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Substrate Competition Studies Demonstrate Oxidative Metabolism of Glucose, Glutamate, Glutamine, Lactate and 3-Hydroxybutyrate in Cortical Astrocytes from Rat Brain

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Abstract

It is well established that astrocytes can utilize many substrates to support oxidative energy metabolism; however, use of energy substrates in the presence of other substrates, as would occur in vivo, has not been systematically evaluated. Substrate competition studies were used to determine changes in the rates of $^{14}\text{CO}_2$ production use since little is known about the interaction of energy substrates in astrocytes. The rates of $^{14}\text{CO}_2$ production from 1 mM D-[6- ^{14}C]glucose, L-[U- ^{14}C]glutamate, L-[U- ^{14}C]glutamine, D-3-hydroxy[3- ^{14}C]butyrate, L-[U- ^{14}C]lactate and L-[U- ^{14}C]malate by primary cultures of astrocytes from rat brain were determined to be 1.17 ± 0.19 , 85.30 ± 12.25 , 28.04 ± 2.84 , 13.55 ± 4.56 , 14.84 ± 2.40 and 5.20 ± 1.20 nmol/hr/mg protein (mean $\pm$ SEM), respectively. The rate of $^{14}\text{CO}_2$ production from glutamate oxidation was higher than that of the other substrates. Addition of unlabeled glutamate significantly decreased the rates of $^{14}\text{CO}_2$ production from all other substrates studied; however, glutamate oxidation was not altered by the addition of any of the other substrates. The rate of $^{14}\text{CO}_2$ production of glutamine was decreased by glutamate, but not altered by other substrates. The rate of $^{14}\text{CO}_2$ production from glucose was significantly decreased by the addition of unlabeled glutamate, glutamine or lactate, but not by 3-hydroxybutyrate or malate. Addition of unlabeled glucose did not significantly alter the $^{14}\text{CO}_2$ production from any other substrate. $^{14}\text{CO}_2$ production from lactate was decreased by the addition of unlabeled glutamine or glutamate and increased by addition of malate. The $^{14}\text{CO}_2$ production from malate was decreased by the addition of unlabeled glutamate or lactate, but was not altered by the other substrates. The substrate utilization for oxidative energy metabolism in astrocytes is very different than the profile previously reported for synaptic terminals. These studies demonstrate the potential use of multiple substrates including glucose, glutamate, glutamine, lactate and 3-hydroxybutyrate as energy substrates for astrocytes. The data also provide evidence of interactions of substrates and multiple compartments of TCA cycle activity in cultured astrocytes.

Keywords

astrocytes; energy metabolism; glucose; glutamate; lactate; glutamine; 3-hydroxybutyrate; malate; substrate competition; compartmentation

Introduction

Recent studies reporting that astrocyte metabolism accounts for ~30% of total oxygen consumption in brain *in vivo* demonstrate that considerable oxidative metabolism is taking place in astrocytic mitochondria [1–4]. Indeed, the rates of oxidative glucose metabolism in astrocytic mitochondria, which includes both energy metabolism via the pyruvate dehydrogenase complex and the pyruvate carboxylase pathway, have been shown to equal those in neuronal mitochondria [1, 5]. Although many groups have studied the metabolism of glucose and other substrates by cultured astrocytes from rodent brain over the past 2–3 decades [6–19] relatively little is known about how use of individual substrates by these cells changes when multiple energy substrates are present.

Astrocytes are important cells that play crucial roles in neurotransmission and overall homeostasis in brain. These cells have a flexible and perhaps opportunistic metabolism that can utilize, and synthesize and release many metabolites. For example, astrocytes can use glutamine for energy and also produce glutamine from neurotransmitter glutamate removed from the synaptic cleft, as well as from precursors such as glucose and fatty acids [13, 20–24]. The relative amount of synthesis versus utilization of a number of key substrates including glutamine [20, 22, 25, 26], ketone bodies [27–29], and lactate [11, 20, 30, 31] is greatly influenced by the concentration of other compounds within the cells and in the extracellular milieu. When considering the question of metabolic fuel for astrocytes the potential importance of substrates generated within the brain for supporting oxidative metabolism in these cells has been underestimated and often ignored. This may be due in part to the fact that different groups have focused on specific substrates, but comparison of substrate use and competition of substrates has not been studied extensively under the same conditions. Astrocytic metabolism is not homogeneous and can vary with brain region [32, 33]. Heterogeneity in the distribution of transporters and metabolic enzymes in astrocytes has been reported [1, 34, 35]. Considerable evidence from different labs demonstrates that TCA cycle activities in cultured astrocytes are heterogeneous, indicative of different compartments containing mitochondrial with different metabolic capabilities, presumably due to different compositions of enzymes and transporters.

It is well established that glucose is the primary energy substrate taken up and utilized by adult brain, and is also essential for developing brain [9, 36–38]. Metabolism of glucose via the pyruvate carboxylase pathway in astrocytes functions to add net carbons to the TCA cycle which ultimately provides the carbon skeleton to replenish neuronal glutamate [7, 39, 40]. Metabolism of glucose via glycolysis, pyruvate dehydrogenase and the TCA cycle in astrocytes has been reported by many groups [6, 9, 11, 16, 41]; and it is well established that glucose metabolism in brain is highly regulated [20]. Glucose and lactate metabolism in astrocytes have been studied extensively [6, 7, 9, 11, 16, 32, 42–48]. Studies by Waagepetersen and colleagues [6, 16] reported differential compartmentation (e.g. labeling patterns from) of glucose and lactate metabolism in astrocytes that was attributed to the metabolic capabilities of different populations of mitochondria within these cells. Astrocytes have an extremely high capacity for uptake and oxidative metabolism of lactate [9, 11, 31, 49] as well as extensive distribution of lactate through the glial syncytium via astrocytic gap junctions [5, 49, 50].

