Mapping Extracellular pH in Rat Brain Gliomas in Vivo by 1H Magnetic Resonance Spectroscopic Imaging: Comparison with Maps of Metabolites

María-L. García-Martín, Gwenaëlle Hérigault, Chantal Rémy, Régine Farion, Paloma Ballesteros, Jonathan A. Coles, Sebastián Cerdán, and Anne Ziegler


ABSTRACT

The value of extracellular pH (pHe) in tumors is an important factor in prognosis and choice of therapy. We demonstrate here that pHe can be mapped in vivo in a rat brain glioma by 1H magnetic resonance spectroscopic imaging (SI) of the pH buffer (±2-imidazole-1-yl-3-ethoxycarbonylpropionic acid (IEPA)). 1H SI also allowed us to map metabolites, and, to better understand the determinants of pHe, we compared maps of pHe, metabolites, and the distribution of the contrast agent gadolinium1,4,7,10-tetraazacyclododecane-N,N',N",N"-tetraacetic acid (Gd-DOTA). C6 cells injected in caudate nuclei of four Wistar rats gave rise to gliomas of ~10 mm in diameter. Three mmols of IEPA were injected in the right jugular vein from 0 to 60 min after injection. On average, the echo time of 40 ms in a 2.5-mm slice including the glioma (nominal voxel size, 2.2 µl) 1H resonances were detected only within the glioma and were intense enough for pHe to be calculated from the chemical shift of the H2 resonance in almost all voxels of the glioma. 1H SI also allowed us to map metabolites: lactate, choline-containing compounds (tCho), phosphocreatine/creatine, and N-acetylaspartate. Preliminary results had shown that systemically delivered IEPA infiltrates the extracellular space of tumors in rat brain. The tool we used is a new probe molecule, which has pH-dependent 1H resonances detectable by 1H NMR spectroscopy. This molecule is IEPA. It has been shown that IEPA does not enter erythrocytes (compound 9 in Ref. 16), and it appears to remain extracellular in a tumor model in a mouse mammary fat pad where the 1H signal was sufficient for SI (17). Preliminary results had shown that systemically delivered IEPA infiltrates the extracellular space of C6 gliomas in brain and allows mapping of pHe (18).

In addition to providing a signal:noise ratio sufficient to allow imaging of pHe rather than just measurement of the average value in a volume including the tumor, detection by 1H magnetic resonance spectroscopy has an additional advantage: using the same radiofrequency coil, the distributions of various endogenous compounds with 1H resonances can be readily imaged in the same experiments. These include compounds of which the concentrations might be causally related to the value of pHe, notably lactate. Hence, in this paper, we not only report the use of IEPA to image the distribution of pHe within C6 gliomas, but we have compared this distribution with the distribution of lactate and also of tCho, NAA, and tCr. The results lead us to consider the reasons why pHe in C6 gliomas should be acid. Both normal brain tissue (19) and tumor cells in particular (20, 21) produce lactate even under aerobic conditions. Lactate is exported from cells in association with protons, within the gliomas, no evidence was observed that pHe was significantly lower where lactate concentration was higher. These results suggest that lactate is produced mainly in viable, well-perfused, tumoral tissue from which proton equivalents are rapidly cleared.

INTRODUCTION

pH4 in tumor cells is normal or slightly alkaline; in contrast, pHe is usually acid compared with normal tissue and, unlike pHe, appears to vary with the type of tumor (1–3) so that measurement of pHe is potentially more informative than measurement of pH2. Knowledge of pHe is important not only for diagnosis but also for choosing chemotherapeutic agents, because most are weak bases or weak acids, and their accumulation within cells and, hence, their efficacy depends on the transmembrane pH gradient (1, 4–7). In addition, the effectiveness of thermoradiotherapy has been reported to correlate with the extracellular acidity (8). Therefore, noninvasive measurement of tumor pHe might be useful for diagnosis, choice of therapy, and prognosis.

