**ORIGINAL ARTICLE**

**Glycolysis and the pentose phosphate pathway after human traumatic brain injury: microdialysis studies using 1,2-13C2 glucose**

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Increased ‘anaerobic’ glucose metabolism is observed after traumatic brain injury (TBI) attributed to increased glycolysis. An alternative route is the pentose phosphate pathway (PPP), which generates putatively protective and reparative molecules. To compare pathways we employed microdialysis to perfuse 1,2-13C2 glucose into the brains of 15 TBI patients and macroscopically normal brain in six patients undergoing surgery for benign tumors, and to simultaneously collect products for nuclear magnetic resonance (NMR) analysis. 13C enrichment for glycolytic 2,3-13C2 lactate was the median 5.4% (interquartile range (IQR) 4.6–7.5%) in TBI brain and 4.2% (2.4–4.4%) in ‘normal’ brain (P < 0.01). The ratio of PPP-derived 3-13C lactate to glycolytic 2,3-13C2 lactate was median 4.9% (3.6–8.2%) in TBI brain and 6.7% (6.3–8.9%) in ‘normal’ brain. An inverse relationship was seen for PPP-glycolytic lactate ratio versus PbtO2 (r = 0.5, P = 0.04) in TBI brain. Thus, glycolytic lactate production was significantly greater in TBI than ‘normal’ brain. Several TBI patients exhibited PPP–lactate elevation above the ‘normal’ range. There was proportionally greater PPP-derived lactate production with decreasing PbtO2. The study raises questions about the roles of the PPP and glycolysis after TBI, and whether they can be manipulated to achieve a better outcome. This study is the first direct comparison of glycolysis and PPP in human brain.

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**INTRODUCTION**

An increase in the proportion of glucose undergoing ‘anaerobic’ (non-oxygen consuming) metabolism is observed after traumatic brain injury (TBI).¹–³ This has been postulated to provide the energy in the form of adenosine triphosphate (ATP), produced via glycolysis, needed to restore ionic and neurochemical gradients, which become disturbed after TBI.⁴,⁵ Uprogulation of the pentose phosphate pathway (PPP) has also been suggested as a possible contributor to increased anaerobic glucose metabolism after TBI, based on rat models, and indirect (arteriovenous-jugular difference) studies in human patients.⁶–⁸

Glycolysis consists of the Embden–Meyerhof pathway from glucose to pyruvate, which does not use oxygen, and generates 2 moles of ATP per mole of glucose. Pyruvate can then be metabolized via the mitochondrial tricarboxylic acid cycle (TCA) cycle coupled to mitochondrial electron transport chains, which utilize oxygen in oxidative phosphorylation. The theoretical overall yield is 36 moles of ATP per mole of glucose. Alternatively, pyruvate can be converted to lactate, which recycles NADH (reduced nicotinamide-adenine dinucleotide) to NAD⁺, enabling further glucose molecules to be processed by glycolysis.

The PPP, also termed the hexose monophosphate shunt, is a biosynthetic pathway that constitutes a complex detox bypassing some of the steps of glycolysis in the metabolism of glucose. The key enzyme for the PPP, glucose-6-phosphate dehydrogenase, which is responsible for the rate-limiting step, is present in most tissues and cell types, and is regarded as a ‘housekeeping’ enzyme.⁹,¹⁰ The PPP does not consume or produce ATP and does not require molecular oxygen. In the early ‘oxidative phase’ of the PPP, during which the first carbon of the glucose skeleton is lost as carbon dioxide, nicotinamide adenine dinucleotide phosphate (NADPH) is converted to NADPH. The latter is a reducing agent that participates in reductive biosynthetic reactions, such as lipid and steroid synthesis, and in the production of the reduced form of...
glutathione and thioredoxin. Glutathione and thioredoxin are cofactors for glutathione peroxidase enzymes and peroxiredoxins respectively, both of which scavenge hydroperoxides, thereby combating oxidative stress. Among the many intermediates of the later ‘non-oxidative’ phase of the PPP is ribose 5-phosphate, used for nucleic acid synthesis. Hence, the proportion of glucose metabolized via the PPP is greatest in tissues with high lipid- and steroid-synthetic roles (for example, liver and lactating mammary glands), also in cells with a high oxidative load (for example, red blood cells), and is thought to be one of the mechanisms supporting high cell proliferation rates in cancer.