Both early and more recent studies by Hertz [12, 13] underscore the use of glutamate as an energy substrate for astrocytes that can offset the high cost of glutamate uptake. Microdialysis studies by Zielke [51] confirm that oxidative metabolism of glutamate occurs in rat brain *in vivo*. Studies from our group [26, 52–54] and others [7, 12, 13, 17, 55, 56] have reported oxidative metabolism of glutamate in astrocytes. Hertz [12] referred to this metabolism as “cataplerotic” underscoring the potential importance of glutamate as energy

source for astrocytes. Although the importance of astrocytes in removal of neurotransmitter glutamate from the synaptic cleft is well established [17, 18, 57–62]; the use of glutamate as an energy source for astrocytes is still not universally accepted.

In the present studies addition of unlabeled substrates has been used to obtain specific information about the $^{14}\text{CO}_2$ release from a number of ^{14}C -labeled substrates (including glutamate, glucose and lactate) and to provide insight into whether there is differential compartmentation of substrate metabolism in astrocytes. As stated in a recent review by Kreft et al. [63] “metabolic compartmentation at the level of the single cell is defined as the presence of multiple, distinct intracellular pools of identical metabolites that are not in equilibrium”. The first evidence of *intracellular* compartmentation within astrocytes was our demonstration of malate compartmentation, shown by biphasic oxidation kinetics and the differential response of high and low concentrations of malate to substrates and other effectors [10]. Studies using ^{13}C -NMR spectroscopy to determine labeling of intracellular metabolites as well as labeling of metabolites released into the medium have greatly enhanced the ability to determine the presence of compartmentation in the metabolism of different substrates. Using this technique Sonnewald et al. (1993) and Schousboe et al. (1993) showed evidence of metabolic compartmentation due to differential labeling patterns in the synthesis, and differential regulation of the oxidation of glutamate and glutamine. Previous studies from our group, using astrocytes from rat brain, demonstrated that endogenous glutamate formed intracellularly from glutamine entered the TCA cycle primarily via transamination, and did not dilute the pool of TCA cycle intermediates (e.g. enter the same TCA cycle compartment) as exogenous glutamate taken up from the extracellular milieu [25]. Whereas, extracellular glutamate entered the TCA cycle in astrocytes via both transamination and GDH, and competed with (e.g. diluted the pool of TCA cycle intermediates) the oxidation of the endogenous glutamate produced from glutamine intracellularly via glutaminase [25]. Schousboe and coworkers [18, 64] concluded that glutamate and glutamine oxidation are compartmentalized in astrocytes, at least during the initial phase(s) of metabolism. Many reports have documented compartmentation in astrocytes including evidence that lactate originating from glycogen is not in equilibrium with the pool of lactate derived from glucose metabolism [32], and that the lactate labeled from the metabolism of glutamate in the TCA cycle and partial pyruvate recycling is not in equilibrium with the lactate from glucose metabolism [54].

Previous reports by our group [10, 25, 52, 54, 65–67] and others [6, 16, 22, 63] have shown that the metabolism of individual substrates can be significantly altered by the presence of other compounds that may normally be found in the extracellular and/or intracellular milieu, and that such interactions can provide information about the compartmentation of metabolism in brain. Our group [10, 25, 52, 65, 66, 68], and others [6, 12, 16, 18] have used competition studies to determine if energy substrates are metabolized in different compartments (e.g. have no effect on $^{14}\text{CO}_2$ release), or in the same compartments (e.g., dilute the same TCA cycle pool) in brain. This approach was utilized to assess substrate use and to obtain additional data supporting multiple compartments of energy metabolism in primary cultures of cortical astrocytes from rat brain.

EXPERIMENTAL PROCEDURES

Materials

Tissue culture flasks (Nunc) were purchased from Vanguard International (Neptune, NJ, USA). Culture media (minimum essential medium (MEM) with Earle's salts and nonessential amino acids), Dulbecco's phosphate buffered saline (PBS) and fetal bovine serum were purchased from Paragon Biotech (Baltimore, MD, USA). Nylon screening cloth (Nitex) was obtained from Tetko (Elmsford, NY, USA). Timed-pregnant rats were

purchased from Zivic Miller Laboratories (Zelienople, PA, USA). L-Glutamine, L-glutamate, L-aspartate, oxaloacetic acid, L- α -ketoglutarate, L-lactate (lithium salt), L-malate, and methylbenzonium hydroxide were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The fluor used for liquid scintillation counting (Ready Solve EP) was purchased from Beckman Instruments, Inc. (Fullerton, CA, USA). D-3-hydroxy-butyrate was purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI, USA). Solutions for the BCA microreagent protein assay were purchased from Pierce (Rockford, IL). All reagents and chemicals were of the highest analytical grade. The radioactive compounds L-[U- ^{14}C]lactate and L-[U- ^{14}C]malate were purchased from Amersham Corporation (Arlington Heights, IL, USA), and D-[6- ^{14}C]glucose, D-3-hydroxy[3- ^{14}C]butyrate, L-[U- ^{14}C]glutamine and L-[U- ^{14}C]glutamate were purchased from New England Nuclear-Dupont (Boston, MA, USA).