Tumor pHe has been measured mainly by invasive techniques that measure it at a single point, namely microelectrodes (9, 10) and miniature optical probes (11). The value of these measurements depends on how uniform pH2 is throughout the tumor. Mean pH2 within tumors has also been measured noninvasively by NMR using extracellular probe molecules containing 31P or 23Na (6, 12–14). Spatial variations in pHe have been measured over distances in the order of 100 µm in the tissue between blood vessels in the exposed superficial layers of a s.c. tumor by optical techniques (15). In the present work, we have made maps of pH2 on a larger scale throughout sections of C6 gliomas in rat brain. The tool we used is a new probe molecule, which has pH-dependent 1H resonances detectable by 1H NMR spectroscopy. This molecule is IEPA. It has been shown that IEPA does not enter erythrocytes (compound 9 in Ref. 16), and it appears to remain extracellular in a tumor model in a mouse mammary fat pad where the 1H signal was sufficient for SI (17). Preliminary results had shown that systemically delivered IEPA infiltrates the extracellular space of C6 gliomas in brain and allows mapping of pHe (18).

In addition to providing a signal:noise ratio sufficient to allow imaging of pHe rather than just measurement of the average value in a volume including the tumor, detection by 1H magnetic resonance spectroscopy has an additional advantage: using the same radiofrequency coil, the distributions of various endogenous compounds with 1H resonances can be readily imaged in the same experiments. These include compounds of which the concentrations might be causally related to the value of pHe, notably lactate. Hence, in this paper, we not only report the use of IEPA to image the distribution of pHe within C6 gliomas, but we have compared this distribution with the distribution of lactate and also of tCho, NAA, and tCr. The results lead us to consider the reasons why pHe in C6 gliomas should be acid. Both normal brain tissue (19) and tumor cells in particular (20, 21) produce lactate even under aerobic conditions. Lactate is exported from cells in association with protons, within the gliomas, no evidence was observed that pHe was significantly lower where lactate concentration was higher. These results suggest that lactate is produced mainly in viable, well-perfused, tumoral tissue from which proton equivalents are rapidly cleared.

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The abbreviations used are: pHe, intracellular pH; NMR, nuclear magnetic resonance; pHe, extracellular pH; NAA, N-acetylaspartate; SI, spectroscopic imaging; tCho, total choline-containing compounds; tCr, creatine and creatine phosphate; CHESS, chemical-shift selective excitation; OVS, outer volume saturation; TE, echo time; TR, repetition time; T2*, transverse relaxation time; pHe, arterial pH; Gd-DOTA, gadolinium1,4,7,10-tetraazacyclododecane-N,N',N",N"-tetraacetic acid; IEPA, (±)2-imidazole-1-yl-3-ethoxycarbonylpropionic acid; ppm, parts per million; WSI, water spectroscopic imaging.
MATERIALS AND METHODS

IEPA was synthesized as described previously, with the preparation more than 99.9% pure as determined by gas chromatography and mass spectrometry (16). C6 cells were a gift from P. Canioni, Bordeaux University, and DMEM culture medium was purchased from Life Technologies, Inc. Isoulorane was obtained from Abbott Laboratories (Abbott Park, IL). Other reagents were from Sigma Chemical Co. (St. Louis, MO).

Calibration of the pH Dependence of the Chemical Shift of the IEPA H2 Resonance. Fifteen vials containing solutions of 20 mM IEPA and 100 mM of the HEPEs buffer in rat plasma were titrated at 37°C with HCl or NaOH to pHs in the range 4.5–8.5. The chemical shift of the IEPA H2 hydrogen and its T2 were measured at 37°C using an 8.4 Tesla magnet (Oxford Instruments, United Kingdom) interfaced with an AM-360 NMR spectrometer (Bruker, Karlsruhe, Germany). We used a single pulse sequence with a 1 s presaturating pulse on the water resonance to decrease the water resonance intensity. The excitation pulse duration was 7 µs, data size 16384 points, and acquisition time 0.95 s. pH titrations of the H2 chemical shift were fitted using SigmaPlot v 4.0 (SPSS Inc., Chicago, IL) with the Henderson-Hasselbalch equation:

\[ pH = pK_a + \log \left( \frac{\delta_i - \delta}{\delta - \delta_0} \right) \]

where \( \delta \) is the chemical shift and \( \delta_i > \delta_0 \) are the asymptotic values.

