Hyperpolarized ^{13}C magnetic resonance spectroscopy reveals the rate-limiting role of the blood-brain barrier in the cerebral uptake and metabolism of L-lactate in vivo

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ABSTRACT

The dynamics of L-lactate transport across the blood-brain barrier (BBB) and its cerebral metabolism are still subject to debate. We studied lactate uptake and intracellular metabolism in the mouse brain using hyperpolarized ^{13}C magnetic resonance spectroscopy (MRS). Following the intravenous injection of hyperpolarized [1-^{13}C]lactate, we observed that the distribution of ^{13}C label between lactate and pyruvate, which has been shown to be representative of their pool size ratio, is different in NMRI and C57BL/6 mice, the latter exhibiting a higher cerebral lactate dehydrogenase A (*Ldha)* expression. Based on this observation, and on an additional set of experiments showing that the cerebral conversion of [1-^{13}C]lactate to [1-^{13}C]pyruvate increases after exposing the brain to ultrasound irradiation that reversibly opens the BBB, it was concluded that lactate transport is rate-limited by the BBB, with a 30% increase in lactate uptake after its disruption. It was also deduced from these results that hyperpolarized ^{13}C MRS can be used to detect a variation in cerebral lactate uptake of less than 40 nmol in a healthy brain during an *in vivo* experiment lasting only 75 s, opening new opportunities to study the role of lactate in brain metabolism.

KEYWORDS

Hyperpolarization; magnetic resonance spectroscopy; dynamic nuclear polarization; ultrasound; pyruvate; bicarbonate;

INTRODUCTION

The role of lactate as a source of energy for the mammalian brain has repeatedly been a subject of debate[1-3], but it is established that lactate utilization by the brain increases upon elevation of lactate plasma concentration following lactate injection or exercise. While glucose is thought to be preferentially taken up by astrocytes[4], a large number of observations show that lactate is predominantly taken up by neurons and transformed, via lactate dehydrogenase (LDH), into pyruvate for mitochondrial oxidation [5]. In mice, it has been demonstrated that lactate is metabolized by the intact brain in an activity-dependent manner[3].

L-lactate can cross the blood-brain barrier (BBB) relatively easily and is taken up by cells in the mammalian brain either via monocarboxylate transporters (MCTs) in the plasma membrane or by non-saturable diffusion[6]. There is an equilibration between blood and brain concentrations[1, 7], and at high plasma lactate levels, the transport is dominated by non-facilitated mechanisms[8]. Earlier studies in rats[9] and in patients[10] had shown that this equilibration is not immediate, leaving unsettled the question of the kinetics of lactate transport through the BBB. After its uptake into brain cells, lactate rapidly equilibrates with pyruvate through the action of LDH. The conversion is a near-equilibrium reaction governed by the relation: $[pyruvate]/[lactate]= K_{LDH}[NAD^+]/([NADH][H^+])$, where K_{LDH} is the equilibrium constant for lactate dehydrogenase.

^{13}C magnetic resonance spectroscopy (MRS) has been widely used to investigate the kinetics of substrate utilization in cerebral intermediary metabolism, in particular following the fate of infused ^{13}C-glucose and ^{13}C-lactate. However, this technique is limited by poor sensitivity, which precludes directly probing metabolic transformations taking place within the first minute of injection. With the advent of dissolution dynamic nuclear polarization (DNP) [11], hyperpolarized (HP) ^{13}C MRS has become a powerful technique for monitoring fast metabolic conversions *in vivo* by enhancing the sensitivity of MRS signals up to 4 orders of magnitude[12]. It is assumed that the lactate-to-pyruvate ratio derived from hyperpolarized ^{13}C-pyruvate can be a suitable marker of LDH activity[13-15]. Studies using hyperpolarized ^{13}C-pyruvate have also

shown that the limited transport of pyruvate across the BBB can be a significant constraint for cerebral metabolic studies based on hyperpolarized ^{13}C MRS[16].