Our aim was to evaluate glycolysis and the PPP as routes of glucose metabolism, directly in TBI patients’ brains. Our novel approach was to perfuse the brain using a microdialysis catheter with 1,2-13C2 glucose and measure the ensuing labeling patterns in lactate collected in the emerging microdialysates by analyzing them using high-resolution nuclear magnetic resonance (NMR) spectroscopy. For comparison, we also carried out the same technique in the ‘normal’ brain, and in muscle as a non-CNS tissue. The present study is the first direct comparison of glucose metabolism via glycolysis and PPP in human brain.

MATERIALS AND METHODS

Patients

The Cambridge Central Local Research Ethics Committee approved this study. Informed consent was obtained from all participants in the control ‘normal’ brain and muscle groups and assent from the relatives of those patients who had suffered a TBI. The study was carried out in conformance with the spirit and the letter of the Declaration of Helsinki.

TBI brain microdialysis patients. The TBI patients were defined as those experiencing cranial trauma with consistent computed tomography (CT) scan findings and requiring invasive intracranial monitoring as part of their clinical management. Patients were managed according to local TBI-management protocols, which include endotracheal intubation, ventilation, sedation, and muscular paralysis.

Control ‘normal’ brain microdialysis patients. Patients undergoing a craniotomy for the resection of a benign lesion whereby a microdialysis catheter could be safely placed into radiographically normal brain were considered for inclusion. Patients undergoing resections of acoustic neuromas, also in cells with a high oxidative load (for example, liver and lactating mammary glands), also in cells with a high oxidative load (for example, red blood cells), and is thought to be one of the mechanisms supporting high cell proliferation rates in cancer. The present study is the first direct comparison of glucose metabolism via glycolysis and PPP in human brain.

Microdialysis Technique

CMA 71 microdialysis catheters (membrane length 10 mm, nominal molecular weight cutoff 100 kDa) (M Dialysis AB, Stockholm, Sweden) were placed either via a triple lumen cranial access device (Technicam, Newman Abbot, UK) or during a craniotomy for a traumatic lesion, along with an intracranial pressure monitor (Codman, Raynham, MA, USA) and a Liquox intracranial pressure monitor (Microsensor UK). The control patients were awake with the spirit and the letter of the Declaration of Helsinki. Patients undergoing resections of acoustic neuromas, also in cells with a high oxidative load (for example, liver and lactating mammary glands), also in cells with a high oxidative load (for example, red blood cells), and is thought to be one of the mechanisms supporting high cell proliferation rates in cancer. The present study is the first direct comparison of glucose metabolism via glycolysis and PPP in human brain.

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identified on the $^{13}$C and $^1$H spectra were determined using combined Gaussian and Lorentzian line-shape fitting. Peak areas relative to the DSS internal standard were used with reference to calibration curves for each signal derived from standards (see above) of known concentrations, which showed linear relationships between peak areas (ratio to DSS internal standard) and concentrations. Fractional enrichment (%) is defined as $100 \times ([^{13}C]/([^{12}C]+[^{13}C]))$ where square brackets indicate concentrations of the relevant species. $[^{13}C]$ was determined from the $^{13}$C NMR spectra and $[^{12}C]$ from the $^1$H spectra, using the calibration method above. The natural abundance of $^{13}$C is 1.1% of all carbon atoms, and $^{13}$C results presented for lactate (see Results section) were expressed after subtracting this natural background from the $^{13}$C singlet signals. $^{13}$C doublet signals were not background-subtracted because the probability of two $^{13}$C atoms occurring next to each other naturally is 0.01%.

Interpretation of the NMR results was based on biosynthetic pathways summarized schematically in Figure 1, which shows the lactate-labeling patterns corresponding to glycolysis, which produces 2,3-$^{13}$C$_2$ lactate, and the PPP, which produces 3-$^{13}$C lactate.

**Figure 1.** Simplified schematic of steps in glycolysis and the pentose phosphate pathway (PPP) showing $^{13}$C labeling patterns resulting from 1,2-$^{13}$C$_2$ glucose substrate. Red fills indicate $^{13}$C atoms. Glc-6-P, glucose-6-phosphate; 6PGL, 6-phosphogluconolactone; F6P, fructose-6-phosphate; G3P, glyceraldehyde-3-phosphate; PYR, pyruvate. Figure originally published in Carpenter et al.© 2015 ISCBFM Journal of Cerebral Blood Flow & Metabolism (2015), 111–112 under a Creative Commons Attribution License.