Cortical Astrocyte Preparation

Astrocytes were prepared from brains from newborn rats as described by McKenna et al. [10, 54, 69]. Cerebral hemispheres were removed, placed in media (MEM with Earle's salts and nonessential amino acids) with 10% fetal bovine serum, cleaned of meninges and trimmed to retain the neopallium. In the newborn animal this consists of the subventricular zone containing most of the proliferating astrocyte precursor cells plus underlying cortex containing some dividing astroblasts [70]. The isolated neopallia were transferred to a 100mm dish containing 10 mls of fresh media until all of the dissection was completed. The medium was removed and the tissue was minced and resuspended in fresh medium (1 ml per brain), mechanically disrupted by vortexing (60 seconds) to destroy most of the neurons, and filtered through sterile nylon Nitex screening cloth (80 and 10 μm pore size) to remove blood vessels and aggregated cells. The cell suspension, enriched in astrocytes was seeded in 12.5 cm^2 Falcon plastic T-flasks at a density of 6 flasks per brain for substrate competition studies. Portions of the preparations were also grown in 24-well cluster plates and on sterile cover slips for microscopic and immunocytochemical examination for identification of cell types and purity. The astrocytic nature of the cells prepared by this method has been documented [10, 26, 71]. In our laboratory the cells were identified as astrocytes on the basis of their morphology by microscopic examination and by positive staining for immunoreactivity with antibodies against glial fibrillary acidic protein in at least 95% of the cells. The cells were incubated at 37°C in an atmosphere of 95% air/5% CO_2 , with 90% humidity. The culture medium containing 5 mM glucose was replaced after 3 days and twice weekly thereafter. No dBcAMP was added to these cultures. All experiments were done on day 10 or 11 after initiation of the cultures.

Measurement of the Rate of $^{14}\text{CO}_2$ Production from ^{14}C -Labeled Compounds

The rates of $^{14}\text{CO}_2$ production from ^{14}C -labeled substrates by astrocytes were determined by methods previously described [25, 54]. All oxidation rates were determined using 4–5 replicates that were averaged to obtain the rate for each of 6–9 different astrocyte preparations. The cells were rapidly washed three times with Dulbecco's PBS, and the final wash was immediately replaced with reaction mixture containing 1 mM of either D-[6- ^{14}C]glucose, D-3-hydroxy[3- ^{14}C]butyrate, L-[U- ^{14}C]glutamine and L-[U- ^{14}C]glutamate, L-[U- ^{14}C]lactate and L-[U- ^{14}C]malate as indicated in Results. The reaction mixture contained the following final concentrations of other components: 4.38 mM KCl, 0.24 mM EDTA, 1.1 mM KH_2PO_4 , 4.03 mM MgSO_4 , 122.1 mM NaCl, 80.7 mM Tris, pH 7.4. The rates of $^{14}\text{CO}_2$ production from the metabolism of labeled substrates were also measured in the presence and absence of 1 mM unlabelled substrates to determine if the unlabeled substrate decreased or increased the rate of $^{14}\text{CO}_2$ production from the radioactive substrate, and thus obtain information about compartmentation of substrate metabolism in cultured astrocytes. The flasks were placed on the platform of a rotary shaker and incubated at 37°C

for 1 hr with continuous shaking. Metabolic activity was stopped by the addition of 0.3 ml of 10% (v/v) trichloroacetic acid. The samples were shaken for an additional 45 minutes and the $^{14}\text{CO}_2$ released was trapped in center wells containing filter paper saturated with methylbenzoniun hydroxide and counted in a liquid scintillation spectrometer. Protein was determined by the Pierce BCA protein assay microreagent system [72]. The rates of $^{14}\text{CO}_2$ production (nmol/mg protein/h) from the ^{14}C -labeled substrates were calculated as dpm/mg protein/h, then divided by the specific activity (dpm/nmol) of the substrate in the medium. These rates represent minimal values because the specific activity of the intracellular substrate pools may be lower than that of the medium. Earlier studies by our group [9, 10] demonstrated that the rates of $^{14}\text{CO}_2$ production from all of the labeled substrates were linear with time up to 2 hours.

Statistical Analysis

Data comparing the rates of $^{14}\text{CO}_2$ production from all of the ^{14}C -labelled substrates by astrocytes were analyzed using Analysis of Variance using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA). Data from the competition studies comparing the rate of $^{14}\text{CO}_2$ production in the presence of each added unlabelled substrate to the control rate of $^{14}\text{CO}_2$ production without added substrates were tested for significance using a one way Analysis of Variance and Dunnet's post hoc test to compare the rates of oxidation of each ^{14}C -labelled substrate with unlabeled added substrates to the rate $^{14}\text{CO}_2$ production from the ^{14}C -labelled substrate with no added substrates (control).

RESULTS

The location of $^{14}\text{CO}_2$ release by the different substrates used in this study is depicted in Figure 1. $^{14}\text{CO}_2$ release from some substrates occurs in one to three reactions (e.g. glutamate, lactate and glutamine); whereas, the release of $^{14}\text{CO}_2$ from [6- ^{14}C]glucose occurs in the 3rd turn of the TCA cycle far downstream from the initial steps in metabolism. Thus the absolute rates of $^{14}\text{CO}_2$ release these substrates must be considered in the context of such factors as dilution of metabolite pools by other intermediates and greater trapping of label from some substrates.