Several recent studies have shown that hyperpolarized ^{13}C-lactate can also be used to investigate the lactate-to-pyruvate conversion *in vivo*[17-22]. Compared to pyruvate, hyperpolarized lactate has the advantage that a bolus injection does not greatly alter its circulating concentration, since lactate has a substantially higher physiological concentration than pyruvate in the blood[23].

It has previously been demonstrated that the ^{13}C-lactate signal detected *in vivo* following the injection of hyperpolarized ^{13}C-pyruvate mostly originates from the rapid ^{13}C label exchange catalyzed by LDH and that the net production of lactate is nearly negligible [24, 25]. The intensity of the lactate signal is therefore expected to be directly related to the endogenous lactate pool size and this property can be used to detect tumorous tissue, which is known to have a higher lactate concentration than healthy tissue[26]. The same holds true for the ^{13}C-pyruvate signal measured following the injection of hyperpolarized ^{13}C-lactate, the intracellular pyruvate pool being labeled by exchange catalyzed by LDH[20]. With both hyperpolarized ^{13}C-substrates, the detected pyruvate-to-lactate ^{13}C signal ratio is representative of the local pyruvate-to-lactate concentration ratio since LDH governs the equilibrium between these two metabolites.

The aim of the present study was to evaluate the rate-limiting role of the BBB on equilibration between plasma and brain within the first minute following the intravenous injection of hyperpolarized [1-^{13}C]lactate. We first established that the kinetics of the observed cerebral [1-^{13}C]pyruvate signal can provide a measure of cerebral [1-^{13}C]lactate uptake through its rapid intracellular equilibration catalyzed by LDH, by performing hyperpolarized ^{13}C MRS experiments in two mouse strains, namely C57BL/6 and NMRI, that exhibit differences in *Ldha* expression. The rate-limiting role of the BBB on lactate transport was then assessed by disrupting the BBB with ultrasound and microbubbles, following a protocol described in an earlier study[27].

RESULTS AND DISCUSSION

The hyperpolarized ^{13}C magnetic resonance (MR) signals originating from the injected substrate, [1-^{13}C]lactate (183 ppm), and its metabolites, [1-^{13}C]alanine (176 ppm) and [1-^{13}C]pyruvate (171 ppm), were detected in all the experiments (n = 19) (Fig. 1). [^{13}C]bicarbonate (161 ppm) was also observed in nearly all the experiments (18 out of 19). The additional peak observed around 176.5 ppm corresponds to an impurity overlapping with the [1-^{13}C]alanine peak and was confirmed by high-resolution ^{13}C NMR of the purchased solution (data not shown). Compared to NMRI mice, the pyruvate-to-lactate ratio was clearly smaller in C57BL/6 mice ($p<0.05$), while the bicarbonate-to-lactate ratio was not significantly different between the two strains (Fig. 2).

The origin of the higher pyruvate-to-lactate signal ratio observed in NMRI mice as compared to C57BL/6 mice could possibly be explained by a difference in cerebral MCTs between the two strains. The quantitative real-time PCR analyses did however not exhibit any significant difference in *Mct* expression (Fig. 3). Since MCTs activity was not measured, it is nevertheless not possible to completely rule out that a difference in cerebral MCTs may have had an impact on the observed pyruvate-to-lactate signal ratios. The results of the quantitative real-time PCR analyses highlighted that *Ldha* expression in NMRI mice brain was lower than in C57BL/6 mice ($p < 0.05$), while no significant difference was found in *Ldhb* expression between the two strains (Fig. 3). It was previously proposed that increased *Ldha* might lead to an increased lactate concentration in the brain [(28, 29)]. Although the p-value did not reach significance, it was also previously observed in a ^{1}H MRS study that C57BL/6 have a higher lactate brain concentration than NMRI[(30)].

