Hyperpolarized $^{13}$C Spectroscopy Detects Early Changes in Tumor Vasculature and Metabolism after VEGF Neutralization

Sarah E. Bohndiek, Mikko I. Kettunen, De-en Hu, and Kevin M. Brindle

Abstract
No clinically validated biomarkers exist to image tumor responses to antiangiogenic therapy. Here, we report the utility of hyperpolarized $^{13}$C magnetic resonance spectroscopy (MRS) to detect the early effects of anti-VEGF therapy. In two colorectal cancer xenograft models, displaying differential sensitivity to VEGF blockade, we compared hyperpolarized MRS with measurements of tumor perfusion using dynamic contrast agent–enhanced (DCE)-MRI and tumor cellularity using diffusion-weighted MRI of the apparent diffusion coefficient (ADC) of tissue water. In tumors sensitive to anti-VEGF therapy, $^{13}$C flux between hyperpolarized $[^{1-}{^{13}}C]$/lactate and $[^{1-}{^{13}}C]$/pyruvate and $[^{1-}{^{13}}C]$/fumarate increased in parallel with reduced perfusion. Production of $[^{1,4-}{^{13}}C]$/malate from hyperpolarized $[^{1,4-}{^{13}}C]$/fumarate increased in parallel with tumor cell necrosis, preceding any change in tumor ADC. In contrast, tumors that were less sensitive to anti-VEGF therapy showed an increase in $^{13}$C flux from hyperpolarized $[^{1-}{^{13}}C]$/pyruvate and an increase in uptake of a gadolinium contrast agent, whereas tumor ADC decreased. Increased label flux could be explained by vascular normalization after VEGF blockade, increasing delivery of hyperpolarized $[^{1-}{^{13}}C]$/pyruvate as observed. Despite the minimal response of these tumors to treatment, with only a minor increase in necrosis observed histologically, production of $[^{1,4-}{^{13}}C]$/malate from hyperpolarized $[^{1,4-}{^{13}}C]$/fumarate in therapy-resistant tumors also increased. Together, our findings show that hyperpolarized $^{13}$C MRS detects early responses to anti-VEGF therapy, including vascular normalization or vascular destruction and cell death.

Introduction
Angiogenesis, the growth of new blood vessels from surrounding host vasculature, can be a rate limiting process in tumor development and progression. VEGF is a key proangiogenic factor that stimulates endothelial cell proliferation, migration, and survival. Sustained and excessive exposure of tumors to angiogenic factors, including VEGF, leads to a chaotic neovasculature, composed of immature blood vessels that are often tortuous and highly permeable (1). Targeting the tumor vasculature is an attractive treatment option, with angiogenic agents providing a means not only to prune immature vessels but also to induce a window of "vascular normalization" before ultimately reducing the tumor vasculature to inadequacy (2).

Bevacizumab is a monoclonal antibody that binds VEGF and blocks signal transduction through the VEGFR1 and VEGFR2 receptors (3). In the preclinical setting, treatment with bevacizumab leads to sustained changes in vascular function, including reduced microvessel density (MVD) and permeability (4). These changes have also been reproduced in clinical trials (5, 6) within 24 hours of VEGF blockade (7) but are often transient, rather than sustained, and frequently reverse upon cessation of treatment (2, 8). The most promising results in the clinic have been observed by combining bevacizumab with conventional cytotoxic therapy (8), in which a 5-month increase in overall survival in metastatic colorectal cancer patients (9) led to the first U.S. Food and Drug Administration approval in 2004. Bevacizumab was subsequently approved for treatment of metastatic renal cell carcinoma (10), non–small-cell lung cancer (11), and glioblastoma (12). However, this success has recently been confounded by results in metastatic breast cancer, in which accelerated approval (13) was rescinded after 2 subsequent studies failed to show the same improvement in overall survival (14). The rapid adoption of bevacizumab in the clinic has led to an urgent need to develop biomarkers that can select patients that will best respond to the therapy, direct drug dose, and sensitively detect response to treatment (15–17); such biomarkers have remained elusive (14). Dynamic contrast agent–enhanced MRI (DCE-MRI) of the tumor vasculature has proved promising in this regard (17), with patients whose tumors undergo a 50% or greater reduction in contrast agent uptake within the first cycle of treatment usually attaining stable or better...
Hyperpolarized $^{13}$C Detects Early Response to Bevacizumab

Hyperpolarized $^{13}$C Detects Early Response to Bevacizumab (27).