The T2 of IEPA H2 and H5 peaks were also determined in the same samples used for the pH titration. The Hahn spin-echo sequence was modified to include a 1-s selective presaturating pulse, a jump and return excitation pulse (interpulse delay, 210 µs), and a binomial refocusing pulse (interpulse delay, 420 µs; Ref. 25). Ten different values of TE in the range 2–400 ms were used to obtain T2 at different pHs. T2(H2) showed a minimum of 43 ms at pH 6.5 increasing to 81 ms at pH 4.5 and to 100 ms at pH 8.5. Although this variation in T2 does not affect the calculation of pH from the chemical shift, it would affect the apparent distribution of IEPA. Therefore, the distribution of IEPA was calculated from the H5 peak of which the T2 did not vary significantly over the pH range 5.5–7.5 (T2 = 57 ± 4 ms).

Animal Preparation. All of the procedures involving animals conformed to the guidelines of the French Government (decrees 87–848, October 19, 1987, licenses 006683 and A38071). To prepare the glioma model, Wistar rats (200–230 g) were anesthetized with chloral hydrate (400 mg/kg), and –10 C6 glioma cells (26) in DMEM were injected stereotaxically in the right caudate nucleus as in Refs. 27, 28 (but without the use of agar). Gliomas developed, which, after 3 weeks, occupied 30–50% of the right hemisphere of the brain. All of the rats were females except for one male included in the results shown in Fig. 4.

To prepare the rats for NMR experiments, anesthesia was induced with 4% isoflurane in air enriched with oxygen to 30% (v/v). All of the rats were females except for one male included in the results shown in Fig. 4.

NMR Measurements. Experiments were carried out with a SMIS console (Surrey Medical Imaging Systems Ltd., Guildford, United Kingdom) equipped with a 20-cm horizontal bore, 7 Tesla magnet (Magnex Scientific Ltd., Abington, United Kingdom), and actively shielded gradients (Magnex). Standard NMR images were processed with the standard SMIS image-processing package. Raw NMR SI data were transferred to a Unix workstation (Sun Microsystems Inc., Palo Alto, CA) for off-line analysis. The processing software was developed in the Interactive Data Language programming environment (Research Systems International, Boulder, CO).

The rat was prone, its head secured by ear bars, and a 25-mm diameter surface coil located directly above the brain was used for radiofrequency transmission and reception. After radiofrequency coil matching and tuning, the magnetic field homogeneity was coarsely adjusted to obtain a line width for water <40 Hz in a 5-mm horizontal slice of the brain. Additional fine adjustments were carried out in the volume of interest for each separate NMR measurement. An idealized scheme of the experiment is shown in Fig. 1.

Spin-Echo NMR Imaging. To assess glioma development in each rat and to select a slice including the tumor for subsequent single voxel or SI experiments, scout spin-echo images were obtained (slice thickness, 1 mm; TR, 3 s; TE, 80 ms; 128 × 128 pixels, 5 horizontal slices and 7 coronal slices).

Single Voxel Spectroscopy. To follow changes in IEPA concentration and pH within the glioma, during the infusion of IEPA and the subsequent 2 h from a volume in the center of the glioma, experiments were made on three rats using point resolved spectroscopy (Ref. 29; TR, 3 s; TE, 40 ms; voxel size, 5 × 5 × 5 mm, 64 scans) taken at 5-min intervals. At the end of the NMR measurements, the brains were excised and the tumor dissected and frozen. The concentration of IEPA in an aqueous extract of each tumor was measured by high-resolution 1H NMR spectroscopy.

1H SI. The pulse sequence is shown in Fig. 2. 1H spectroscopic images were acquired on four rats using a spin-echo sequence with OVS (four slices), and CHESS excitation (30) for water saturation (3-lobe sinc pulse, 8 ms duration, followed by crusher gradients of 2 ms, 20 mT/m). For IEPA mapping (TE, 40 ms), a binomial pulse (31, 32) with a frequency response centered on 8 ppm was used for excitation, and the refocusing pulse was selective for a 2.5-mm horizontal slice (5-lobe sinc pulses, 6 kHz). For mapping other metabolites, both excitation and refocusing pulses were slice selective (TE, 136 ms).

