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The Feasibility of Assessing Branched-Chain Amino Acid Metabolism in Cellular Models of Prostate Cancer with Hyperpolarized [1-¹³C]-Ketoisocaproate

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Abstract

Recent advancements in the field of hyperpolarized ¹³C magnetic resonance spectroscopy (MRS) have yielded powerful techniques capable of real-time analysis of metabolic pathways. These non-invasive methods have increasingly shown application in impacting disease diagnosis and have further been employed in mechanistic studies of disease onset and progression. Our goals were to investigate branched-chain aminotransferase (BCAT) activity in prostate cancer with a novel molecular probe, hyperpolarized [1-¹³C]-2-ketoisocaproate ([1-¹³C]-KIC), and explore the potential of branched-chain amino acid (BCAA) metabolism to serve as a biomarker. Using traditional spectrophotometric assays, BCAT enzymatic activities were determined *in vitro* for various sources of prostate cancer (human, transgenic adenocarcinoma of the mouse prostate (TRAMP) mouse and human cell lines). These preliminary studies indicated that low levels of BCAT activity were present in all models of prostate cancer but enzymatic levels are altered significantly in prostate cancer relative to healthy tissue. The MR spectroscopic studies were

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conducted with two cellular models (PC-3 and DU-145) that exhibited levels of BCAA metabolism comparable to the human disease state. Hyperpolarized [1-¹³C]-KIC was administered to prostate cancer cell lines, and the conversion of [1-¹³C]-KIC to the metabolic product, [1-¹³C]-leucine ([1-¹³C]-Leu), could be monitored via hyperpolarized ¹³C MRS.

Keywords

hyperpolarized carbon-13; dynamic nuclear polarization; magnetic resonance spectroscopy/spectroscopic imaging; prostate cancer; branched-chain aminotransferase

Introduction

Prostate cancer is the second most frequently diagnosed cancer in men worldwide [1]. It is currently diagnosed by blind biopsy, prompted by elevated serum prostate specific antigen levels [2], but nearly 20% of these procedures result in false negatives [3]. There is also no reliable indicator for establishing the aggressiveness of prostate tumors [4]. These deficiencies have resulted in an increased number of painful biopsies, over-treatment of the disease and undesired side effects (e.g. impotence) for patients. The accurate non-invasive characterization of prostate cancer remains a critical unmet clinical challenge.

The development of imaging methods could find immediate application in prognostication and treatment planning in prostate cancer [5]. Current protocols for prostate magnetic resonance imaging (T₂-weighted MRI) provide anatomic details, but are limited by the failure to accurately assess tumor invasion and tumor aggressiveness [6]. Proton (¹H) MR spectroscopy, exploiting metabolic characteristics, has also been advocated as a potential method of diagnosing and monitoring prostate cancer [7]. However, ¹H-MR spectroscopy currently provides only moderately increased sensitivity and specificity beyond conventional T₂-weighted MRI, diffusion-weighted MRI, and dynamic contrast-enhanced MRI. Although molecular imaging techniques have also been advanced, the best molecular target identified to date, α-methyl CoA racemase, has shown minimal utility [8], and ¹⁸F-FDG PET has had limited clinic applicability in the examination of prostate tumors due largely to poor uptake and rapid excretion of the tracer [9]. PET and PET/CT imaging techniques employing [¹¹C]- and [¹⁸F]-labeled choline have also been used for the detection of prostate cancers and have shown particular benefit with the evaluation of disease recurrence [10].

The recent advent of hyperpolarized ¹³C MRS [11], which achieves dramatically enhanced signal-to-noise ratios using dynamic nuclear polarization (DNP), provides unprecedented opportunities for real-time imaging of *in vivo* metabolic pathways critical to the identification and evaluation of cancer [12]. To date, the leading *in vivo* hyperpolarized metabolic imaging candidate is [¹³C]-labeled pyruvate ([¹³C]-Pyr). Several important applications with this agent have been proposed including measurement of high glycolytic rates in tumors, and metabolic abnormalities in ischemic heart disease and inflammatory processes [13]. Studies of small and large animal models are currently ongoing, and [¹³C]-Pyr is the first substrate for hyperpolarized MRS to enter clinical trials [14].