These observations coupled to the fact that no significant difference in blood lactate concentration was found between C57BL/6 (1.1 ± 0.2 mM) and NMRI (1 ± 0.1 mM), led us to the conclusion that the variation in pyruvate-to-lactate ratio is most likely not due to a difference in transport kinetics but rather a reflection of the endogenous lactate pool size. Our observation therefore seems to be a confirmation that the pyruvate-to-lactate signal ratio is correlated to the ratio between the two metabolite pool sizes and it shows that it is possible to

determine variations in lactate pool size using hyperpolarized [1-^{13}C]lactate. Since the detected pyruvate signal originates from cerebral tissue only and we did not observe any difference in lactate blood concentration between the two strains, we could conclude that our observation is related to unequal intracellular concentrations.

To assess the role of the BBB on the kinetics of L-lactate transport into the brain, we measured the cerebral pyruvate signal pre- and post-ultrasound irradiation. The analysis is based on the assumption that LDH will nearly instantaneously equilibrate the ^{13}C distribution between the two pools as soon as lactate has entered the brain. This assumption is reasonable given that the transport kinetics across the BBB is at least one order of magnitude slower than the apparent rate constant associated with intracellular transport and LDH activity in the rodent brain[16, 31]. Because the pyruvate-to-lactate ratio determined from our measurements was larger in NMRI mice than in C57BL/6 mice, the study was performed on the former strain. To assess the effect of ultrasound on the BBB, T_1-weighted images of the brain were acquired in NMRI mice after administration of either Gd^{3+} (Fig. S1) or Mn^{2+} (Fig. S2). The striking change in contrast observed in the images, particularly in the lateral ventricles which exhibit a hyperintense signal following ultrasound irradiation, confirmed the opening of the BBB after the injection of microbubbles and the application of ultrasound.

To quantify the increase in intracellular cerebral lactate after ultrasound application, we propose the following model illustrated in Fig. 3: in the absence of ultrasound (US) exposure, the pyruvate-to-lactate ^{13}C signal ratio can be written as follows,

$$\left|\frac{S_{pyr}}{S_{lac}}\right|_{No\ US} = \frac{S_{pyr}^{brain}}{S_{lac}^{blood} + S_{lac}^{brain}}, \qquad (1)$$

where the [1-^{13}C]pyruvate signal (S^{brain}_{pyr}) originates exclusively from the brain cells. If we assume that the ^{13}C fractional enrichment for both pyruvate and lactate brain pools are equal due to the rapid exchange through LDH,

$$\left|\frac{S_{pyr}}{S_{lac}}\right|_{No\ US} = \frac{S_{lac}^{brain} \cdot \frac{[pyr]}{[lac]}}{S_{lac}^{blood} + S_{lac}^{brain}}, \qquad (2)$$

where [pyr] and [lac] are the pyruvate and lactate brain concentrations, respectively. When the brain is exposed to ultrasound irradiation, S_{lac}^{brain} can be replaced by $S_{lac}^{brain} \cdot (1+\delta)$, with δ the portion of ^{13}C signal originating from the increase in brain lactate during the measurement. We obtain

$$\left|\frac{S_{pyr}}{S_{lac}}\right|_{US} = \frac{S_{lac}^{brain} \cdot (1+\delta)\frac{[pyr]}{[lac]}}{S_{lac}^{blood} + S_{lac}^{brain}}, \quad (3)$$

where S_{lac}^{blood} is assumed to be identical in both cases because the reduction of blood lactate due to higher cerebral uptake is negligible, and that $S_{lac}^{blood} + S_{lac}^{brain} >> \delta \cdot S_{lac}^{brain}$ to approximate the denominator from $S_{lac}^{blood} + S_{lac}^{brain} \cdot (1+\delta)$ to $S_{lac}^{blood} + S_{lac}^{brain}$. Therefore, the change in ^{13}C signal ratio induced by the application of ultrasound is reduced to

$$\left|\frac{S_{pyr}}{S_{lac}}\right|_{US} = \left|\frac{S_{pyr}}{S_{lac}}\right|_{No\ US} \cdot (1+\delta). \quad (4)$$

The pyruvate-to-lactate ratio measured in mice exposed to ultrasound irradiation was significantly higher than in mice that did not experience ultrasound exposure (Fig. 5). The 28±3% increase ($p<0.05$) post ultrasound demonstrates that the BBB limits the transport of lactate into the brain. Although the difference in bicarbonate-to-lactate ratio did not quite reach significance ($p=0.45$), most likely because of low SNR, the observed trend towards increased conversion also points in the direction of an increased lactate transport rate post ultrasound.