0.5 M IEPA infusion

<table>
<thead>
<tr>
<th>WSI</th>
<th>40 ms-TE SI</th>
<th>Waiting time</th>
<th>WSI</th>
<th>40 ms-TE SI</th>
<th>Readjustments</th>
<th>136 ms-TE SI</th>
<th>WSI</th>
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<tr>
<td>8 min</td>
<td>40 min</td>
<td>45 min</td>
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<td>BS2</td>
<td>BS3</td>
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Fig. 1. Scheme showing the order in which the different kinds of NMR images were acquired after the rat was placed in the magnet. First, a WSI was acquired over 8 min, then a 40-ms TE SI was acquired to obtain baseline spectra for subsequent subtraction from spectra including IEPA signals, then IEPA was infused, and so forth. Metabolic SI had 136-ms TE; T1-weighted images (30 s each) were acquired after GdDOTA injection.
Comparison with the histology confirmed that large central parts of the glioma from which the signal was weak corresponded to necrotic regions. Before the injection of Gd-DOTA, IEPA had been infused during 60 min into the right jugular vein and \(^1H\) spectra with a 40-ms TE obtained from each voxel of a slice (see Fig. 1). After infusion of IEPA, the spectra from the contralateral hemisphere were unchanged (Fig. 3a) but new peaks appeared in the glioma. A double peak near 7.2 ppm corresponded to the H4 and H5 resonances of IEPA, which are relatively insensitive to pH, and a peak near 8 ppm corresponded to the pH-sensitive H2 resonance (arrow in Fig. 3b; Refs. 16, 17). Analysis of the spectra showed that within the brain, signals from IEPA reached our criterion amplitude (5 times the noise) only within the glioma. The area under the H5 peak was measured for each voxel and a map made (Fig. 3d). To relate the chemical shift of the H2 peak to pH, we made an in vitro calibration curve for IEPA in plasma at 37°C, which showed that the chemical shift of the IEPA H2 proton varied from 7.72 ppm (\(\delta_{ij}\) to 8.85 ppm (\(\delta_{ij}\)) with a pK<sub>a</sub> of 6.566 ± 0.035 (Fig. 3e). By use of this curve, pH<sub>c</sub> was calculated for each voxel and a map constructed (Fig. 3f). This could only be done where IEPA was present, so there are no valid measurements for normal tissue or for the center of the necrotic core. The maps showed variations in pH<sub>c</sub> within the glioma over a range of ~0.4 pH units (Figs. 3f and 6h), but no marked and significant general trend was observed (e.g., from the center to the periphery). The mean value of pH<sub>c</sub> measured in the regions infiltrated by IEPA in each glioma was calculated; the mean value for the four gliomas was 7.084 ± 0.017 (±SE). This is well below the pH of the arterial blood, which was monitored in all of the experiments and found to remain within the range 7.33–7.41 during the IEPA imaging. In almost all of the voxels, only one H2 peak was detected. In a few voxels there were two peaks, but both peaks always corresponded to pHs more acidic than that of the circulating blood (~7.4). We failed to detect any association of the presence of two peaks with any other feature of the gliomas. In agreement with the majority of previous results in other tumors and tumor models made using other methods of measurement, pH<sub>c</sub> in the C6 glioma was lower than the values most frequently reported for normal brain tissue (~7.3; Ref. 6).

Fig. 4 presents results that relate to the possibility that the presence of IEPA in the extracellular clefts modified pH<sub>c</sub>, pH<sub>e</sub> was measured in a single large voxel covering about one-half the volume of the glioma. During the infusion of IEPA, as the quantity of IEPA, these results show that a high mean concentration of IEPA in the glioma increased 3-fold, pH<sub>e</sub> changed little and may have corresponded to well-perfused regions with leaky blood vessels (35).

**RESULTS**

**Imaging pH<sub>c</sub> in a Glioma.** Fig. 3c is a T<sub>2</sub>-weighted image of a horizontal section of a glioma-bearing rat brain acquired over 32 s starting ~80 s after injection of a bolus of Gd-DOTA in the right jugular vein. The marked hyperintensity in the periphery of the glioma corresponded to well-perfused regions with leaky blood vessels (35).
contaminated the lactate peak in vivo. However, the IEPA methyl group peak is closer to the methylene group peak of the lipids (at 1.26 ppm). In the absence of certainty that the lipid peak was free of contamination by the IEPA peak, we have not considered the lipid distribution in this paper.