**RESULTS**

Demography

Fifteen severe TBI patients (10 male and 5 female patients) aged 16–59 years (median 27 years) were studied using 1,2-$^{13}$C$_2$ glucose (4 mmol/L) perfusion via the microdialysis catheter, with simultaneous collection of microdialysates for NMR analysis. For comparison, macroscopically normal-appearing brain was studied using the same $^{13}$C-labeled microdialysis method in six patients (age range 59–73 years; three male and three female patients) undergoing surgery for benign brain tumors. For a non-CNS tissue comparison, thigh (quadriceps) muscle was similarly studied in four patients (age range 20–61 years; three male and one female patients) who underwent surgery for acoustic neuroma resection. A further seven patients (two ‘normal’ brain and five muscle) were studied by microdialysis but with plain unsupplemented perfusion fluid (without 1,2-$^{13}$C$_2$ glucose). The 15 TBI patients who received 1,2-$^{13}$C$_2$ glucose (see above) were also studied for a baseline period with plain unsupplemented perfusion fluid (without 1,2-$^{13}$C$_2$ glucose). Demographic details of all patients are presented in Supplementary Table 1.

Baseline Results Compared with 1,2-$^{13}$C$_2$ Glucose Perfusion Period

Microdialysate measurements (using the ISCUS analyzer) of lactate, pyruvate, glutamate, and glycerol are shown in Figure 2. These were acquired in the 15 TBI patients during periods when the microdialysis catheter was perfused with plain unsupplemented CNS perfusion fluid and also during the 24-hour period of perfusion with 1,2-$^{13}$C$_2$ glucose (4 mmol/L). In ‘normal’ brain, the ISCUS analysis was performed for two patients who received plain unsupplemented perfusion fluid and for six patients who received 24-hour perfusion with 1,2-$^{13}$C$_2$ glucose (4 mmol/L). In muscle, the ISCUS analysis was performed for five patients who received plain unsupplemented perfusion fluid, and for four patients who received 8-hour perfusion with 1,2-$^{13}$C$_2$ glucose (4 mmol/L). The 8-hour period for muscle was necessitated because of clinical practicalities of limb movement—microdialysis was limited to periods during the acoustic neuroma surgery.

Significant increases ($P < 0.05$) in microdialysate glucose concentration (measured on the ISCUS analyzer) between baseline (unsupplemented) and the 1,2-$^{13}$C$_2$ glucose perfusion period were seen for all three groups, as follows (medians): TBI brain (from 1.0 to 3.8 mmol/L), ‘normal’ brain (from 1.9 to 3.9 mmol/L), and muscle (from 2.8 to 5.3 mmol/L). The only other statistically significant, but not thought to be biologically significant, changes in ISCUS results...
between baseline and the 1,2-$^{13}$C$_2$ glucose perfusion period were for lactate/pyruvate ratio in TBI brain (from 22.2 to 24.8), for glycerol in TBI brain (from 52.0 to 69.6 μmol/L), and for glutamate in TBI brain (from 3.4 to 4.4 μmol/L) and muscle (from 7.9 to 29.7 μmol/L). Concentrations of 1,2-$^{13}$C$_2$ glucose (median and IQR) measured with $^{13}$C NMR in the microdialysates from TBI brain (24-hour perfusion period), normal brain (24-hour perfusion period), and muscle (8-hour perfusion period) were, respectively, 2.14 (1.45–2.43), 0.93 (0.83–1.83), and 0.95 (0.59–1.25) mmol/L, with TBI significantly different from normal brain ($P = 0.008$) or muscle ($P = 0.016$).

Throughout the 24-hour period, during which the cerebral microdialysis catheter was perfused with 1,2-$^{13}$C$_2$ glucose, the median serum glucose concentration measured in TBI patients was 7.3 mmol/L and all values were within the range 4–12 mmol/L except for one time point in patient 11 in which glucose was 1 mmol/L (Supplementary Figure 1). The median serum lactate concentration was 1.1 mmol/L (min 0.7, max 3.0 mmol/L; Supplementary Figure 1).