It has been shown that the intracellular and extracellular hyperpolarized [1-^{13}C]lactate signals can be separated *in vitro*[32]. It was however not possible to achieve the required spectral resolution in the present *in vivo* study. As a consequence, we could not deduce the Michaelis-Menten constants associated with BBB transport. However, because of the rapid exchange catalyzed by LDH, the detection of cerebral pyruvate provides an indirect readout of lactate transport into the brain. In fact, the increase in pyruvate-to-lactate ratio following ultrasound irradiation corresponds to a quantitative measurement of the increased cerebral lactate uptake, δ (see Equation 4). We conclude that the application of ultrasound led to an

increase of 28±3% of cerebral lactate within the 75 s experiment. It was previously demonstrated that lactate flux through the BBB via non-facilitated transport is roughly identical to the flux via MCTs in the rat brain across a large lactate plasma concentration[33]. Assuming the uptake via MCTs was not substantially affected by ultrasound, non-facilitated transport would then account for the nearly 30% increase of lactate uptake after opening the BBB. Note that because the pyruvate and bicarbonate signals originate from the intracellular compartment, their intensity will not be affected by a potential variation in extracellular pH that may result from the opening of the BBB.

Using V_{max} = 300 nmol·g^{-1}·min^{-1} and K_M = 13 mM as the Michaelis-Menten constants for the kinetics of lactate transport across the BBB[8], we estimate for a 700 mg mouse brain (42 g body weight corresponds to 0.017 · 42 = 0.7 g brain weight for a male[34]), with 13 mM lactate in the blood, that approximately 150 · 0.7 · 75 / 60 = 130 nmol of lactate entered the brain during the 75 s experiment. This is consistent with the results obtained in NMRI mice using thermally polarized [3-^{13}C]lactate, where 516 nmol was estimated to be taken up by the brain within 5 min of a bolus injection (with a nearly 5 times higher lactate dose than in the present study)[35]. It can therefore be concluded that the method herein presented allows for the detection of a 130 · 0.28 $\cong$ 36 nmol increase in lactate uptake upon BBB opening by ultrasound, demonstrating the high sensitivity of hyperpolarized ^{13}C MRS for studying cerebral metabolism[36]. For future hyperpolarized ^{13}C MR imaging studies aiming at correlating the spatio-temporal evolution of the BBB opening with hyperpolarized ^{13}C MRI, it would be necessary to determine the spatio-temporal delivery of small molecules like ^{13}C-lactate as was done by Choi et al. with Gd-based contrast agents[37].

The dynamics of substrate transport across the BBB has previously been studied using hyperpolarized ^{13}C MR. Hurd *et al.* looked at the cerebral metabolism of [1-^{13}C]pyruvate[16] and ethyl [1-^{13}C]pyruvate[31]. Because the flux through MCTs is on the order of 4 times slower for pyruvate than for lactate[38], and the pyruvate plasma concentration largely exceeded K_M, it can be assumed that MCTs were saturated following the injection of hyperpolarized ^{13}C-