Materials and Methods

Animal preparation and treatment

Tumors were established in female severe combined immunodeficient mice by subcutaneous injection of $5 \times 10^7$ LoVo or HT29 human colorectal adenocarcinoma cells. LoVo and HT29 cells of the same passage were genotyped with the AmpFLSTR Kit (Applied Biosciences) and were a 90% and 100% match with American Type Culture Collection standards, respectively. The cell lines were obtained from the European Collection of Cell Cultures (LoVo, catalog no. 8706101; HT29, catalog no. 91072201). Drug-treated animals received bevacizumab (Avastin; Roche) at 5 mg/kg twice weekly (4, 9). Drug dosing was carried out on day 1 and 3, after baseline imaging on day 0; time points referred to in this study are 24 hours following each drug administration (24 and 72 hours). Procedures were conducted in accordance with project and personal licenses issued under the United Kingdom Animals (Scientific Procedures) Act, 1986.

MRI

Animals were anesthetized with 10 mL/kg of a 5:4:1 mixture of Hypnorm (VetPharma Ltd.), Hypnovel (Roche) and saline. A catheter was inserted into a tail vein and the animal was positioned in a cradle, which was placed in a quadrature $^1$H-tuned volume coil (Varian) in a 9.4T vertical wide-bore magnet (Oxford Instruments). The body temperature of the animals was maintained using a flow of warm air. For hyperpolarized experiments, a 24-mm diameter surface coil tuned to $^{13}$C (100 MHz) was positioned over the tumor. In all experiments, transverse $^1$H "pilot" images were acquired for tumor localization using a spin-echo pulse sequence [repetition time (TR), 500 milliseconds; echo time (TE), 10 milliseconds; field-of-view (FOV), $35 \times 35$ mm$^2$] in a data matrix of $256 \times 256$; 21 slices of thickness 2 mm]. All data analysis was done using custom routines written in MATLAB (MathWorks).

Hyperpolarized $^{13}$C

- $[1-^{13}C]$pyruvic acid and $[1,4-^{13}C_2]$-fumaric acid (99% $^{13}$C; Cambridge Isotope Laboratories) were hyperpolarized as described previously (26, 28, 33). Samples were polarized in a GE Healthcare DNP prototype hyperpolarizer for 45 minutes before dissolution in 6 mL of buffer (26).
- Final concentrations were $75 \text{ mmol/L}$ for pyruvate and $20 \text{ mmol/L}$ for fumarate, with polarizations in the range of 20% to 30%. A slice-selective excitation "pulse and acquire" sequence was used for $^{13}$C spectroscopy following injection of 0.2 mL of hyperpolarized substrate. The slice, which was 6 mm thick, was placed within the subcutaneous tumors, which protruded above the body of the animal and into the surface coil loop. There was minimal inclusion of underlying tissue. Treatment with bevacizumab was started at the same time after tumor cell implantation (15 days). Because the HT29 tumors grew slightly faster than the LoVo tumors, this meant that the MR measurements and treatment with bevacizumab were initiated when the tumors had slightly different sizes (Length $\times$ Width = $88 \pm 16$ mm$^2$ and $138 \pm 18$ mm$^2$ for LoVo and HT29, respectively).
- For hyperpolarized pyruvate, $160^{13}$C spectra were acquired at 1-second intervals with a nominal flip angle of 10 degrees. Approximately 1 hour after the pyruvate experiment, hyperpolarized fumarate was injected into the same animal, and following a delay of 20 seconds to allow production of hyperpolarized malate, 32 spectra were acquired from the same tumor slice at 5-second intervals with a nominal flip angle of 60 degrees. The integrated peak intensities of hyperpolarized $[1-^{13}C]$pyruvate and $[1-^{13}C]$lactate were fitted to the modified Bloch equations for 2-site exchange to obtain apparent rate constants for flux of label from pyruvate to lactate ($k_p$) and the reverse flux ($k_l$), assuming equal spin lattice relaxation rates ($1/T_1$) for both metabolites. The rate constant $k_p$ was shown previously to be a robust measure of pyruvate-lactate exchange, regardless of the assumptions that were made in the process of data fitting (26).
- Given the relatively low level of $[1,4-^{13}C_2]$malate detected in the fumarate experiments, the ratio of malate to fumarate in the first spectrum (25 seconds postinjection)
was used as a metric, as this was found to be an equivalent marker of response in previous work (26).