NMR Results for Lactate Production by Glycolysis and PPP, and Relationship with Brain Tissue Oxygen

Illustrative examples of $^{13}$C NMR spectra of the microdialysates are shown in Figure 3. As a result of 1,2-$^{13}$C$_2$ glucose perfusion, TBI brain and 'normal' brain microdialysates included a clearly visible doublet (red stars in expansion of $^{13}$C spectra) for the lactate C3 methyl group, and likewise a doublet for lactate C2, indicating glycolysis-derived 2,3-$^{13}$C$_2$ lactate, and a smaller C3 singlet (green stars in expansion of $^{13}$C spectra) representing PPP-derived 3-$^{13}$C lactate plus endogenous lactate. Qualitatively similar-appearing
spectra were seen for muscle microdialysates resulting from $^{1,2}$-$^{13}$C$_2$ glucose perfusion. Unlabeled TBI microdialysates (with plain (unsupplemented) perfusion fluid) showed singlets for endogenous lactate C3 and C2 (Figure 3).

Natural abundance of $^{13}$C was assumed to be 1.1%, and $^{13}$C fractional enrichment values for lactate were expressed after subtracting this naturally occurring $^{13}$C background from the $^{13}$C singlet signals. In TBI brain microdialysates, $^{13}$C fractional enrichment (%) in 2,3-$^{13}$C$_2$ lactate, indicative of glycolysis, was median 5.4% (IQR 4.6–7.5%) measured using the C3 doublet and this was significantly greater than in ‘normal’ brain (Figure 4). Fractional enrichment was based on quantification of the lactate C3 doublet signal ($^{13}$C spectrum) to measure $^{13}$C at the C3 position and the lactate methyl protons (attached to C3) triplet signal ($^1$H spectrum) to quantify $^{12}$C at the C3 position. Similar $^{13}$C fractional enrichment to that of lactate C3 doublet was obtained using the lactate C2 doublet, as expected (Figure 4). In muscle, $^{13}$C fractional enrichment in 2,3-$^{13}$C$_2$ lactate was lower than in TBI and ‘normal’ brain (Figure 4).

On the basis of the lactate C3 methyl group, $^{13}$C fractional enrichment (%) in 3-$^{13}$C lactate indicative of the PPP in TBI brain microdialysates was median 0.24% (IQR 0.17–0.61%; Figure 4), which was thus much less than the $^{13}$C fractional enrichment in glycolytic 2,3-$^{13}$C$_2$ lactate (above). The ratio in TBI brain microdialysates of PPP-derived 3-$^{13}$C lactate to glycolytic 2,3-$^{13}$C$_2$ lactate was median 4.9% (IQR 3.6–8.2%) (Figure 4). Four of the fifteen TBI patients exhibited ratios of PPP-derived to glycolytic lactate (11.4, 11.5, 11.9, and 14.0%) that were greater than the range observed in the present study in normal brain (min 5.9, max 10.4%).

In muscle, microdialysis perfusion with $^{1,2}$-$^{13}$C$_2$ glucose (4 mmol/L, for 8 hours) resulted in lactate C3 doublet and C2 doublet, indicating glycolytic 2,3-$^{13}$C$_2$ lactate, with $^{13}$C enrichment at the C3 median 1.2% (range 1.0–1.4%). However, there was no $^{13}$C enrichment above background natural abundance for lactate C3 singlet; therefore, PPP production of lactate was not detected in muscle.

The concentration of 2,3-$^{13}$C$_2$ lactate (indicative of glycolysis) showed a nonsignificant inverse trend ($r = -0.4$, $P = 0.1$) with brain tissue oxygen concentrations (PbtO$_2$ expressed in mm Hg) measured in the vicinity of the microdialysis catheter in TBI brain. A significant inverse correlation ($r = -0.5$, $P = 0.04$) was seen for the ratio of PPP-lactate to glycolytic lactate versus PbtO$_2$ (Figure 5). Local tissue oxygen concentration was not measured in normal brain or muscle.

Five TBI patients had a second 24-hour period of $^{1,2}$-$^{13}$C$_2$ glucose (4 mmol/L) microdialysis perfusion. There were no significant differences in the production of glycolytic lactate or PPP-derived lactate between these two time periods.