pyruvate. Consequently, the non-saturable flux could be considered as the main contribution to transport across the BBB, but the more hydrophobic precursor ethyl [1-^{13}C]pyruvate crossed the BBB much faster and resulted greater cerebral [1-^{13}C]lactate production. Although the results of this previous study are of high importance in the context of hyperpolarized ^{13}C MR because ^{13}C-pyruvate is by far the most widely used substrate and to date the only one used in humans, the authors highlighted that the contribution of extracellular ^{13}C lactate to the measured brain signal is difficult to estimate. For this reason, injecting hyperpolarized ^{13}C-lactate is an interesting alternative because the signal from its metabolic product pyruvate is purely intracellular. In fact, both pyruvate and lactate concentrations stay near physiological range following the injection of a bolus of hyperpolarized ^{13}C-lactate. The disadvantages of using hyperpolarized ^{13}C-lactate are that the fractional ^{13}C enrichment of brain lactate during the timeframe of the experiment is limited and the pool size of pyruvate is small, leading to lower sensitivity as compared to experiments done with hyperpolarized ^{13}C-pyruvate. It is however worth noting that the bicarbonate signal intensity is not substantially larger in experiments with hyperpolarized ^{13}C-pyruvate with similar ^{13}C polarization level using the same equipment [39]. It can also be mentioned here that the use of sodium lactate instead of lactic acid, which is considerable less stable, highly simplifies sample preparation and precludes the need for adding sodium hydroxide to reach physiological pH in the hyperpolarized ^{13}C-lactate solution prior to injection. Note finally that alternative hyperpolarized ^{13}C MR approaches based on flow[40], chemical shift[32, 41], and diffusion weighting measurements[42, 43] could be used to study the impact of ultrasound on transport dynamics of small molecules across the BBB.

CONCLUSION

We demonstrated that L-lactate transport across the BBB can be observed using hyperpolarized ^{13}C-lactate and we deduced that its transport is limited by the BBB. Our findings show that the equilibration between plasma and brain lactate is not instantaneous and that the BBB can restrict diffusion into the brain by nearly 30% at high plasma lactate

concentration. We also deduced that, although an exact determination of metabolite concentrations is challenging using hyperpolarized ^{13}C, variations of less than 40 nmol in cerebral lactate uptake can be detected. Our study shows that [1-^{13}C]lactate is a promising molecule for studying cerebral metabolism by hyperpolarized ^{13}C MRS since the injected substrate and its metabolites, namely pyruvate and bicarbonate, remain and can be detected at near physiological concentration. Hyperpolarized ^{13}C-lactate could also be potentially considered as a non-toxic alternative to lanthanide-based contrast agents for diagnosing brain tumors through the evaluation of BBB integrity by MRI.

MATERIALS AND METHODS

All experiments were conducted in accordance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines and were approved by the local regulatory body of the Canton Vaud, Switzerland (Service de la consommation et des affaires vétérinaires, Affaires vétérinaires, Canton de Vaud, Suisse). All animals were purchased from Charles River Laboratories (Châtillon-sur-Chalaronne, France).

Animal preparation and injection protocol

A total of 7 C57BL/6J mice (29.1 ± 1.5 g, 10 – 24 weeks, male) and 17 NMRI mice (41.7 ± 1.4 g, 10 - 24 weeks, male) were purchased for the magnetic resonance (MR) experiments. All mice were anesthetized using 1.5-2% isoflurane in air containing 50% oxygen (1 L/min). A 12-cm long home-made catheter was introduced into the femoral vein 60 min before each hyperpolarized ^{13}C MRS experiment. Animal physiology, e.g. body temperature and respiration rate, were monitored and maintained stable during the experiments by controlling the temperature of circulating warm water and the amount of isoflurane delivered to the animal. The animals were euthanized in accordance with local guidelines at the end of each hyperpolarized ^{13}C MRS experiment.

Preparation of hyperpolarized [1-^{13}C]lactate solution

A mixture of sodium [1-^{13}C]lactate solution (45-55 % (w/w) in H_2O, 99 atom % ^{13}C) and d_8-glycerol (1:1 w/w) doped with 50 mM 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl (TEMPOL) radical was warmed for 15 min in a water bath at 50°C[17-22]. All chemicals were purchased from Sigma-Aldrich (Buchs, Switzerland). A total volume of 0.2 mL of 2 ± 0.5 µL frozen beads were prepared in liquid nitrogen and placed into a polytetrafluoroethylene (PTFE) sample cup described in a previous publication[44]. The cup was then loaded into a 5 T home-built DNP polarizer and polarized at 1 ± 0.05 K and 140.18 GHz with a millimeter-wave power output of 50 mW[45]. The solid-state ^{13}C polarization build-up was monitored by applying a 5° radiofrequency (rf) pulse every 5 min.