**Dynamic contrast-enhanced imaging.** DCE-MRI data were acquired as described previously (26) using a T$_1$-weighted spin-echo pulse sequence. Spin-lattice relaxation rates (R$_1$) were measured using an inversion recovery fast low angle shot (FLASH) pulse sequence; one R$_1$ map, and 6 baseline images were collected precontrast. Gd$^{3+}$-DTPA (diethylenetriaminepentaacetic acid; 200 μmol/kg; Magnevist, Schering) was diluted in sterile saline (0.9% sodium chloride) and injected as a bolus through a tail vein catheter over 2 to 3 seconds. Dynamic imaging was done for 10 minutes postinjection. Inversion recovery data were fitted, pixel-by-pixel, to a monoexponential function and spin-echo images were converted to relaxation rate maps (26). The Gd$^{3+}$-DTPA concentration curve was evaluated in all tumor-containing slices with a manually delineated region of interest (ROI), and the integrated area under the curve up to 60 (AUC$_{60}$) or 600 seconds was calculated for each ROI (34, 35). Data shown are from the inner slice.

**Diffusion- and T$_2$-weighted imaging.** The apparent diffusion coefficient (ADC) of tissue water was mapped using diffusion weighted MRIs (DW-MRI), acquired with a navigated dual-echo spin-echo pulse sequence, as described previously (26), in 9 slices of 2 mm thickness. Diffusion-sensitizing gradients equivalent to b-values of 0, 68, 271, 609, and 1,082 s/mm$^2$ were applied along the slice axis (gradient length 5, 7.5 milliseconds; delay between gradients Δ, 13 milliseconds). The spin–spin relaxation time, T$_2$, was then mapped using a dual-echo spin-echo pulse sequence for TE values of 10, 20, 30, 40, and 50 milliseconds (FOV, 35 × 35 mm$^2$; data matrix, 128 × 128; TR, 1.0 seconds; 9 slices, thickness 2 mm). ADC and T$_2$ maps were generated for each slice on a pixel-by-pixel basis (26). Histograms were generated for each tumor-containing slice, inside a manually de

**Ex vivo analysis**

**Biochemical assays.** Tumors were freeze clamped 30 seconds after intravenous injection of a 0.2-mL bolus containing 75 mmol/L pyruvate and 20 mmol/L fumarate. Freeze-clamped tumors were used to determine, spectrophotometrically, LDH (EC1.1.1.27) and fumarase (EC4.2.1.2) activities, and tumor concentrations of pyruvate and lactate (26, 36). Citrate synthase (EC2.3.1.3) activity was determined according to Srere (37). Briefly, tissue was homogenized on ice in a Teflon-glass Potter-Schomiggoner in extraction buffer (20 mmol/L Tris-HCl pH 7.6, 0.25 mol/L sucrose, 40 mmol/L KCl, 2 mmol/L EGTA and EDTA). Homogenates were centrifuged to remove cell debris, extracted with 1% Triton X-100 then centrifuged again; the supernatant was removed and assayed immediately at 405 nm in a DTNB-linked assay.