**DISCUSSION**

This study has shown that $^{1,2}$-$^{13}$C$_2$ glucose infusion via the microdialysis catheter results in $^{13}$C-labeling in lactate (Figure 3) in the emerging microdialysates, enabling us to compare glycolysis and PPP as routes by which lactate is derived. This is the first time this comparison has been performed directly in human brain.
Glucose Metabolism via Glycolysis and the Pentose Phosphate Pathway

Clear evidence for glycolysis being the main route of lactate production from glucose, as expected, was seen as diagnostic doublet signals for both C3 and C2 of lactate (Figure 3) indicating 2,3-13C2 lactate, a hallmark of the pathway (Figure 1). These doublets were seen for all patients, in TBI brain, 'normal' brain and muscle. This is in accord with the recognized glycolysis pathway consisting of the Embden–Meyerhof pathway from glucose to pyruvate, followed by conversion to lactate by the action of lactate dehydrogenase (Figure 1). This 13C enrichment was significantly higher in the TBI brain, suggesting greater glycolytic activity than in 'normal' brain and muscle. The doublet signals provide a distinctive signature that in effect avoids the issue of natural abundance 13C background (1% of all carbon atoms) because the probability of two endogenous 13C atoms occurring next to each other naturally is 0.001%.

The PPP loses the first carbon of 1,2-13C2 glucose as carbon dioxide and thus gives rise to 3-13C lactate with a singlet signal for C3 (Figure 1). Whereas 13C singlet signals for lactate C3 were clearly visible in all patients' microdialysates (Figure 3), these were smaller than the C3 doublet. Furthermore, it emerged that much of the C3 singlet signal intensity (peak area) was because of endogenous natural abundance 13C. Small 13C fractional enrichments for lactate C3 singlet above this background were seen in microdialysates from TBI brain and 'normal' brain, indicating PPP-derived lactate, but were negligible in muscle. Although there was no statistically significant difference in 13C fractional enrichment for lactate C3 between 'normal' brain and TBI brain, the higher upper range of the latter suggests that in certain TBI individuals lactate production via the PPP is elevated above that in 'normal' brain. In the four TBI patients with the most elevated PPP lactate, the ratio (%) of PPP lactate to glycolytic lactate was 11.4–14.0%.

Our finding of increased PPP activity in some patients as a result of brain injury is supported by experimental studies. In rat models relevant to TBI, Bartnik et al6,7 infused 1,2-13C2 glucose intravenously after fluid percussion injury and cortical contusion injury, and performed NMR analysis of brain tissue extracts.6,7 They found an increase in the proportion of 3-13C lactate (indicative of PPP) relative to 2,3-13C2 lactate (indicative of glycolysis) in brain-injured rats compared with control rats, although with glycolysis remaining dominant. In humans, Dusick et al8 infused 1,2-13C2 glucose intravenously, with 13C labeling in lactate assessed by...
arteriovenous-jugular difference. This study in TBI patients and controls, although it did not sample the brain directly and could only yield information on brain lactate labeling during periods when the brain was a net exporter of lactate into the vasculature, led to similar conclusions to the rat studies above.

Significance of the PPP

The PPP is a complex biosynthetic network generating many other species besides lactate (Figure 1) and the balance between these species can potentially shift depending on the local biology and pathology. Some PPP-derived species are potentially reparative, for example, ribose (for nucleic acid synthesis) and NADPH (to provide reducing equivalents for fatty acid synthesis), and neuroprotective for example, NADPH, used for producing the reduced form of glutathione and thioredoxin, which are cofactors for glutathione peroxidases and peroxidins, respectively, enzymes that combat oxidative stress. Accordingly, Herrero-Mendez et al. demonstrated that if one of the key regulatory enzymes of glycolysis, phosphofructokinase, is activated in neurons so that more glucose is funneled through glycolysis at the expense of the PPP, apoptosis soon ensues because of oxidative stress. The PPP, which does not involve molecular oxygen and does not generate ATP, can thus be regarded as sacrificing some of the cell's supply of glucose molecules, which might otherwise have been used for ATP synthesis via glycolysis and the TCA cycle, for the sake of generating more reducing power (NADPH) and the ability to protect, repair, or build cells.