***In vivo* hyperpolarized ^{13}C MRS**

All *in vivo* MR acquisitions were performed with a Direct Drive spectrometer (Agilent, Palo Alto, CA, USA) interfaced to an actively-shielded 9.4-T magnet with a 31-cm horizontal bore (Magnex Scientific, Abingdon, UK) using a home-built dual-channel surface coil consisting of 12-mm diameter quadrature ^{1}H loops and an 8-mm diameter ^{13}C surface coil. After having positioned the animal inside the magnet, series of axial, sagittal and coronal 2D images were acquired using a gradient echo sequence (TR = 50 ms, TE = 3 ms, field of view = 30 × 30 mm, matrix =128 × 128, flip angle = 30°) from which the volume of interest (VOI) was selected. The static magnetic field was shimmed in a 75 µL (3 × 5 × 5 mm^3) voxel to reduce the localized proton line width to 20 Hz using the FAST(EST)MAP protocol[46].

After 2 h of polarization, the frozen beads were rapidly dissolved in 6 mL of superheated D_2O and transferred into the separator/infusion pump[44, 47], which was located inside the magnet bore in 2 s. A ^{13}C polarization of 10 ± 2% was measured at the time of injection inside the pump as described in a previous publication[47]. The concentration of the infusate, measured afterwards in a high-resolution NMR system, was 110 ± 20 mM. Either 200 µL (for 7 C57BL/6 mice) or 300 µL (for 12 NMRI mice) of hyperpolarized [1-^{13}C]lactate solution was injected within 5 s, corresponding to a volume-to-weight ratio of about 7 µL/g. The blood

concentration at the end of the injection was estimated to be 13±3 mM. Starting 5 s after dissolution, 40 single-pulse ^{13}C acquisitions were sequentially recorded every 3 s using 30° adiabatic rf pulses (BIR4) with ^{1}H decoupling during acquisition (WALTZ-16). Localization was achieved by placing the surface coil on top of the mouse head. The adiabatic pulse offset and power were set such as to ensure a homogeneous 30° excitation of substrate and metabolite resonances within the entire VOI.

After inducing the BBB opening in NMRI mice, hyperpolarized [1-^{13}C]lactate was injected using the same protocol as the one used without ultrasound application.

Quantitative real-time PCR analysis and blood lactate measurement

A set of 7 C57BL/6J mice (28.4 ± 0.5 g, 10 weeks, male) and 7 NMRI mice (42.4 ± 1.0 g, 10 weeks, male) were dedicated to PCR analyses. Quantitative real-time PCR was performed to measure the expression levels of *Ldha*, *Ldhb*, *Slc16a1* (MCT1), *Slc16a7* (MCT2), and *Slc16a3* (MCT4) transcripts associated with the metabolism of [1-^{13}C]lactate for both C57BL/6 mice and NMRI mice. Plasma lactate was measured with the lactate oxidase method using a multi-assay analyzer (GW7 Micro-Stat, Analox Instruments, London, UK). For the QPCR measure, mice were rapidly killed with a guillotine and brains extracted. 40 mg of brain cortex was directly mixed in lysis solution. RNA was extracted using the Maxwell 16 LEV simplyRNA Cells kit (Promega, ref AS1270) following manufacturer instructions. Reverse transcriptions were performed with the High Capacity RNA-to-cDNA Kit (Life Technologies) according to the manufacturer's instructions. Each reaction was done in 20 µl with 400 ng of mRNA. Quantitative determination of the targeted mRNA sequences were performed with the fast real time PCR system Applied Biosystem 7900HT (Applied Biosystens, Rotkreuz, Switzerland). The following primers (5' to 3') were used: mActinBF031 GCT TCT TTG CAG CTC CTT CGT; mActinbRE94 ATA TCG TCA TCC ATG GCG AAC; mLDHAFo427 TTG TCT CCA GCA AAG ACT ACT GTG T; mLDHARe536 TTT CGC TGG ACC AGG TTG AG; mLDHBFo312 GCA GCA CGG GAG CTT GTT; mLDHBRe389 CAA TCT TAG AGT TGG CTG TCA CAG A; mMCT1Fo1361 AAT GCT GCC CTG TCC TCC TA; mMCT1Re1441 CCC AGT ACG TGT