**Histology.** Tumors excised post mortem were fixed in 10% formalin and paraffin embedded. Hematoxylin and eosin (H&E) staining was done for assessment of cell death. CD31 antibody (BD Pharmingen) and Jackson Biotinylated anti-rat secondary antibody (Stratech) were used with the Bond Intense B Detection kit (VisionBioSystems) for immunohistochemistry. Slides were scanned using an AperioXT (Aperio Technologies), and quantitative analysis was done using ImageScope v10.2 software. For H&E stained slides, the fractional area containing fragmented nuclei of dead cells in 2 slices per tumor was measured. For CD31-stained slides, the Microvessel Analysis algorithm v1.0 was integrated into the ImageScope software, and the measurement parameters were optimized to evaluate MVD and perimeter in five 1 mm$^2$ fields of view in at least one slice from each tumor. A minimum of 4 tumors were analyzed at each treatment time point, which equated to a minimum number of vessels for analysis of 1,692 and 540 for LoVo and HT29 tumors, respectively.

**Statistical analysis**

Results are expressed as mean ± SE unless stated otherwise. All experiments were repeated independently in at least 3 animals. Statistical significance was tested using Prism (Graphpad) with a 2-sided t test at the 95% confidence level (unpaired unless repeated measures) or 1-way ANOVA.

**Results**

Intravenous administration of hyperpolarized [1-13C] pyruvate resulted in readily detectable signals from both [1-13C]pyruvate at 173 ppm and [1-13C]lactate at 185 ppm (Fig. 1). The differences in signal-to-noise in these spectra reflected differences in the levels of polarization. The flux of 13C label between these species decreased in LoVo tumors following treatment with bevacizumab (Fig. 1A), whereas it continued to increase after treatment in HT29 tumors (Fig. 1B). For LoVo tumors, the apparent rate constant describing label flux between pyruvate and lactate ($k_p$) was reduced by 50% at 24 hours (0.031 ± 0.001 vs. 0.063 ± 0.007/s pretreatment, $P=0.03$) and was maintained at this level at 72 hours (0.039 ± 0.005/s; $P = 0.03$ vs. pretreatment, $P = 0.34$ vs. 24 hours; Fig. 1C). In HT29 tumors, $k_p$ instead tended to increase over the time course (0.025 ± 0.006/s; pretreatment; 0.043 ± 0.011/s, 24 hours; 0.049 ± 0.024/s, 72 hours; Fig. 1D). On the basis of tumor size changes, neither tumor type would be considered to have responded over the treatment time course, as neither displayed a reduction in tumor size [LoVo: Length × Width = 105 ± 20 mm$^2$ at 72 hours (day 5) vs. 88 ± 16 mm$^2$ pretreatment (day 0), $P = 0.1$; HT29: 164 ± 20 mm$^2$ at 72 hours (day 5) vs. 138 ± 18 mm$^2$ pretreatment (day 0), $P < 0.001$]. However, the growth curves for the drug-treated and vehicle-treated tumors showed that both had responded to bevacizumab treatment, although this response was greater for the LoVo as compared with the HT29 tumors (Fig. 2).

The fumarase-catalyzed hydration of hyperpolarized [1,4-13C$_2$]fumarate (177 ppm) to [1,4-13C$_2$]malate (182, 184 ppm), which was barely detectable before treatment, was seen to increase following treatment in both tumor models (Fig. 3). In the LoVo tumors (Fig. 3A), the ratio of malate to fumarate at 25 seconds postinjection was 0.04 ± 0.01 before treatment and rose dramatically (>10-fold) after treatment to 0.49 ± 0.04
(P = 0.03) at 24 hours and 0.32 ± 0.14 (P = 0.01) at 72 hours (the difference observed between 24 and 72 hours values was not significant; P = 0.49). The ratio in the HT29 tumors was much lower than that of the LoVo tumors (Fig. 3B) but still increased over the treatment time course (0.010 ± 0.004 at 24 hours, 0.047 ± 0.003 at 72 hours vs. 0.002 ± 0.001 pretreatment, P < 0.05; no significant difference between the 24 and 72 hours values; Fig. 3C and D).