NADPH is involved in many other biochemical reactions, for example, NADPH is a cofactor for NADPH oxidase and nitric oxide synthase and, if these become dysregulated, oxidative stress may ensue, with adverse consequences. Zuurbier et al. reported that inhibition of the so-called ‘oxidative’ phase of the PPP (the early steps of the PPP responsible for NADPH production), by means of administering 6-aminonicotinamide, was cardioprotective in an ischemia-reperfusion rat heart model. Interestingly, when Tyson et al. administered 6-aminonicotinamide to inhibit the PPP in rats in a study of brain using intravenous 2-13C glucose as the substrate, they found that despite achieving PPP inhibition, evidenced by a build-up of 6-phosphogluconate, there appeared to be a feedback effect of PPP inhibition on glycolysis, such that both pathways decreased in a constant ratio.

Role of Brain Tissue Oxygen Concentration in Brain Metabolism

In the literature, ‘hyperglycolysis’ and the elevation of lactate production in the injured brain have been attributed to hypoxia and/or mitochondrial dysfunction, although the exact nature of the latter remains unclear. In a combined positron emission tomography (oxygen-15 and fluoride-18) and microdialysis study, Vespa et al. reported that ‘metabolic crisis’ (defined as lactate/pyruvate ratio > 40) occurred in 7 out of 19 TBI patients studied, although only one case showed regional ischemia judged by positron emission tomography. In a microdialysis study of 24 TBI patients in conjunction with PbtO2 measurement and perfusion CT, Sala et al. concluded that the majority of lactate production was ‘glycolytic’ (rather than hypoxic), albeit without evidence from carbon labeling.

In the present study, we measured PbtO2 alongside the microdialysis catheter in the TBI patients and found that there was a nonsignificant trend towards greater glycolytically generated 2,3-13C2 lactate with decreasing PbtO2. It is important to note that there were only three cases that could be described as hypoxic (defined as PbtO2 < 20 mm Hg), and the remaining 14 data points ranged from 22 to 43 mm Hg. Interestingly, the ratio of PPP-derived lactate to glycolytic lactate showed a significant inverse correlation with PbtO2 (r = -0.5, P = 0.04; Figure 5). Thus, although PPP-derived lactate was always much less than glycolytic lactate, the balance between the two appeared to shift towards the PPP with lower PbtO2. Similar to glycolysis, the PPP does not utilize oxygen. Studies in adult rats and in brain slices have also suggested that hypoxia increases the PPP. In contrast, Brekke et al. found evidence for a decrease in PPP in neonatal rats after hypoxic–ischemic injury, which the authors discussed as paradoxical. Moreover, they postulated that the apparent inability to upregulate the PPP in this situation might render the neonatal rats vulnerable. An analogous situation might conceivably exist in human TBI, whereby low ability to upregulate the PPP might result...
in increased risk of damage in those individuals’ brain tissue (either globally or locally). Therapeutic upregulation of the PPP in this context might be beneficial. Apart from hypoxia (mentioned above), a few other upregulators of the PPP have been identified. Insulin has been shown to increase the expression of glucose-6-phosphate dehydrogenase mRNA in primary rat hepatocytes.24 Dehydroascorbate, the oxidized form of vitamin C, has been shown to stimulate the activity of several PPP enzymes, increase glutathione levels, and inhibit hydrogen peroxide-induced changes in mitochondrial transmembrane potential in Jurkat cells (a cancer cell line).25 Although these two latter studies were not performed in the brain, they nevertheless illustrate the principle that the PPP can be deliberately upregulated with measurable biochemical and biological effects.

Significance of Lactate in the Brain

Lactate has been conventionally regarded as a waste product of glucose metabolism, although a more recent idea is that it can act as a brain fuel—neurons take up lactate (produced from glucose by glia), metabolize lactate to pyruvate, which is transported into the mitochondria and converted to acetyl CoA, which enters the tricarboxylic acid (TCA) cycle. This has become known as the astrocyte–neuron lactate shuttle hypothesis.26 Animal studies have provided evidence for brain utilization of intravenously administered 3,13C2 lactate via the TCA cycle.27 In the human brain, direct evidence for utilization of lactate was first obtained in a cerebral microdialysis study in TBI patients.28 In the latter study, administration of 3,13C2 lactate via the microdialysis catheter, and simultaneous collection of the emerging microdialysates, with 13C NMR analysis, revealed 13C labeling in glutamate consistent with lactate utilization via the TCA cycle.28 Recently, an intravenous lactate supplementation study in TBI patients revealed evidence for a beneficial effect judged by surrogate end points.29 Studies of brain microdialysates (without supplementation) in 223 patients show a statistical association between high extracellular lactate and unfavorable outcome.30 Taken together, available evidence suggests that where neurons are too damaged to utilize the lactate produced from glucose by astrocytes, that is, uncoupling of neuronal and glial metabolism, high extracellular levels of lactate would accumulate, explaining one potential mechanism behind the association between high extracellular lactate and poor outcome.