In these studies addition of an unlabeled substrate has been used to obtain specific information about the $^{14}\text{CO}_2$ release from each labeled substrate and to provide insight into whether there is differential compartmentation of substrate metabolism in astrocytes. For example, the $^{14}\text{CO}_2$ from [U- ^{14}C]lactate is released at the pyruvate dehydrogenase step and also at multiple points in the TCA cycle. D-3-hydroxybutyrate enters the TCA cycle after the PDH step so it could not dilute the label from lactate in that reaction (except for any 3-HOB metabolized via the pyruvate recycling pathway); however, if unlabeled 3-HOB were to enter the same TCA cycle pool labeled from lactate metabolism it would dilute the pool and lead to decreased $^{14}\text{CO}_2$ release from the [U- ^{14}C]lactate. In addition, when labeled D-3-hydroxy[3- ^{14}C]butyrate is used, the acetyl CoA formed from the addition of unlabeled lactate would be incorporated into citrate and dilute the label incorporated from D-3-hydroxy[3- ^{14}C]butyrate if the label from lactate were to enter the same pool of TCA cycle intermediates. However, as can be seen in Figure 2d and 2f, neither of these added unlabeled substrates leads to significant dilution of the $^{14}\text{CO}_2$ released by the other labeled substrate. This is interesting considering that both substrates are taken up via monocarboxylic acid transporters and both are robustly metabolized by astrocytes [9, 28, 31]. The logical conclusion from this competition is that there is differential compartmentation of lactate and 3-HOB metabolism; e.g. they are not simultaneously metabolized within the same TCA cycle. Our group has previously demonstrated that there is active metabolism of malate in multiple simultaneous TCA cycles in astrocytes [10]. There is considerable evidence of heterogeneity of both astrocytic and neuronal mitochondria [6, 9, 10, 25, 32, 42, 64, 66, 68,

73–75]. However, the question of whether there is heterogeneity of mitochondria within individual astrocytes is not known at this time.

It is important to note that ATP demand (ADP availability) ultimately drives substrate utilization. This is also apparent in figures 2d and 2f by the significant decrease in $^{14}\text{CO}_2$ production from both L-[U- ^{14}C]lactate and D-3-hydroxy[3- ^{14}C]butyrate when unlabeled glutamate is added. Uptake of glutamate by astrocytes is very energy demanding because co-transport of Na^+ along with glutamate uptake increases the demand for ATP to extrude the Na^+ via Na^+ , K^+ ATPase [61]. However, rather than increasing $^{14}\text{CO}_2$ release from the other substrates, glutamate dilutes the pool(s) of TCA cycle intermediates produced from both the ^{14}C -labeled lactate and 3-hydroxybutyrate leading to decreased $^{14}\text{CO}_2$ from both substrates, and providing evidence that glutamate can enter and dilute the pools of TCA cycle intermediates labeled by both of these substrates. Such competition from glutamate is not surprising given the cost of glutamate transport and the fact that rapid equilibration of glutamate with α -ketoglutarate would serve to “pull” glutamate in to the TCA cycle [63, 73]. This would be facilitated by multiple mitochondrial glutamate transporters since both the aspartate-glutamate carrier [1, 74] and the glutamate-hydroxyl carrier [75] are present on astrocytic mitochondria.

The rates of $^{14}\text{CO}_2$ production from ^{14}C -labeled substrates by primary cultures of cortical rat brain astrocytes are shown in Table 1. The rate of $^{14}\text{CO}_2$ production from L-[U- ^{14}C]glutamate by rat brain astrocytes was significantly higher than the rates from all other substrates, presumably because co-transport of Na^+ along with glutamate uptake increases the demand for ATP to extrude the Na^+ . In contrast, the rate of $^{14}\text{CO}_2$ production from glutamine was considerably lower than that of glutamate (~38% of glutamate oxidation) [25], but was higher than the rates of $^{14}\text{CO}_2$ production from the other substrates.

The rate of $^{14}\text{CO}_2$ production from [6- ^{14}C]glucose was significantly lower than the rates from other substrates but comparable to rates reported for this compound [9]. It should be noted that $^{14}\text{CO}_2$ from carbon 6 position is not lost until the 3rd turn of the TCA cycle so use of this compound (specifically selected in this study since it determines $^{14}\text{CO}_2$ released only in the TCA cycle) underestimates the overall rate of glucose oxidation in astrocytes because it does not determine loss of carbons at the pyruvate dehydrogenase step or in the pentose phosphate pathway. The rates of $^{14}\text{CO}_2$ production from L-[U- ^{14}C]lactate and D-3-hydroxy[3- ^{14}C]butyrate were ~11–12-fold higher than from glucose. The rate of $^{14}\text{CO}_2$ production from L-[U- ^{14}C]malate was several fold higher than the rate from glucose. $^{14}\text{CO}_2$ production from malate oxidation by astrocytes was about 38% of the rates from lactate and 3-hydroxybutyrate, and ~5 and 16-fold less than the rates from glutamine and glutamate, respectively. Rates of $^{14}\text{CO}_2$ production from these individual substrates were comparable to rates published by our group [9, 10, 25] and others [15]. As noted above and discussed below, the differences in rates of $^{14}\text{CO}_2$ production are due in part to label retention in different metabolic pools, differences in transport and metabolic rates, and differences in regulation of pathways that oxidize these substrates.

Substrate Competition Experiments

Competition experiments were carried out to determine the extent to which the oxidation of each ^{14}C -labeled substrate by cortical rat brain astrocytes was altered by the presence of other compounds and energy substrates that could be present in brain [6, 10, 25, 66, 67]. For these studies, the rate of $^{14}\text{CO}_2$ production from 1 mM of a ^{14}C -labelled substrate was determined in the absence and presence of each unlabelled substrate (1 mM). This concentration was selected because during neurotransmission glutamate in the synaptic cleft can transiently reach concentrations as high as ~1–3 mM [76]. Addition of the other

substrates would not be expected to directly alter metabolic demand, but they may alter pathway fluxes and substrate utilization by altering levels of metabolites in various pools.