Fig. 6 illustrates for a sample glioma the seven kinds of maps that were made. As known for necrotic gliomas (35), the contrast agent Gd-DOTA, or, more strictly, the water proton hypersignal it produces on T1-weighted images, is predominantly confined to the outer parts (Figs. 3c and 6, a and b). The distribution of IEPA (Fig. 6c) has some similarity to that of Gd-DOTA. Comparison of the Gd-DOTA distribution (Fig. 6, a and b) and the histology with maps of endogenous compounds detected by 1H SI with a TE of 136 ms show that NAA was at a low concentration in gliomas (Fig. 6d). tCho or tCr tended to be present in the periphery (Fig. 6, e and f). Lactate was strongly present in outer parts of the gliomas (Fig. 6g); its distribution in the necrotic core could not be determined because of overlap of the (negative) lactate peak and the (positive) lipid peak. Note that Fig. 6g has not been corrected for this interference.
Voxel-by-Voxel Comparison of Distributions. Because the resonance peaks of the $^1$H spectra were normalized with respect to the water peak, the results for the four gliomas were pooled. The various signals measured in the rectangular areas including the gliomas (as in Fig. 6a) were then compared, voxel-by-voxel, for the various compounds and for pH$_e$. Sample scatter diagrams are shown in Fig. 7. Fig. 7a shows that tCho and tCr, both indicative of metabolizing tissue, had, as expected, somewhat similar distributions. Lactate posed a problem because of the overlap with the lipid peak. Simulations of the two peaks showed that in voxels with no distinct lipid peak, the maximum error in the area of the lactate peak was $\leq 10\%$, and, for quantitative comparisons, we took into account only these voxels with no detectable lipid. In those voxels where lactate could be measured (i.e., where lipids were not detected), it correlated with Gd-DOTA, a marker of extravasation through a ruptured blood-brain barrier (Fig. 7b).

The correlations were quantified by calculating the Pearson correlation coefficients (Eq. 2), which are given in Table 1. The coefficients for NAA are not given, because coexistence of NAA and a tumor marker occurred in only a small number of voxels at the periphery of the gliomas, which probably included both tumoral and normal tissue. Correlations for lactate were calculated only for voxels where no lipid signal was apparent. The correlations in Table 1 will be considered in the “Discussion.”

DISCUSSION

Validity of the Use of IEPA to Measure pH$_e$. Ojugo et al. (6) have raised the question of the extent to which signals from an extracellular probe molecule, such as IEPA, designed to measure pH in the interstitium, are contaminated by signals from the probe in the blood, which lead to an erroneously alkaline estimate of pH$_e$. We suggest that the error, if any, is very small. van der Sanden et al. (36) have found that in 9L gliomas the blood plasma volume fraction in well-perfused parts is 0.75–1.2%, and the volume fraction of the interstitium is typically 25%, so the ratio, plasma volume:interstitium, is $\leq 0.05$. During most of the time of the IEPA imaging, IEPA was being cleared from the glioma (Figs. 1 and 4) so the concentration of IEPA in the plasma must have been less than that in the nearby interstitium. Hence, although the blood volume fraction in C6 gliomas may be higher than in 9L gliomas, the quantity of plasma IEPA in a voxel was almost certainly $\leq 10\%$ of the quantity of interstitial IEPA.

In the hypothetical case of no exchange of IEPA molecules between the blood compartment and the interstitial compartment within a voxel, one would observe, in the absence of noise, two H2 peaks with two distinct chemical shifts corresponding to the two pHs, $\sim 7.4$ in the plasma and $\sim 7.1$ in the interstitium. However, with the signal:noise ratios of our spectra, the small peak corresponding to the plasma compartment would have been excluded from the analysis, and, indeed, no peak corresponding to pH $\sim 7.4$ was detected. The other extreme case, of exchange between the plasma and interstitial compartments at a rate that is high compared with the frequency difference corresponding to the two pH values is improbable but readily analyzed. In this case, there would be a single peak at a frequency corresponding to the weighted mean of the frequencies of the two component peaks (37). With our worst case assumption that the...
quantity of IEPA in the plasma is as great as 10% of the quantity in the interstitium, then the single peak would be at the interstitium frequency shifted 0.1 of the way toward the plasma frequency. Because the calibration curve (Fig. 3e) is nearly linear over the pH range 7.1–7.4, this shift corresponds to 0.03 pH units. This is a very conservative upper limit for the possible error introduced by the IEPA in plasma.