Fate of Lactate

Regarding the possible fate of labeled lactate produced from 1,2,13C2 glucose, a likely scenario is that some of the labeled lactate is processed via the TCA cycle, and some of it exported into the bloodstream. Because we were microdosing the brain with 1,2,13C2 glucose locally via the microdialysis catheter, it seemed highly unlikely that any of the ensuing doubly labeled lactate would have been detectable in the bloodstream, given the inevitable dilution both with endogenous unlabeled lactate and with the volume of blood; therefore, 13C NMR analyses of blood were not performed previously.

Previously, using 3,13C lactate as the substrate, we detected singly labeled glutamine (and/or glutamate) in TBI brain microdialysates, indicating the utilization of lactate via the TCA cycle.28 In the present study, if 2,3,13C lactate (produced from the substrate 1,2,13C2 glucose) entered the TCA cycle in brain cells, this would in theory have resulted in doublets in the 13C NMR spectra, because of 4,5,13C glutamine and 2,3,13C glutamine (and/or glutamate in each case) on the first turn of the cycle, and, on the second turn, doublets because of 2,13C glutamine and a singlet because of 3,13C glutamine.28,31 Experimental models of brain injury in rodents, using 1,2,13C2 glucose as the substrate, with analysis of brain tissue extracts representing total intracellular and extracellular molecules, confirm double labeling in glutamate and glutamine.6,7,31 In our present study, small singlet signals, but not doublets, corresponding to glutamine were seen in the 13C NMR spectra of some of the microdialysates from ‘normal’ brain (4/6 patients) and TBI (2/15 patients), verified by 2D NMR methods, including 1H,13C heteronuclear single quantum correlation (Supplementary Figure 2). In theory, pyruvate cycling,32 which breaks the 13C–13C bond might explain the glutamine singlets. In addition, in theory, PPP-derived 3,13C lactate might enter the TCA cycle forming singly labeled glutamate (and/or glutamate), although accompanied by doubly labeled glutamine from glycolytic 2,3,13C2 lactate, as found in vitro by Brekke et al.33 However, in the present study, quantification of the small glutamate singlets revealed no significant 13C enrichment above natural abundance.

Microdialysis only samples the extracellular compartment and therefore cannot measure the intracellular distribution of 13C. Even so, our 13C-labeled microdialysis method provides a useful means for measuring ‘early’ glucose metabolism (glycolysis and PPP) as sufficient labeled lactate is exported into the extracellular compartment to allow detection. However, in the subsequent metabolism of lactate, the downstream dilution of 13C label with endogenous unlabeled intermediates probably explains why we did not observe significant 13C enrichment of extracellular metabolites derived from the TCA cycle. In our previous study, when 3,13C lactate (4 mmol/L) or 2,13C acetate (4 mmol/L) was perfused via the microdialysis catheter in the TBI brain, labeling in glutamate consistent with TCA cycle was seen in the emerging microdialysates.28 However, in the same study, perfusion with 1,13C glucose (2 mmol/L) resulted in negligible labeling in glutamine, consistent with the present study.

Strengths and Limitations

This 13C-labeled microdialysis method, performed in parallel with local brain tissue oxygen measurement, provides a method of measuring glycolytic conversion of glucose into lactate and at the same time distinguishing hypoxic from non-hypoxic glycolysis, as well as evaluating the contribution of the PPP. Even though our evidence indicates a minor PPP-derived lactate (3,13C lactate), it must be remembered that the PPP is a complex network of biosynthetic reactions (Figure 1) and it is conceivable that if heavy recycling is operating within the PPP, less lactate might emerge, although with elevated production of NADPH and intermediates utilized in neuroprotection and repair of cells.