Novel substrates are also emerging for this powerful imaging technology [15]. As first reported by Karlsson et al., [1-¹³C]-2-ketoisocaproate ([1-¹³C]-KIC) is a promising substrate for hyperpolarized ¹³C MRS studies (Fig. 1) [16–17]. [1-¹³C]-KIC is metabolized to [1-¹³C]-leucine ([1-¹³C]-Leu) by branched-chain aminotransferases (BCAT). In humans, BCAT has two major isoforms, BCAT1 (cytosol) and BCAT2 (mitochondria), and the enzyme also catalyzes the transamination of other branched-chain amino acids (BCAA) including isoleucine and valine [18]. BCAT, first identified as an overexpressed gene product in a mouse teratocarcinoma cell line [19], is a target of the proto-oncogene *c-myc* and a putative marker for metastasis [16,20]. Following the bolus injection of hyperpolarized [1-¹³C]-KIC, the metabolic production of [1-¹³C]-Leu has been recently shown to correlate with BCAT levels in murine lymphoma (EL4), a tumor with high BCAT activity [16].

Although unstudied using hyperpolarized ¹³C MRS techniques, recent reports have demonstrated the critical role of BCAAs in the proliferation of tumorigenic prostate tissue [21]. In particular, a variety of cancerous tissues are characterized by altered BCAA availability and elevated rates of BCAA oxidation [22]. BCAA metabolism is primarily altered in malignant tissue in order to meet the demands of *de novo* protein synthesis [23]. BCAAs can alternatively be utilized for energy production through a catabolic pathway mediated initiated by BCAT. Several other lines of evidence support the potential importance of BCAT metabolism in prostate cancer. In a recent clinical PET study, anti-1-amino-3-¹⁸F-fluorocyclobutane-1-carboxylic acid (anti-¹⁸F-FACBC), a synthetic leucine analog, was demonstrated to be a promising radiotracer for imaging prostate cancer with significant uptake in both primary and metastatic disease [24]. Although hyperpolarized [1-¹³C]-KIC has shown initial promise for investigating BCAA metabolism, this agent has yet to be thoroughly explored in other cancer models. In this report, BCAT activity is investigated in various models of prostate cancer and the ability of hyperpolarized [1-¹³C]-KIC to probe BCAA metabolism is examined.

Methods

Imaging Agent

The [1-¹³C]-KIC free acid was prepared from the sodium salt, [1-¹³C]-ketoisocaproic acid (Cambridge Isotopes, Andover, MA) [16] by the following procedure: [1-¹³C]-ketoisocaproic acid, sodium salt (250 mg, 1.63 mmol) was charged into a 10-mL glass vial and dissolved in water (3 mL). The solution was acidified to pH = 1 with 1-M hydrochloric acid (0.50 mL) and the aqueous layer was extracted with diethyl ether (3 × 3 mL). The combined organic layers were dried with sodium sulfate, filtered, and concentrated to afford [1-¹³C]-KIC (214 mg, 96 % yield) as a colorless oil.

Polarization of [1-¹³C]-KIC

The polarized samples consisted of 20 μL of a mixture of 8 M [1-¹³C]-KIC and 11 mM Ox063 trityl radical. Dotarem (1 μL (1:50 dilution) Guerbet, France) was added just prior to polarization. The samples were polarized via DNP using a HyperSense system (Oxford Instruments Molecular Biotools, Oxford, UK), for 1–1.5 h each, to achieve liquid-state

polarization at dissolution of 15%. The polarized sample was initially dissolved in a buffered solution (80 mM NaOH, 40 mM TRIS, 50 mM NaCl, 0.1 g/L EDTA- Na_2) followed by further dilution with non-basic buffered solution leading to a 4 mM solution of the hyperpolarized substrate with a pH of ~7.5.

Prostate Cancer Cell Lines

Prostate cancer cell lines (PC-3 and DU-145) were purchased from American Type Culture Collection (Manassas, VA). LNCaP and LAPC-4 were donated by the Stanford Canary Center. Cell line was cultured with DMEM supplemented with 10% FBS and 1% penicillin/streptomycin and grown to >80% confluence prior to *in vitro* studies.

Spectrophotometric BCAT assays

Homogenates were prepared from both healthy and malignant patient prostate tissues, and BCAT activity levels were determined spectrophotometrically ($n = 4$ for both healthy and malignant tissues) [25]. Protein concentrations were determined via the Bradford assay. Tissues had been collected after patients signed informed consent under a Stanford University Institutional Review Board approved protocol. A series of enzymatic assays ($n = 4$) were also performed with transgenic adenocarcinoma of the mouse prostate (TRAMP) mice (Charles River Laboratories, Wilmington, MA). For cellular experiments, BCAT activities in the human prostate cancer cell lines (PC-3, DU-145, LAPC-4 and LNCaP) were employed ($n = 3$). All BCAT activities are expressed as mean $\pm$ ste.