Treatment response was also assessed using clinical MRI methods. For DCE-MRI measurements, changes in perfusion were assessed from the integrated area under the Gd-DTPA uptake curve (AUC) at 60 and 600 seconds. Drug treatment of LoVo tumors produced significant (P < 0.05) antivascular effects at 24 hours (Fig. 4A and B), with AUC60 falling from 0.52 ± 0.09 to 0.25 ± 0.04 mmol/L and AUC600 from 6.4 ± 0.4 to 4.9 ± 0.5 mmol/L. By 72 hours, there was some recovery, with AUC60 rising to 0.35 ± 0.07 and AUC600 to 7.0 ± 0.5 mmol/L. By contrast, no antivascular effects were observed in the HT29 tumors; in fact, there was evidence for increased contrast agent uptake after treatment (Fig. 4C). The AUC60 remained close to the pretreatment value of 0.67 ± 0.09 mmol/L (0.59 ± 0.12 and 0.58 ± 0.10 mmol/L at 24 and 72 hours, respectively), but there was a significant increase at later time points in the Gd-DTPA uptake curve (Fig. 4C), with the AUC600 (Fig. 4D) increasing from 10.0 ± 0.7 to 12.6 ± 1.1 mmol/L at 24 hours (P < 0.05) and 14.0 ± 1.6 mmol/L at 72 hours (P < 0.05). We observed a similar differential response with MRI measurements of Gd-DTPA uptake in a previous study, in which these tumors were treated with an antivascular drug (31). Drug-induced decreases in vascular permeability could also affect the Gd-DTPA uptake curves and the tumor uptake of a Gd-DTPA–albumin conjugate has been used previously to measure bevacizumab-induced changes in vascular permeability (38). However, changes in tumor contrast observed with low molecular weight contrast agents, such as the Gd-DTPA used here, are influenced primarily by flow (39), as opposed to vascular permeability, and considering only the initial rate of contrast agent uptake minimizes the effects of changes in permeability (40).

Maps of tumor ADC and the transverse relaxation time (T2) were used to determine the mean and median values of these parameters (Table 1). Average ADC histograms for each time point are shown in Fig. 5. There was no significant change in the ADC or T2 of LoVo tumors following bevacizumab treatment (Fig. 5A–C, Table 1). HT29 tumors did not exhibit any change in T2, but there was a 15% decrease in median ADC (Table 1) and narrowing of the ADC histogram (Fig. 5D–F), from (0.67 ± 0.04) × 10−3 mm2/s to (0.57 ± 0.03) × 10−3 mm2/s by 72 hours (P = 0.04).

Tumor enzyme and substrate levels were assayed in extracts from freeze-clamped tumor tissue (Fig. 6). Measurements on
LoVo tumor samples revealed a transient increase in lactate dehydrogenase (LDH) activity of more than 20% at 24 hours, from 2.3 \pm 0.2 to 2.8 \pm 0.1 \text{ mmole/min/mg protein \,(P \leq 0.03),}
followed by a return to baseline at 72 hours (2.1 \pm 0.4 \text{ mmole/min/mg protein; Fig. 6A}).
There was also an increase in lactate concentration, which rose by 30% from 4.2 \pm 0.4 to 5.6 \pm 0.5 \text{ mmol/g (wet weight) at 24 hours \,(P \leq 0.08) and by a further 30% to 7.1 \pm 0.4 \text{ mmol/g at 72 hours \,(P < 0.01; Fig. 6B).}
These changes in LDH activity and lactate concentration were consistent with an increase in hypoxia in the LoVo tumors (29, 41).
There was no change in fumarase activity from the pretreatment level of 0.40 \pm 0.04 \text{ mmole/min/mg protein (Fig. 6C).}
The tumor pyruvate concentration tended to decrease (Fig. 6D), and this was significant at 72 hours (0.30 \pm 0.06 \text{ mmol/g vs. 0.43 \pm 0.03 mmol/g pretreatment; \(P = 0.04\), in part, reflecting the reduced tumor perfusion observed in DCE-MRI measurements.
By contrast, the HT29 tumor extracts showed no significant changes in tumor lactate concentration (pretreatment level, 8.6 \pm 0.4 \text{ mmol/g}), pyruvate concentration (0.36 \pm 0.02 \text{ mmol/g}), or LDH activity (1.8 \pm 0.2 \text{ mmole/min/mg protein}), although the pretreatment lactate level was approximately double that found in LoVo tumors (Fig. 6B). Interestingly, fumarase activity decreased by approximately 50%, from 0.19 \pm 0.04 \text{ mmole/min/mg protein pretreatment, to 0.10 \pm 0.04 and 0.08 \pm 0.03 mmole/min/mg protein at 24 and 72 hours, respectively \,(P < 0.05) (Fig. 6C). As fumarase exists in both cytosolic and mitochondrial forms, we also measured the activity of a mitochondrial marker enzyme, citrate synthase; this fell by approximately 20% in HT29 tumors, from 0.12 \pm 0.01 to 0.10 \pm 0.01 \text{ mmole/min/mg protein at 72 hours, but the change was not significant \((P = 0.13)\).
Histologic assessment of tumor sections obtained post mortem showed that both tumors contained small abnormal or necrotic areas (Fig. 7A) by 24 hours after the first drug treatment (HT29, 8 \pm 1%, LoVo, 14 \pm 3%), with a lower level of necrosis in untreated tumors (HT29, 5 \pm 1%; LoVo, 9 \pm 1%). At 72 hours, the necrotic area had increased substantially in LoVo tumors (26 \pm 2%; \(P < 0.001\), although it remained diffuse concentration.