The strategy of double labeling (using 1,2,13C2 glucose as the substrate) provides a characteristic signature that appears in glycolytic lactate (2,3,13C2 lactate) showing that the 13C–13C covalent bond stays intact. Moreover, the doublet signal is essentially free from endogenous lactate because the probability of two 13C atoms being adjacent to each other naturally is 0.01%. Similar to all cerebral microdialyses, the 13C-labeled microdialysis method is invasive, so it is only suitable for severe TBI patients or those requiring brain surgery (for example, for tumors), and it is a focal technique.

This 13C-labeling method has the potential to be a useful adjunct to existing methodologies of positron emission tomography and unlabeled microdialysis in monitoring and studying TBI patients. The 13C-labeled microdialysis method does not involve disruption of the patient’s standard medical care on the neurocritical care unit and does not involve radioactivity or moving the patient to a scanner. The 1,2,13C2 glucose microdialysis method is reasonably inexpensive and convenient to perform because 1 or 2 g of the labeled material are enough to formulate enough perfusion fluid for multiple patients, and the formulation can be stored in individual sterile ready-to-use sealed glass vials in a refrigerator. The concentration (4 mmol/L) of 1,2,13C2 glucose administered via microdialysis locally into the brain corresponds to the upper end of the ‘normal’ range found in...
brain extracellular fluid.\textsuperscript{13} Whereas conventional microdialysis performed with unsupplemented perfusion fluid allows us to measure endogenous extracellular lactate, the unsupplemented method cannot inherently distinguish between ‘old’ lactate and recently synthesized lactate. An advantage of the \textsuperscript{1,2-}$^{13}$C$_2$ glucose microdialysis method is that it enables the measurement of labeled lactate production within a known timeframe—at present this is 24 hours because of current practical NMR considerations (see below).

A current limitation to our \textsuperscript{13}C-labeled microdialysis method is the amount of material necessary for performing NMR analysis. We have combined brain microdialysates for a 24-hour period for each patient to facilitate \textsuperscript{13}C NMR spectroscopy within a convenient acquisition time on the spectrometer. Our Bruker Avance 500 MHz spectrometer is equipped with a cryoprobe, as used in the present study and in our previous study,\textsuperscript{18} which is more sensitive than non-cryo probe technology. Even so, the practical time frame we can analyze in terms of microdialysis perfusion.

microdialysis with \textsuperscript{1,2-}$^{13}$C$_2$ glucose as substrate may thus have combined brain microdialysates for a 24-hour period for optimizing perfusion and mitochondrial function. This is the first time that a comparison between glycolysis and the PPP has been made.\textsuperscript{19} 1,2-\textsuperscript{13}C$_2$ glucose microdialysis may have future potential for metabolic kinetic modeling of local glucose consumption rates and glycolytic- and PPP-production rates of lactate. Commercially available NMR microcryoprobes, which take smaller volumes, may be useful in future. Analysis with mass spectrometry might also be useful in future, enabling smaller volumes to be analyzed, although at the expense of at least some of the detailed information on precise intramolecular position of label.

CONCLUSION

Here we have shown that \textsuperscript{13}C-labeled microdialysis can be used to interrogate glucose metabolism via glycolysis and the PPP. The major pathway, glycolytic lactate production, was significantly greater in the TBI brain than in the normal brain. The minor pathway, PPP-derived lactate production, was statistically not significantly different in the TBI brain than in normal brain. However, several of the TBI individuals showed PPP-derived lactate elevation above the range observed in the normal brain. There was a shift in glucose metabolism from glycolysis to PPP with decreasing brain tissue oxygen concentrations. The findings raise interesting questions about the roles of the PPP and glycolysis after TBI, and whether they can be manipulated to enhance the potentially reparable and antioxidant role of the PPP and achieve a better outcome for the patient. The \textsuperscript{13}C methodology developed here provides a means of distinguishing recently synthesized lactate and its biosynthetic origin, and at the same time measuring local oxygen tension alongside. \textsuperscript{12}C-labeled microdialysis with 1,2-\textsuperscript{13}C$_2$ glucose as substrate may thus find a methodological role in studies of hyperoxia or strategies to optimize perfusion and mitochondrial function. This is the first time that a comparison between glycolysis and the PPP has been carried out directly in the human brain.

DISCLOSURE/CONFLICT OF INTEREST

J.D.P. and P.J.H. are the Directors of Technicam.

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