The rate of $^{14}\text{CO}_2$ production from glutamate was considerably higher than the rates from any other substrates (Table 1). None of the other unlabeled substrates added had any effect on the rate of $^{14}\text{CO}_2$ production from $[\text{U-}^{14}\text{C}]\text{glutamate}$ by cultured astrocytes (Figure 2a). This finding underscores the capability of astrocytes to readily utilize available glutamate for energy [5, 12, 25, 54, 77]. It is interesting that 1 mM unlabeled glucose had no effect of the rate of $^{14}\text{CO}_2$ production from $[\text{U-}^{14}\text{C}]\text{glutamate}$; Schousboe et al. [73] reported that glycolytically derived ATP production was required for glutamate uptake by astrocytes and that pyruvate and lactate were not able to replace glucose as the energy substrate for glutamate uptake. However, it has also been suggested that the decrease in cytosolic glucose in the presence of exogenous glutamate reflects diversion of glucose towards glycogen synthesis [63].

The addition of unlabeled glutamate decreased the rate of $^{14}\text{CO}_2$ production from $[\text{U-}^{14}\text{C}]\text{glutamine}$ by 75% ($p < 0.001$), as shown in Figure 2b. In contrast, there was no effect on the rate of $^{14}\text{CO}_2$ production from $[\text{U-}^{14}\text{C}]\text{glutamine}$ when unlabeled glucose, lactate, malate or 3-hydroxybutyrate were added. These data demonstrate that exogenous glutamate can clearly enter the compartment in which glutamine is metabolized and dilute the intracellular pool of endogenously formed glutamate in astrocytes.

Several of the unlabeled substrates significantly decreased the rate of $^{14}\text{CO}_2$ production from $[\text{6-}^{14}\text{C}]\text{glucose}$ by astrocytes (Figure 2c). The addition of unlabeled glutamate and glutamine significantly decreased the rate of $^{14}\text{CO}_2$ production from $[\text{6-}^{14}\text{C}]\text{glucose}$ by 75% and 45% ($p < 0.001$), respectively. Added unlabeled lactate significantly decreased the rate of $^{14}\text{CO}_2$ production from $[\text{6-}^{14}\text{C}]\text{glucose}$ by ~41% ($p < 0.01$). However, it should be noted that glucose metabolism in brain is highly regulated and that pool of intracellular pyruvate from glucose metabolism is relatively small and thus would be diluted considerably by lactate uptake [20]. The addition of unlabeled 3-hydroxybutyrate and unlabeled malate had no effect on the rate of $^{14}\text{CO}_2$ production from $[\text{6-}^{14}\text{C}]\text{glucose}$. These data suggest that glutamate, glutamine and lactate are at least partially metabolized in the same compartment as glucose in cultured rat brain astrocytes.

The effect of unlabeled substrates on the rate $^{14}\text{CO}_2$ production from $[\text{U-}^{14}\text{C}]\text{lactate}$ was interesting because it was different from the pattern seen with glucose metabolism (Figure 2d). The addition of unlabeled glutamine and glutamate decreased $^{14}\text{CO}_2$ production from $[\text{U-}^{14}\text{C}]\text{lactate}$ by 30% and ~25%, respectively (both $p < 0.05$). The addition of unlabeled glucose and unlabeled 3-hydroxybutyrate both had no significant effects. In contrast to all of the other substrates, the addition of unlabeled malate *increased* the rate of $^{14}\text{CO}_2$ production from $[\text{U-}^{14}\text{C}]\text{lactate}$ by 38% ($p < 0.05$).

The addition of unlabeled glutamate decreased $^{14}\text{CO}_2$ production from $[\text{U-}^{14}\text{C}]\text{malate}$ by 64% ($p < 0.001$) as shown in Figure 2e. The addition of unlabeled lactate decreased the rate $^{14}\text{CO}_2$ production from $[\text{U-}^{14}\text{C}]\text{malate}$ by ~28% ($p < 0.05$), consistent with earlier data reported by our group [10]. The addition of unlabeled glucose, 3-hydroxybutyrate and glutamine had no effect on $^{14}\text{CO}_2$ production from $[\text{U-}^{14}\text{C}]\text{malate}$.

The addition of unlabeled glutamate decreased the rate of $^{14}\text{CO}_2$ production from 3-hydroxy[3- $^{14}\text{C}]\text{butyrate}$ by 60% (Figure 2f). Although added glutamine had a tendency to decrease $^{14}\text{CO}_2$ production from 3-hydroxybutyrate; there was no significant effect because of variability. The addition of unlabeled glucose, lactate and malate had no effect on the rate of $^{14}\text{CO}_2$ production from 3-hydroxy[3- $^{14}\text{C}]\text{butyrate}$. These data suggest that glutamate enters and is metabolized in the same TCA cycle compartment and thus dilutes the $^{14}\text{CO}_2$

production from 3-hydroxy[3-¹⁴C]butyrate, whereas lactate, glucose and malate are clearly not metabolized in the same compartment or do not alter the size of the label trapping pool.

Discussion

Use of Energy Substrates by Cultured Rat Brain Astrocytes

Recent studies provide evidence that metabolism in astrocytes accounts for ~30% of total oxygen consumption in brain [2–4, 78]. The ability of cultured astrocytes to utilize a variety of substrates for energy has been demonstrated by many groups over the past two decades [6–18, 29, 42, 58, 64, 77, 79]. Despite the large number of studies to date the interactions and competition of substrate use by astrocytes has not been studied in depth.