Figure 2. Growth curves for LoVo and HT29 tumors over the course of treatment with bevacizumab. Day 0 is the first day of imaging in untreated animals. Tumors were first treated on day 1; the growth of treated tumors is represented by dashed lines. Both tumor types exhibit growth inhibition with bevacizumab, although this is more pronounced for LoVo than HT29 tumors. The error bar for treated LoVo tumors on day 5 is within the symbol representing the data point.

Figure 3. \(^{13}\text{C}\) MR spectra acquired from a 6-mm tumor slice at 25 seconds after intravenous administration of hyperpolarized \([1,4-^{13}\text{C}_2]\text{fumarate (177.2 ppm)}\) show readily detectable signals from \([1,4-^{13}\text{C}_2]\text{malate (182.2, 183.6 ppm)}\) after treatment with bevacizumab in both LoVo (A and C) and HT29 (B and D) tumors. The vertical scale of the spectra from HT29 tumors has been increased 5-fold compared with the spectra from LoVo tumors.

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throughout the tumor. This was not the case in HT29 tumors (10 ± 3% at 72 hours; P = 0.68). CD31 staining of tumor blood vessels (Fig. 7B) showed that in LoVo tumors, vessel density declined by 40%, from (2.1 ± 0.4) × 10^{-4}/μm² to (1.2 ± 0.1) × 10^{-4}/μm² at 24 hours (P = 0.03) and further to (0.8 ± 0.1) × 10^{-4}/μm² at 72 hours (P < 0.001). There was no significant change in vessel density in HT29 tumors from the pretreatment level of (0.5 ± 0.1) × 10^{-4}/μm². Vessel perimeter in the untreated LoVo tumor sections was 63.6 ± 0.7 μm, significantly larger than that in HT29 tumor sections (43.9 ± 1.5 μm; P < 0.001), which is consistent with a higher level of VEGF expression in these tumors. The perimeter of LoVo tumor vessels declined after treatment to 59.2 ± 4.5 μm at 24 hours and 54.8 ± 2.1 μm at 72 hours (P = 0.03), but no change was observed in the perimeter of HT29 tumor vessels (43.0 ± 2.0 and 48.7 ± 4.4 μm at 24 and 72 hours, respectively).

Discussion

The early success of bevacizumab in improving progression-free survival has been marred by more recent failures to show significant improvements in overall survival. The recent introduction of hyperpolarized 13C MRSI into the clinic (32) offers a new way to investigate the action of these drugs and to detect response to them. In this preclinical study, we sought to detect early metabolic and vascular changes induced by bevacizumab treatment in 2 colorectal cancer xenograft models with differential sensitivity to VEGF blockade. LoVo tumors display a higher dependence on VEGF, with greater vessel permeability (31) and lower vessel pericyte coverage (29) than HT29 tumors, therefore upon VEGF blockade will exhibit reduced MVD and perfusion, along with marked expansion of hypoxic regions (29). Conversely, HT29 tumor cells are less sensitive to VEGF...
blockade (30), requiring high doses of bevacizumab (42) or receptor tyrosine kinase inhibitors (43) to display a similar response. We have shown here that the growth of these tumors has differential sensitivity to VEGF blockade and that this differential response can be detected early during treatment by MR measurements of metabolism, vascular function, and the ADC of tissue water. These measured changes in tumor biochemistry and physiology occur in the absence of a decrease in tumor size, and in HT29 tumors, precede a significant change in tumor growth rate.