MR experiments

In vitro MRS studies ($n = 3$ for each cell line) were conducted on the human prostate cancer cell lines utilizing a clinical 3T GE Signa MRI scanner (GE Healthcare, Waukesha, WI, USA). Immediately before dissolution, approximately 1×10^8 PC-3 or DU-145 cells were trypsinized and resuspended in 2 ml culture media. This was immediately followed by an injection of 2 ml of 4 mM hyperpolarized $[1-^{13}\text{C}]\text{-KIC}$ solution. MR measurements were performed using a custom-built carbon-13 surface coil ($\varnothing_{\text{inner}} = 28$ mm), operating at 32.16 MHz, used for both radiofrequency (RF) excitation and signal reception. A dynamic free induction decay spectroscopy sequence (spectral width, 5,000 Hz; spectral points, 2048) with non-selective RF pulse excitations (pulse width, 40 μs ; nominal flip angle, 10°) was used to acquire spectra with 3 s of temporal resolution (total $T_{\text{acq}} = 4:00$ min).

The acquired data sets were apodized by a 10-Hz Gaussian filter and zero-filled by a factor of 4 in the spectral dimension. After a fast Fourier transform (MATLAB, Mathworks Inc., Natick, MA), the metabolite peaks were integrated in absorption mode after zero-order phase correction to quantify time-curves of the metabolites. For the display of spectra, both a zero- and a first-order phase correction were performed and the baseline was subtracted by fitting a spline to the signal-free regions of the smoothed spectrum. $[1-^{13}\text{C}]\text{-Leu-to-}[1-^{13}\text{C}]\text{-KIC}$ ratios were calculated by summing the first 30 time-points (90 s) of each metabolite's time-curve and taking ratios of the metabolite signal intensities.

Results

In vitro assessment of BCAT Activity in human prostate tissue

Healthy prostate tissue was found to display modest levels of BCAT activity (2.96 ± 0.10 U/gram of protein) (Fig. 2a). However, prostate cancer homogenates proved to have a significant decrease in enzyme activity as 1.68 ± 0.48 U/gram of protein was detected ($P = 0.0045$). Although BCAT activity is not at high levels in either state, malignant tissue displayed a unique metabolic profile relative to healthy prostate tissue.

In vitro assessment of BCAT Activity in models of human prostate cancer

In *ex vivo* experiments, homogenates of TRAMP prostate tissues were found to possess an enzyme activity of 0.84 ± 0.17 U/gram of protein (Fig. 1b); therefore, TRAMP mice display a decreased level of BCAT activity relative to human disease ($P = 0.051$). A variety of human prostate cancer cell lines were also examined in order to determine whether they would serve as appropriate models for MR spectroscopic studies. BCAT assays were conducted with four cell lines: PC-3, DU-145, LNCaP and LAPC-4 (Fig. 1c) [26]. In the experiments among the human prostate cancer cell lines, the PC-3 cell line displayed the highest level of BCAT activity (1.05 ± 0.39 U/gram of protein) followed by the DU-145 cell line (0.97 ± 0.16 U/gram of protein) with no statistical difference between the two models ($P = 0.42$). Both cell lines were found to have increased enzyme activity in comparison to the TRAMP mouse model (PC-3: $P = 0.31$; DU-145: $P = 0.28$). Low levels of BCAT activity were detected *in vitro* from both the LNCaP and LAPC-4 cell lines (PC-3/LNCaP: $P = 0.043$; PC-3/LAPC-4: $P = 0.052$; DU-145/LNCaP: $P = 0.0001$; DU-145/LAPC-4: $P = 0.0001$). Importantly, the PC-3 and DU-145 cell lines displayed significantly lower BCAT activity than healthy human prostate tissue (PC-3/Healthy Prostate: $P = 0.0001$; DU-145/Healthy Prostate: $P = 0.0001$), but only moderate differences from malignant prostate tissue were observed (PC-3/Malignant Prostate: $P = 0.22$; DU-145/Malignant Prostate: $P = 0.13$).