ATT TGT AGT CTC CAT; mMCT2Fo755 CAG CAA CAG CGT GAT AGA GCT T; mMCT2Re830 TGG TTG CAG GTT GAA TGC TAA T; mMCT4Fo320 TCT GCA GAA GCA TTA TCC AGA TCT A; mMCT4Re407 ATG ATG AGG GAA GGC TGG AA. Gene expression data were analyzed using an Excel macro from Frontiers in Genetics (RT-PCR analysis-macro version 1.1). The average quantities were normalized to a normalization factor obtained by calculating the geometric mean of the most stable reference gene[(48)]. For the normalization factor, we tested the β-actin, cyclophilin, hypoxanthine guanine phosphoribosyl transferase and TATA box binding protein genes and chose β-actin as it was the most stable gene in our study.

Protocol for BBB opening

The BBB was opened using a previously described method[(49)]. In brief, a circular single-element ultrasound transducer (model A306S-SU, Olympus NDT) with a diameter of 13 mm and a center frequency of 2.25 MHz was used in this study. Using a stereotaxic instrument, the transducer was positioned at its natural focal distance (58 mm) in a column of degassed water (contained by a thin plastic film) placed directly over the mouse brain. The transducer was driven by a 47 dB power amplifier (model RF0510-200; RFPA, France), which was fed by a periodic pulse sequence from a signal generator (model 33220A, Agilent, Santa Clara, CA). The pulse sequence consisted of bursts of 2.15 MHz sinusoidal pulses with 50,000 cycles per burst and a burst period of 64 ms. The pulse amplitude was calibrated to generate negative acoustic pressure peaks of 0.5 MPa at the center of the natural focus of transducer. Power output of the ultrasonic transducer was calibrated in a water bath using a needle hydrophone (1-mm diameter needle hydrophone probe containing a 28-μm thick gold electroded polyvinylidene fluoride film, Precision Acoustics Ltd, UK).

Prior to the application of ultrasonic excitation, hair was removed from the mouse scalp using an electric trimmer. Ultrasound gel was placed on the scalp and the water column was lowered onto the head. A 100μL bolus of sulfur hexafluoride microbubbles with phospholipid

shell (SonoVue, Bracco Imaging, Milan, Italy) was administered through the femoral vein within 5 s, about 1 min before ultrasound irradiation. Following microbubble injection, the ultrasound pulse sequence was initiated and maintained for 10 min. Note that to insure an effective opening of the BBB, we applied a substantially longer pulse (10 min instead of 3 min) than in the original protocol published by Howles et al.[(49)]. We did however not specifically quantify skull attenuation in this study but it was previously demonstrated that the opening of the BBB in mice can be induced with ultrasound without craniotomy by compensating for the about 20% attenuation in pressure amplitude[(50)].

The efficacy of the protocol to open the BBB was evaluated via T_1-weighted MRI scans using either a gadolinium-based contrast agent, Gadoteridol (ProHance, Bracco Imaging, Milan, Italy), injected intraperitoneally 20 min before imaging at a concentration of 2 mmol/kg, or a 100 mM $MnCl_2$ solution (Sigma-Aldrich, Buchs, Switzerland) injected intraperitoneally 30 min before imaging at a concentration of 0.4 mmol/kg[(51)]. We used a three-dimensional spoiled gradient-recalled (SPGR) MRI sequence with the following parameters [(49)]: pulse repetition (TR) = 25 ms; echo time = 6 ms; flip angle = 30°; field of view = 20 × 20 × 16; matrix = 256 × 256 × 32; number of averages = 4.