Our first key finding is that hyperpolarized [1-13C]pyruvate and [1,4-13C2]fumarate are early markers of a positive response to bevacizumab treatment in LoVo tumors. The apparent rate constant describing label exchange between [1-13C]-pyruvate and [1-13C]lactate, kP, fell by more than 50% at 24 hours after treatment. This rate constant can be influenced by several factors, including LDH activity, the concentrations of the coenzymes NAD+ and NADH, as well as pyruvate delivery and transport (33). Tumor lactate concentration and LDH activity, measured by biochemical assays, both increased significantly, which can be attributed to an increase in tumor hypoxia following VEGF blockade and consequent HIF-1–dependent upregulation of glycolytic enzyme expression, including LDH (41). Because both the increase in lactate concentration and LDH activity are expected to produce nearly proportional increases in label flux between pyruvate and lactate (27), the decrease in label flux is most likely due to the decrease in tumor perfusion observed in the DCE-MRI measurements, which will result in reduced pyruvate delivery to the tumor (26). The DCE-MRI measurements showed a 52% reduction in AUC60 at 24 hours after treatment, which recovered a little, to 67% of the pretreatment level, by 72 hours (Fig. 4). The reduction in perfusion at 72 hours was accompanied by a statistically significant decrease in tumor pyruvate concentration, which was measured 30 seconds after intravenous pyruvate injection (Fig. 6). However, the continued suppression of label flux at 72 hours may also reflect cell death as the percentage of tumor necrosis had reached a significant

Table 1. ADC of water and transverse relaxation time (T2) measured over the whole tumor volume

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</table>

aP < 0.05.
The increase in rate was most likely due to increased pyruvate. There was no change in lactate concentration or LDH activity, this increased 2.2-fold after bevacizumab treatment. Because there has been shown previously to be a sensitive indicator of tumor cell necrosis (28). This has been shown in vitro and in vivo in different tumor types, using both cytotoxic and anti-vascular drugs (26, 28). In the LoVo tumors, the hyperpolarized malate/fumarate ratio increased by more than 10-fold after bevacizumab treatment. The increased malate production reflected an increase in necrotic fraction observed in histologic sections, which increased to 26% by the 72 hours time point. The decline in the malate/fumarate ratio observed at 72 hours, although not significant, may reflect some washout of fumarase from necrotic areas. There was no significant change in DW-MRI measurements of tumor ADC, when considering either the median value or histograms of the distribution of ADC values (Table 1 and Fig. 5A–C). The lack of change in tumor ADC is consistent with previous observations that in xenograft tumors with small or diffuse regions of necrosis, there may be no change in ADC with necrotic fractions of up to 40% (44). This shows the sensitivity of the hyperpolarized fumarate experiment for detecting diffuse tumor cell necrosis. Our second key finding is that in the less sensitive HT29 tumors, hyperpolarized [1-13C]pyruvate is affected by vascular normalization induced by bevacizumab treatment. Although histopathology showed no significant change in MVD or vessel perimeter in these tumors, the apparent rate constant for label exchange between [1-13C]pyruvate and [1-13C]lactate, kP, increased 2.2-fold after bevacizumab treatment. Because there was no change in lactate concentration or LDH activity, this increase in rate was most likely due to increased delivery to the tumor. This is supported by the Gd-DTPA uptake data, although there was no change in Gd-DTPA uptake over the first 60 seconds following contrast agent injection (AUC60), which covers the time course of the hyperpolarized experiment (~160 seconds, Fig. 1), there was nevertheless a significant increase in AUC600.