Another possible source of systematic error is the possible modification of pH$_{e}$ by the IEPA itself. pH$_{e}$ is determined by the balance between the net production (and subsequent extrusion) of proton equivalents by the cells and the clearance of the extracellular proton equivalents to the blood by diffusion through the extracellular clefts to the capillaries. pH buffer can contribute to the latter process by facilitating diffusion; flux of proton equivalents occurs not only by the diffusion of H$^+$ ions, which are present at a very low concentration of $\sim$0.1 $\mu$M, but also by diffusion of the buffer molecules, which may typically be present at concentrations $10^2$–$10^3$ times greater (38). Hence, if the concentration of diffusible buffer molecules in the extracellular space was increased (by addition of IEPA), acid equivalents would be transferred more rapidly to the capillaries and the pH in the interstitium would be increased to a value closer to that of the blood (39). A priori, this appears to be a problem. From the measurements of [IEPA] in the glioma (Fig. 4) we estimate the maximum concentration of IEPA in the interstitium during the imaging to have been $\sim$16 mM. The major mobile pH buffer is HCO$_3^-$ /CO$_2$, and the concentration of HCO$_3^-$ in equilibrium with a P$_{CO2}$ of 35 mm Hg at pH 7.1 is $\sim$10.5 mM. Phosphates will contribute an additional 4 mM. Although the diffusion coefficient of IEPA is smaller than those of these endogenous anions ($M_r$ $\sim$211 versus 61 and 100), and it is not at its optimum pH for buffering ($pK_r$ 6.5), IEPA may considerably increase the concentration of diffusible buffers so that pH$_{e}$ is shifted in the alkaline direction. This predicted artifact might be reduced if there exist additional processes undescribed in brain interstitium, such as the contribution to facilitated diffusion by rotating proteins described in vitro by Gros et al. (40). These arguments predict that the greater the concentration of IEPA, the greater the value of pH$_{e}$. However, the experimental result of Fig. 4 shows a change in the opposite direction: as the concentration of IEPA in the glioma rose during infusion and then fell, not only did pH$_{e}$ change very little, but pH$_{e}$ was minimal, not maximal, when the IEPA concentration was maximal (at the end of the 60 min infusion). Another observation of the same type is that there was a significant negative correlation between the spatial distribution of IEPA and pH$_{e}$ (Table 1). In conclusion, we did not observe the predicted alkalinizing effect of IEPA; either the effect was small, or it was masked by other factors that remain to be identified.

**Distributions of IEPA and Gd-DOTA.** Although both IEPA and Gd-DOTA were confined to the gliomas and had their highest intensities in the peripheral regions, more IEPA than Gd-DOTA was present in the center of the glioma, and the distribution of IEPA correlated less than that of Gd-DOTA with the distributions of tCho and tCr (Table 1). These observations are readily explained by the much longer infusion time for IEPA, which would have allowed it to diffuse farther away from the vasculature and into necrotic regions.

**Distributions of tCho and tCr.** tCr, which is associated with storage of high energy phosphate (41) and tCho, which is associated with synthesis and breakdown of membranes and with cell proliferation (42), were strongly correlated (Table 1). As shown previously for human tumors (43), tCho was highest in the periphery of the gliomas (Fig. 6e), and it correlated strongly with Gd-DOTA, indicating that it was in well-perfused regions (Table 1). Although the correlation coefficients were smaller, tCr was also associated with Gd-DOTA, and both tCho and tCr had positive correlations with pH$_{e}$. These results are coherent with the idea that energy metabolism and proliferation are most active in well-perfused, less acid parts of the glioma.