It is well-established that disposal of glutamate from the synaptic cleft to maintain the low resting concentration required for neurotransmission is one of the most essential functions of astrocytes in brain [12, 13, 20, 22, 26, 77, 80–82]. Data from the present study demonstrate that the rate of ¹⁴CO₂ production from L-[U-¹⁴C]glutamate by cultured rat brain astrocytes is considerably higher than from any of the other substrates tested, underscoring that glutamate can be used as an energy source in these cells. Glutamate uptake by astrocytes is an expensive process requiring activation of Na⁺, K⁺-ATPase to pump out the Na⁺ taken up during glutamate transport [61]. A key study by Hertz and Hertz [12] demonstrated the ability of 100 μM glutamate to support oxygen uptake by astrocytes at the same O₂ uptake rate as 7.5 mM glucose. In the same study the combination of glutamate + glucose significantly increased O₂ uptake above the respiration with either substrate alone [12]. This latter finding may be related to the finding of Schousboe et al. [73] that glycolytically derived ATP production was required for glutamate uptake by astrocytes, thus the high glucose present in the study by Hertz and Hertz [12] may have promoted glutamate uptake. Glutamate uptake by astrocytes stimulates glycolysis in some astrocyte preparations [83, 84], but this is not a universal finding because glutamate exposure does not stimulate glycolysis in all astrocyte preparations [85]. In a previous study we reported that addition of 1 mM pyruvate increased the rate of ¹⁴CO₂ production from labeled glutamate consistent with enhanced entry of glutamate into the TCA cycle via transamination in the presence of pyruvate [25]. However, in the present study 1 mM unlabeled glucose had no effect of the rate of ¹⁴CO₂ production from [U-¹⁴C]glutamate.

Hertz and Hertz suggested that through cataplerotic oxidative metabolism glutamate has the ability to “pay its own way” which thus cancels out the high cost of glutamate uptake [12]. In support of this concept an association between glial glutamate transporters with glycolytic enzymes and possibly hexokinase has been reported [86]. A recent study from the Robinson lab [86] demonstrated that the glial glutamate transporter GLT1 can co-compartmentalize with glycolytic enzymes and mitochondria, and that this may provide a mechanism to match energy production to the demands of glutamate transport. Indeed, many studies [12, 13], including some from our group [25, 54] have reported that astrocytes have the capability to oxidize glutamate for energy in a concentration-dependent manner within the range of levels that occur in the synaptic cleft (low millimolar concentration) [76]. It is well established that the carbon skeleton of glutamate can enter the TCA cycle and be incorporated into aspartate and lactate, and that carbons from glutamate can be metabolized via the pyruvate recycling pathway in astrocytes (and neurons albeit to a very small extent) [26, 54, 87–89]. Thus, through the TCA cycle and pyruvate recycling pathway all carbons of a glutamate molecule can be completely oxidized for energy [20]. However, it must be noted that exogenous glutamate taken up by astrocytes would shift the equilibrium between glutamate and α-ketoglutarate and thus lead to entry of more α-ketoglutarate into the TCA cycle and subsequent decarboxylation. Although one could argue that the high oxidation is simply due to mass action, it is interesting to note that work by Hertz and Hertz [12] has shown that the

oxidation of glutamate is as high as the anaplerotic rate of glutamate production. This finding fits well with the concept that the synthesis of glutamate in brain must be balanced by catabolism since this amino acid does not readily exit brain across the blood-brain barrier [12].

The inability of the other unlabeled substrates to decrease the rate of $^{14}\text{CO}_2$ production from $[\text{U-}^{14}\text{C}]\text{glutamate}$ suggests that these other substrates can not rapidly enter and dilute the intermediates of the TCA cycle in which the carbon skeleton of glutamate is metabolized. This may reflect the rapidity of glutamate uptake and conversion to α -ketoglutarate; however, it may also reflect metabolism of substrates in different pools of TCA cycle activity. The existence of multiple compartments of substrate metabolism and of TCA cycle activity have been demonstrated by many groups [6, 10, 18, 25, 54, 77, 90–92].

The rate of $^{14}\text{CO}_2$ production from $[\text{6-}^{14}\text{C}]\text{glucose}$ in the present study was comparable to rates reported by our laboratory for glucose labeled in the C6 position [9]. It should be noted that our group [9, 11] and others [48] have previously reported that rates of oxidation of uniformly labeled glucose by cultured rat brain astrocytes are considerably higher (3.42 ± 0.67 nmol/hr/mg protein and ~ 5 nmol/hr/mg/protein for astrocytes grown in 5 mM and 2 mM glucose, respectively) than that of $[\text{6-}^{14}\text{C}]\text{glucose}$ since $^{14}\text{CO}_2$ production from uniformly labeled glucose also measures oxidation via the pentose phosphate pathway (PPP), and release of label at pyruvate dehydrogenase (PDH) as well as $^{14}\text{CO}_2$ released during the second and third turn of the TCA cycle. Astrocyte metabolism of glucose via glycogen can also trap some of the label from $[\text{6-}^{14}\text{C}]\text{glucose}$. Takahashi et al. [93] recently reported that oxidation of glucose via the pentose phosphate pathway in astrocytes increased significantly with increased extracellular glucose.

The PPP is active in both astrocytes and neurons and is important in providing NADPH for maintaining reduced glutathione (GSH) levels for defense against reactive oxygen species [93–96]. It should be noted that Abe et al. [48] found that astrocytes grown in exceptionally high (22 mM) glucose concentration had a significantly decreased ability to oxidize glucose for energy when determined with 2 mM labeled glucose (~ 2.5 nmol/hr/mg protein for $[\text{U-}^{14}\text{C}]\text{glucose}$, which is 50% of the rate in astrocytes grown in 2mM glucose). A recent study by Halim et al. [97] demonstrated that activity of pyruvate dehydrogenase in astrocytes and neurons is regulated by phosphorylation of the pyruvate dehydrogenase alpha subunit (PDH alpha). Thus differential phosphorylation of the subunit leads to almost maximal activity (and high pyruvate oxidation) in neurons, and tightly inhibited activity (and glucose use) in astrocytes [97]. This may reflect a species difference since Hertz and Hertz [12] reported high oxygen respiration rates in mouse brain astrocytes grown in 7.5 mM glucose.