^{13}C MRS data analysis

A non-linear least-squares quantification algorithm, AMARES, as implemented in the jMRUI software package[(52)], was used to fit the ^{13}C MRS data. The spectra were corrected for phase and DC offset. Soft constraints were imposed to peak frequencies (171.0–171.2 ppm for pyruvate, 161.0-161.4 ppm for bicarbonate and 183.2–183.6 ppm for lactate) and line widths (FWHM = 10–30 Hz) and the relative phases were fixed to zero. The peak areas of [1-^{13}C]lactate, [1-^{13}C]pyruvate and [^{13}C]bicarbonate were quantified, averaged over 25 acquisitions (sum of the 2nd to 26th spectra, corresponding to a total acquisition time of 75 s), and used to compute the pyruvate-to-lactate and bicarbonate-to-lactate ratios. Changes in relative metabolite ratios were used as a measure of kinetic rate changes, because it was demonstrated before that that the kinetic rates obtained from fitting the evolution of the signals

of hyperpolarized substrates and its metabolic products are directly proportional to the ratios of the summed spectral signals[53]. The time range was chosen such as to include only the spectra with a lactate signal-to-noise ratio (SNR) larger than 2.

Statistics

Statistical analyses were performed using the OriginPro 9.0G software. P values were computed using unpaired or paired Student's *t* test, where appropriate. For the statistical analysis of multiple groups, one-way ANOVA was used followed by Tukey's test. A p-value of 0.05 was considered significant. All data are presented as mean ± standard error of the mean (SEM) unless otherwise stated.

ASSOCIATED CONTENT

Supporting information

Supplementary figures displaying two sets of T_1-weighted ^{1}H MRI images demonstrating that the BBB was open after the application of ultrasound on the mouse head as described in the Materials and Methods section.

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Author Contributions

YT, MM, and AC designed the study. YT and TC, JAMB performed the *in vivo* experiments. YT and HAIY analyzed the data. JAMB and BL made critical contribution to the analysis of the kinetics data. SL performed and analyzed the QPCR measurements. AC wrote the manuscript. All authors discussed the results and commented on the manuscript.

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Conflict of Interest

Arnaud Comment is currently employed by General Electric Medical Systems Inc.

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Legends to Figures

Figure 1. Representative dynamic ^{13}C MRS spectra measured in a C57BL/6 mouse (left) and a NMRI mouse (right) head following the injection of hyperpolarized [1-^{13}C]lactate. Along with the substrate resonance at 183 ppm, the three expected metabolites were detected: [1-^{13}C]pyruvate at 171 ppm, [^{13}C]bicarbonate at 161 ppm, and [1-^{13}C]alanine at 176 ppm (overlapping with an impurity peak). The delay between each acquisition was set to 3 s, starting 2 s after the beginning of the injection. A sum of the 2nd to 26th spectra is shown on top.

Figure 2. (A) Pyruvate-to-lactate ratio deduced from the hyperpolarized ^{13}C MRS experiments (sum of the 2nd to 26th spectra) performed in two different mice strains (C57BL/6 and NMRI). The ratio is significantly different for the two groups ($p < 0.05$). (B) Bicarbonate-to-lactate ratio deduced from the same experiments and for the two groups. The difference was not significant. Error bars represent the mean ± S.E.M.

Figure 3. QPCR data for C57BL/6 (n=7) and NMRI mice (n=7). Only the *Ldha* expression level was significantly different between C57BL/6 and NMRI mice brain ($p = 0.025$).

Figure 4. Schematic representation of ^{13}C label distribution, across the detected metabolites in the mouse brain exposed to ultrasound (US; bottom), and not exposed to US (top), 75 s after the intravenous injection of [1-^{13}C]lactate. Each square represents a metabolic pool, with the grey part corresponding to the of ^{13}C labelled fraction. The grey arrows represent the ^{13}C-label flux between the pools. Note that the relative size of the squares or the grey area within each pool are not scaled to the real pool size ratios or proportion of ^{13}C label.

Figure 5. (A) Pyruvate-to-lactate ratio deduced from the hyperpolarized ^{13}C MRS experiments (sum of the 2nd to 26th spectra) performed in NMRI mice with or without ultrasound irradiation. The ratio is significantly different for the two groups ($p < 0.05$). (B) Bicarbonate-to-lactate ratio deduced from the same experiments and for the two groups. The differences were not significant. Error bars represent the mean ± S.E.M.