]Kallinowski F et al Cancer Res. 1998 48:7264-7272. [4]Hall LM Anal. Biochem.1962 3:75-80. [5]Yotagai M et al J. Chem. Soc., Perkin Trans. 1990 1:1826-1828. [6]Marks AR et al JBC. 1992 267:15459-15463. [7]Williamson DH et al Biochem. J. 1967 108:514-527. [8]Hurd RE et al MRM. 2010 63:1137-1143