In the present study we found that added unlabeled glutamate decreased the rate of $^{14}\text{CO}_2$ production from $[\text{6-}^{14}\text{C}]\text{glucose}$ by 75% demonstrating that exogenous glutamate can readily dilute the pool of TCA cycle intermediates thereby decreasing the oxidative glucose metabolism in astrocytes. This effect would be more pronounced with $[\text{6-}^{14}\text{C}]\text{glucose}$ than with $[\text{U-}^{14}\text{C}]\text{glucose}$ since the $^{14}\text{CO}_2$ from the former would not be released until the third turn of the TCA cycle. This is in agreement with the report of Peng et al. [8] that added glutamate decreased the rate of glucose oxidation by 75%. Abe et al. [48] found that addition of 500 μM D-aspartate, which increases the activity of Na^+ , K^+ -ATPase, increased the oxidation of $[\text{U-}^{14}\text{C}]\text{glucose}$ in astrocytes from rat brain by 76% and the rate of lactate oxidation by 83%. It should be noted that D-aspartate is not metabolized but brings Na^+ into the cell, as does glutamate, so it causes an energy demand that would increase glucose oxidation and metabolism [8]. Peng et al. [8] demonstrated that exposure of astrocytes to glutamate did not increase deoxyglucose phosphorylation, whereas D-aspartate exposure did

increase deoxyglucose phosphorylation. Our results are consistent with the findings of Peng et al. [8], and are also not necessarily contradictory to the findings of Abe et al. [48] since the increase in [U- ^{14}C]glucose oxidation in their study may reflect metabolism via the pentose phosphate pathway, PDH reaction, and TCA cycle.

Data from several labs suggests that even in developing brain use of glucose may be more important in neurons than in astrocytes. Indeed, earlier studies by our group reported that the rates of $^{14}\text{CO}_2$ production from [6- ^{14}C]glucose by freshly isolated synaptic terminals from 18 day old and adult brain (8.55 ± 0.78 , and 13.12 ± 1.05 nmol/h/mg protein, respectively) are 7 and 11-fold higher than the rate in cultured astrocytes [9, 11, 66]. These findings are in agreement with ex vivo ^{13}C -NMR studies by Qu et al. [41] which estimated that at least 66% of the acetyl CoA produced from glucose is metabolized in neurons and a maximum of 34% was metabolized in astrocytes. These authors also noted that metabolism via pyruvate carboxylase is about half the rate of metabolism via PDH in astrocytes [41].

The rate of lactate oxidation in the present study is comparable to the rate of ~ 12 nmol/hr/mg protein reported by Abe et al. [48] for lactate oxidation in astrocytes grown in 2 mM glucose, and comparable to rates reported by Edmond et al. [15] and earlier studies by our group [9]. It has been established with microdialysis studies that lactate is oxidized for energy in adult rat brain *in vivo*, with 50% of the oxidation taking place in astrocytes [51]. It is important to note that although lactate can have a high oxidation rate in neurons and synaptic terminals [9, 98], astrocytes have an immense capacity for lactate uptake [31]. The high V_{max} values for lactate uptake by astrocytes 16.55 and 173.84 nmol/min/mg protein), coupled with a low affinity K_m of 11.38 mM, which is considerably higher than that of the neuronal transporter, would allow astrocytes to readily use lactate from the interstitial space in brain [31, 98]. The higher affinity K_m 0.49 mM for astrocytes could also compete effectively with synaptic terminals for lactate uptake and the lower affinity K_m for astrocyte lactate uptake would remain active when the higher affinity transport capacity becomes saturated [31, 98]. A number of groups remain focused on conversion of glucose to lactate in astrocytes and release of this substrate for neurons [83, 99–102], despite the recent *in vivo* studies and modeling that support the concept of lactate release by neurons and uptake by astrocytes [103–105]. Indeed, our data are compatible with the recent modeling studies by Mangia and co-workers [104, 105] supporting a neuron to astrocyte lactate shuttle in human brain and more utilization by astrocytes than neurons. However, as noted above the formation, release, uptake and utilization of lactate by neurons, astrocytes and other brain cells is likely to be very dynamic and would change rapidly in response to the energy level, redox status and concentration of oxygen and energy substrates [44, 85].

In the present study addition of 1 mM glucose did not significantly affect the rate of $^{14}\text{CO}_2$ release from lactate by astrocytes. However, it appears that pathologically high levels of glucose can compromise glial lactate oxidation as Abe et al. [48] found that lactate oxidation was decreased by 60% in astrocytes grown in very high (22 mM) glucose when the lactate oxidation was assayed in the presence of 2 mM glucose. The finding in the present study that the addition of unlabeled malate significantly increased lactate oxidation by astrocytes is consistent with our earlier study of the complex compartmentation of malate metabolism in astrocytes [10]. This effect may reflect increased citrate formation subsequent to increased availability of oxaloacetate (formed from the added malate) for condensation with the acetyl CoA from lactate metabolism. The stimulation of $^{14}\text{CO}_2$ production from [U- ^{14}C]lactate by malate is not likely to reflect any change in malate-aspartate shuttle activity since earlier studies by our group showed that inhibiting the malate-aspartate shuttle with 5 mM aminooxyacetic acid (AOAA) inhibited lactate oxidation only in synaptosomes and not in astrocytes [9].

The addition of either unlabeled glutamate or glutamine decreased the rate of $^{14}\text{CO}_2$ production from $[\text{U-}^{14}\text{C}]\text{lactate}$ in astrocytes by ~25–30%. This finding is somewhat unexpected given the differences in compartmentation of glutamate and glutamine metabolism in astrocytes discussed above [18, 25, 64, 77]. The fact that a high proportion of the label from lactate is still released as $^{14}\text{CO}_2$ in the presence of equal concentrations of either glutamate or glutamine underscores the potential importance of lactate as an energy substrate for astrocytes.