Hyperpolarized ^{13}C MRS of human prostate cancer cell lines

Fig. 3 displays averaged (a) spectra and (b) time-courses obtained after administration of hyperpolarized $[1-^{13}\text{C}]\text{-KIC}$ to PC-3 cells in culture media (substrate concentration = 2 mM). In these experiments, $[1-^{13}\text{C}]\text{-KIC}$ was observed at 172.6 ppm, and $[1-^{13}\text{C}]\text{-Leu}$ was detected at 176.8 ppm. $[1-^{13}\text{C}]\text{-KIC}\cdot\text{H}_2\text{O}$ and $[2-^{13}\text{C}]\text{-KIC}$ (natural abundance) were also present in the spectra but are not related to metabolism. The metabolic product, $[1-^{13}\text{C}]\text{-Leu}$, was immediately formed upon exposure of the prostate cancer cells to hyperpolarized $[1-^{13}\text{C}]\text{-KIC}$ and was detected in all experiments. The maximum product signal was detected after 15–20 s. Higher concentrations (up to 5 mM) of $[1-^{13}\text{C}]\text{-KIC}$ did not result in increased product formation, which may suggest saturation of the BCAT active site at 2 mM. BCAT has a $K_M = 0.14$ mM for KIC and substrate levels should not limit the transamination [25]. In addition, KIC has been demonstrated as an effective substrate for monocarboxylate transporters such as MCT1, which are readily expressed prostate cancer cell lines and prostate tissue [27–29]. Further, although leucine can serve as a co-substrate for the transaminase and potentially could increase isotopic flux, incubation (10 min) of cells with unlabelled leucine (1 mM) prior to $[1-^{13}\text{C}]\text{-KIC}$ administration did not substantially affect $[1-^{13}\text{C}]\text{-Leu}$ signal. Experiments were also performed with DU-145 cells

and analogous metabolic products and time-courses were found (Fig. 4). Ratios of $[1-^{13}\text{C}]\text{-Leu}$ -to- $[1-^{13}\text{C}]\text{-KIC}$ were obtained for PC-3 and DU-145 cell lines from hyperpolarized ^{13}C MRS analysis. The metabolic product was observed over the first 90 s of the experiment, and PC-3 cells displayed a conversion ratio of $1.73 \times 10^{-3} \pm 0.38 \times 10^{-3}$ a.u (mean $\pm$ ste, $n = 3$). The area under the curve for the metabolic product corresponding to $[1-^{13}\text{C}]\text{-Leu}$ production was found to be $2.20 \times 10^{-3} \pm 0.47 \times 10^{-3}$ ratio (mean $\pm$ ste, $n = 3$) for the DU-145 cell line.

Discussion

Hyperpolarized ^{13}C MRS provides an unique opportunity to evaluate metabolism at the molecular level. Karlsson et. al. initially showed that $[1-^{13}\text{C}]\text{-Leu}$ was successfully produced from the injection of hyperpolarized $[1-^{13}\text{C}]\text{-KIC}$ in murine and rat models of cancer [16]. In addition, it was demonstrated that the levels of $[1-^{13}\text{C}]\text{-Leu}$ formation were proportional to the observed BCAT activities. Elevated pool sizes of glutamate and leucine were also detected in the cancerous tissue.

In this report, we investigated BCAT activity in several models of prostate cancer via both traditional spectrophotometric and hyperpolarized ^{13}C MRS methods. While the work of Karlsson et. al. demonstrated the initial observation of metabolite formation from hyperpolarized $[1-^{13}\text{C}]\text{-KIC}$ [16], this study sought to determine whether $[1-^{13}\text{C}]\text{-KIC}$ could serve as a practical method for characterizing malignant prostate tissues. Through initial examination of BCAT activity *in vitro* via conventional spectrophotometric methods, we demonstrated that, despite low levels of BCAT activity, alterations in BCAA metabolism are observed in various prostate cancer models. In particular, healthy human prostate tissue was found to have an elevated BCAT levels relative to malignant tissue. Normal prostate tissue relies upon fatty acid metabolism and glycolysis for energy production because the tricarboxylic acid (TCA) cycle is inhibited due to the high concentration of zinc. However, in prostate cancer, the TCA cycle is restored and can also contribute to meeting the energy requirements of the cell [30]; therefore, the observed changes in BCAT activity are in principle consistent with BCAAs being utilized for protein synthesis rather than for energy production. Importantly, although the observed activity was less in prostate cancer in comparison to healthy tissue, the significant difference between the two states could still provide a clinical opportunity for disease assessment and/or monitoring response to treatment. For example, hyperpolarized $[1,4-^{13}\text{C}_2]\text{-fumarate}$, which displays lower metabolic conversion rate to $[1,4-^{13}\text{C}_2]\text{-malate}$ in tumors relative to normal tissue, has proven to be a valuable clinical tool for assessing cell necrosis and monitoring therapeutic response [31]. In a similar fashion, lower levels of BCAT activity in the disease state could potentially result in detectable decreases in $[1-^{13}\text{C}]\text{-Leu}$ production from hyperpolarized $[1-^{13}\text{C}]\text{-KIC}$ and thus provide a method for evaluating prostate tissue.