**Distribution of Lactate.** In the voxels where the lactate signal was not contaminated by a lipid signal, lactate correlated with tCho, tCr, and Gd-DOTA (Table 1), i.e., it appeared to be present particularly in well-perfused, actively metabolizing parts of the glioma, in agreement with previous work (10, 44). Production of lactate under aerobic conditions is well-known for tumoral tissue (20) and has been reported recently for several kinds of normal tissue, including muscle (45), nerve (46), and brain (19). In C6 gliomas it is known that lactate is rapidly and heavily labeled from $^{13}$C glucose in blood (21), and in C6 cells in culture lactate produced within the cells tends not to enter the TCA cycle (47). Hence, it is indeed to be expected that lactate is produced in the well-perfused parts of the glioma even if they are adequately oxygenated. In the center of the glioma, where we were unable to measure the relative concentration of lactate, we would expect it to be at least as high as the interstitial concentration in the outer parts, even if no lactate were produced there because it would

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**Table 1** Voxel-by-voxel correlations

<table>
<thead>
<tr>
<th>Lactate</th>
<th>tCho</th>
<th>tCr</th>
<th>Gd-DOTA</th>
<th>IEPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH$_{e}$</td>
<td>NS$^a$</td>
<td>0.199$^{b}$</td>
<td>0.128$^{c}$</td>
<td>0.071$^{d}$</td>
</tr>
<tr>
<td>Lactate</td>
<td>0.284$^{a}$</td>
<td>0.117$^{b}$</td>
<td>0.296$^{c}$</td>
<td>NS$^a$</td>
</tr>
<tr>
<td>tCho</td>
<td>0.629$^{b}$</td>
<td>0.591$^{c}$</td>
<td>0.052$^{d}$</td>
<td>NS$^a$</td>
</tr>
<tr>
<td>tCr</td>
<td>0.298$^{b}$</td>
<td>NS$^a$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gd-DOTA</td>
<td>0.230$^{b}$</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ NS, $P > 0.05$ are not significant.
$^b$ $P < 0.0001$.
$^c$ $P < 0.001$.
$^d$ $P < 0.05$. |
tend to be in diffusional equilibrium. However, in the solid parts of the tumor, most of the lactate is expected to be intracellular. This is not only because the intracellular volume fraction is greater than the extracellular volume fraction, but the intracellular concentration of lactate will be higher than the extracellular concentration. Lactate can readily cross the plasma membrane of most cells (including Ehrlich tumor cells; Ref. 48) by passive cotransport with protons, so that the concentrations of the neutral protonated form, \( \text{H}^+ \), \( \text{H}_{\text{lactate}} \), inside and outside the cell tend to equilibrate (22). Within each compartment (intracellular and extracellular) the ratio of [Hlactate]:[lactate] is determined by the pH according to the Henderson-Hasselbalch equation. It follows that because \( \text{[H}^+ / \text{[H}^+]_{\text{e}}} \) is greater than 1, the overall pathway of glycolysis from glucose to lactate is reversed when dephosphorylated to ADP. One glucose molecule also produces two lactate ions, which will be cotransported out of the cell with two protons that just balance the production of \( \text{H}^+ \) by hydrolysis of ATP; however, there is production of \( \text{CO}_2 \) (which reacts with \( \text{H}_2\text{O} \) to give \( \text{H}^+ \)) but at a rate of only 1 \( \text{CO}_2/6 \text{ATP} \) (51). Because a cell requires a certain amount of ATP to function, it is clear that if glycolysis replaces oxidative phosphorylation as a major source of ATP, then more \( \text{H}^+ \) equivalents will be exported to the extracellular space. In general, this is true: \( \text{pH}_e \) is indeed more acid in lactate-producing tumors than in normal tissue. Therefore, it is striking that within the gliomas (at least in the parts with no detectable lipid signal) our results show no significant negative correlation of lactate signal with \( \text{pH}_e \) (Table 1). A possible explanation is that most lactate is produced in well-perfused regions with viable cells and that in these regions \( \text{H}^+ \) can diffuse away to the blood stream more readily than lactate can.

We have demonstrated that a new probe molecule, IEPA, can be used for imaging \( \text{pH}_e \) in a rat brain glioma model in vivo. This new technique made it possible to perform \( ^{1} \text{H} \) NMR in the same experiments. By comparing quantitatively the distributions of different metabolites, Gd-DOTA and \( \text{pH}_e \), no evidence was observed that \( \text{pH}_e \) was significantly lower where lactate concentration was higher, and we reach the conclusion that lactate production is greatest in well-perfused parts of gliomas.

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