That the vascular normalization resulting from VEGF blockade can result in increased delivery of small molecules to tumors has been shown in previous studies. Treatment of a range of tumor xenografts (including colorectal cancer) with an antibody to VEGFR-2 was shown to decrease interstitial fluid pressure (IFP), by improving the integrity and function of the vasculature, which resulted in significantly enhanced penetration of small molecules (45). Treatment of neuroblastoma xenografts with bevacizumab increased the penetration of a chemotherapeutic drug for 1 to 3 days after drug treatment (46), and a similar observation was made in HT29 tumors (47). A reduction in IFP may explain the increase in AUC600 observed here because a negative correlation has been observed between AUC and IFP in cervical cancer, thought to be due to high IFP resulting in increased resistance to tumor blood flow (48). The failure to detect increased pyruvate concentrations in the HT29 tumors following bevacizumab treatment (Fig. 6) may be a reflection of the fact that 30 seconds after pyruvate injection, most of the pyruvate is still in the tumor blood vessels (49). Furthermore, the decrease in median tumor ADC and narrowing of the ADC histogram observed here (Fig. 5) has been observed previously after anti-VEGF treatment in both preclinical (22) and clinical (23, 50) studies, in which it has been attributed to a variety of factors, including cell swelling (22), atypical necrosis, and chronic hypoxia (50), as well as decreased edema (23).

The magnitude of the change in kP is greater than the changes in either ADC or AUC; therefore, the hyperpolarized 13C Detects Early Response to Bevacizumab

Figure 6. Enzyme activities (A, C) and metabolite concentrations (B, D) determined by enzymatic assay in tumor extracts. LDH activity (A) and lactate concentration (B) increased in LoVo tumors after treatment. G. fumarase activity exhibits a marked decline in HT29 tumors after treatment, but there is no significant change for the LoVo tumors. D. pyruvate concentration is decreased in LoVo tumors after the second bevacizumab dose. *, P < 0.05; **, P < 0.01.
The pyruvate experiment is better able to detect not only the antivascular effects in the LoVo tumors but is also more sensitive to vascular normalization in the HT29 tumor, which may provide an avenue to improve scheduling of chemotherapeutic agents in combination with antiangiogenic drugs.

There was little or no signal from hyperpolarized [1,4-13C2]malate in HT29 tumors prior to treatment, but a significant increase was observed following treatment, despite only a relatively minor increase in necrotic area in these tumors and a decrease in fumarase activity. This shows again the sensitivity of the hyperpolarized fumarate experiment for detecting low levels of diffuse necrosis by integrating malate signal over the entire tumor volume. These low levels of necrosis were not detected by either the T2 or DW-MRI measurements. The decrease in fumarase activity reflected, at least in part, a decrease in mitochondrial content, as assessed by measurements of citrate synthase activity. Fumarase exists in both cytosolic and mitochondrial forms, so the membrane disruption that occurs early in the process of necrosis may lead to leakage of the cytosolic form, also causing a reduction in the measured fumarase activity.

In conclusion, we have shown that hyperpolarized 13C MRS sensitively detects early response to antiangiogenic therapy. Although DCE-MRI detects evidence of vascular changes, it cannot determine the downstream effects on tumor metabolism, in which the hyperpolarized pyruvate experiment has the advantage of being sensitive to both perfusion and metabolic changes. In therapies that produce diffuse, low-level necrosis, in which there is no significant change in ADC, the production of malate from hyperpolarized fumarate is still elevated. However, there is clearly an advantage in combining both the metabolic and functional MRI approaches, for example, the early increase in label flux following treatment of HT29 tumors could be explained either by vascular normalization increasing pyruvate delivery to the tumor or an increase in steady-state lactate concentration (27), alternatives which can be resolved by the DCE-MRI experiment. Therefore, the use of hyperpolarized [1-13C]pyruvate and [1,4-13C2]fumarate to detect metabolic response to treatment should prove advantageous, in the clinical assessment of response to antiangiogenic therapies.

Disclosure of Potential Conflicts of Interest

K.M. Brindle received commercial research grant, research support from GE Healthcare; has ownership interest, patents with GE Healthcare on the technology. He also is a consultant and on the advisory board of consultancies with GlaxoSmithKline and Genentech. M.I. Kettunen has ownership interest and patents with GE Healthcare on the technology. The other authors disclosed no potential conflicts of interest.
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