Animal and cellular models are vital tools for exploring the onset and progression of human disease. In addition, a sufficient model, which maintains comparable rates of BCAT activity as human prostate cancer, was necessary for spectroscopic evaluation with hyperpolarized $[1-^{13}\text{C}]\text{-KIC}$. However, there remain a limited number of reports concerning alterations in BCAA demand and metabolism in models of prostate cancer. Through the initial

spectrophotometric examination of the TRAMP mouse and prostate cancer cell lines, PC-3 and DU-145 cell lines were discovered to have relatively similar BCAT activity levels as detected in human disease. These models were then employed in the development of ^{13}C MRS techniques for analyzing BCAA metabolism with hyperpolarized $[1-^{13}\text{C}]\text{-KIC}$ in prostate cancer. We demonstrated that hyperpolarized $[1-^{13}\text{C}]\text{-KIC}$ could be employed *in vitro* for models of prostate cancer. In both the PC-3 and DU-145 cell lines, $[1-^{13}\text{C}]\text{-Leu}$ production was observed after administration of the molecular probe. The $[1-^{13}\text{C}]\text{-Leu}$ -to- $[1-^{13}\text{C}]\text{-KIC}$ ratio also provided insight into the state of BCAA metabolism and, specifically, the propensity for BCAA oxidation, a pathway that can be utilized to drive energy production in proliferating cells. However, the low levels of BCAT activity in the examined prostate cancer cell lines may limit the future application of hyperpolarized $[1-^{13}\text{C}]\text{-KIC}$ in the assessment of the disease.

Conclusions

Based upon our analysis, BCAA metabolism is altered in human prostate cancer relative to healthy tissue. However, only low levels of BCAT activity were found in all animal and cellular models examined. Despite these modest activities, we have demonstrated that $[1-^{13}\text{C}]\text{-Leu}$ production can be determined with hyperpolarized ^{13}C MRS *in vitro* using $[1-^{13}\text{C}]\text{-KIC}$.

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Abbreviations

MRS	magnetic resonance spectroscopy
MRI	magnetic resonance imaging
DNP	dynamic nuclear polarization
BCAT	branched chain aminotransferase
BCAA	branched-chain amino acid
KIC	2-ketoisocaproate
Leu	leucine
Pyr	pyruvate
PC-3, DU-145, LAPC-4, LNCaP	human prostate cancer cell lines
RF	radiofrequency
TRAMP	transgenic adenocarcinoma of the mouse prostate
TCA cycle	tricarboxylic acid cycle

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Fig. 1.
Metabolism of [1-¹³C]-KIC to [1-¹³C]-Leu via BCAT.

Fig. 2. BCAT activity detected from the various prostate sources: (a) human, (b) TRAMP mouse model, and (c) human prostate cancer cell lines. Human prostate cancer displayed significantly lower levels of BCAT activity than normal tissue. All BCAT activities are expressed as mean $\pm$ ste.

Fig. 3.

(a) Representative time-averaged spectra obtained from PC-3 prostate cancer cell line after incubation with 2 mM [$1\text{-}^{13}\text{C}$]-KIC. The signal-to-noise ratio (SNR) for individual ^{13}C -labeled metabolites was 1.31×10^6 ([$1\text{-}^{13}\text{C}$]-KIC) and 2.07×10^3 ([$1\text{-}^{13}\text{C}$]-Leu), respectively. SNR was calculated by taking an integral over individual peaks divided by standard deviation of background noise. (b) Representative time course demonstrate rapid uptake of hyperpolarized substrate and production of metabolic product, [$1\text{-}^{13}\text{C}$]-Leu.

Fig. 4.

(a) Representative time-averaged spectra obtained from DU-145 prostate cancer cell line after incubation with 2 mM [$1-^{13}\text{C}$]-KIC. The SNR for individual ^{13}C -labeled metabolites was 1.60×10^6 ([$1-^{13}\text{C}$]-KIC) and 2.66×10^3 ([$1-^{13}\text{C}$]-Leu), respectively. (b) Representative time course demonstrate rapid uptake of hyperpolarized substrate and production of metabolic product, [$1-^{13}\text{C}$]-Leu.