The rate of $^{14}\text{CO}_2$ production from D-3-hydroxy-[3- ^{14}C]butyrate were comparable to values reported by Edmond et al. [15] and to earlier rates reported by our group [9]. The rates of $^{14}\text{CO}_2$ production from 3-hydroxybutyrate oxidation were ~8-fold higher than glucose oxidation and comparable to the rate of lactate oxidation by astrocytes consistent with earlier reports [9, 15]. The level of the monocarboxylic acid transporter MCT1 in the blood-brain barrier is higher in developing brain, which is consistent with the importance of the ketone bodies 3-hydroxybutyrate and acetoacetate as energy substrates during development [106–108]. 3-hydroxybutyrate is not generally considered to be an important substrate in adult brain; however, studies by Pan et al. [109] showed high metabolism of 3-hydroxybutyrate in human brain after overnight fasting. Earlier studies by our group demonstrated lactate inhibited the initial rates of uptake of 3-hydroxybutyrate by astrocytes by ~20% [28]; however, the addition of unlabeled lactate had in the present study no effect on the rate of $^{14}\text{CO}_2$ production from D-3-hydroxy-[3- ^{14}C]butyrate. The lack of effect of lactate on 3-hydroxybutyrate oxidation is an example of the extensive compartmentation of energy metabolism in astrocytes previously reported by our group and others [6, 10, 16, 52–54, 110]. The lack of effect of malate on 3-hydroxybutyrate is consistent with reports that metabolism of this substrate does not require activity of the malate-aspartate shuttle [9]. It is evident that malate and 3-hydroxybutyrate are metabolized in different compartments in astrocytes since malate can be converted directly to oxaloacetate which would be expected to enhance the formation of citrate from acetyl CoA derived from 3-hydroxybutyrate metabolism if these substrates were metabolized in the same compartments in astrocytes.

The addition of either glutamate or glutamine significantly decreased the rate of $^{14}\text{CO}_2$ production from D-3-hydroxy-[3- ^{14}C]butyrate in astrocytes. These data demonstrate that both exogenous glutamate and glutamate formed endogenously from glutamine can enter and dilute intermediates in the same TCA cycle as 3-hydroxybutyrate. The finding that the competition by glutamine was comparable to that of glutamate is intriguing because of the well-established differences in compartmentation of glutamate and glutamine metabolism in astrocytes [18, 25, 26, 54, 58, 64, 87, 89, 111]. Although both glutamate and glutamine decreased 3-hydroxybutyrate oxidation, it is important to note that the effect was a relatively modest 25–30% decrease which is rather surprising given the ability of glutamate to decrease the other substrates by ~75%. It is somewhat curious that the addition of unlabeled 3-hydroxybutyrate has no effect on the $^{14}\text{CO}_2$ production from either glutamate or glutamine as the acetyl CoA from 3-hydroxybutyrate could facilitate the metabolism of oxaloacetate formed from metabolism of the ^{14}C -labeled glutamate and glutamine in subsequent TCA cycles.

Our group has previously reported that there are multiple compartments of malate metabolism in rat brain astrocytes [10]. The addition of unlabeled glutamate decreased $^{14}\text{CO}_2$ production from $[\text{U-}^{14}\text{C}]\text{malate}$ by 64% demonstrating that there is considerable overlap in the compartmentation of oxidative glutamate metabolism with malate metabolism. The decreased malate oxidation may be due in part to the inhibitory effect of glutamate on mitochondrial malic enzyme in astrocytes previously reported by our group [69]. Unlabeled lactate significantly decreased $^{14}\text{CO}_2$ production from $[\text{U-}^{14}\text{C}]\text{malate}$ in astrocytes, consistent with our earlier study [10]. This effect of lactate may be due in part

to the inhibition of cytosolic malic enzyme (cMDH) by lactate reported previously by our group [65], which is likely due to competition between LDH and cMDH for NAD^+ since these enzymes are known to be in equilibrium with the same pool of NAD^+ [112–114]. The reciprocal effects of lactate and malate on metabolism suggest that these substrates interact within the same compartment in rat brain astrocytes; however, this interaction appears to be quite complex since the addition of unlabeled malate increased, rather than decreased, lactate metabolism. The finding that unlabeled 3-hydroxybutyrate had no effect on the rate of $^{14}\text{CO}_2$ production from $[\text{U-}^{14}\text{C}]\text{malate}$ provides additional evidence of the separate compartmentation of lactate and 3-hydroxybutyrate metabolism in astrocytes. The finding that neither glucose nor glutamine had an effect on malate metabolism is different from the effect of these substrates in synaptosomes where both of these substrates decreased malate oxidation by 25% [66].

Glutamine, like a number of other brain substrates including lactate and 3-hydroxybutyrate, can be both produced by and utilized by astrocytes depending on the concentration of substrates in the extracellular and intracellular milieu and the metabolic requirements of the cells [15, 22, 25]. The addition of unlabeled glutamate decreased the rate of $^{14}\text{CO}_2$ production from $[\text{U-}^{14}\text{C}]\text{glutamine}$ by 75%. In contrast, unlabeled glutamine had no effect on the rate of $^{14}\text{CO}_2$ production from $[\text{U-}^{14}\text{C}]\text{glutamate}$ underscoring the partial overlap of these substrates, and the differences in the compartmentation of glutamate and glutamine in brain [18, 25, 77]. None of the other substrates decreased glutamine oxidation in brain demonstrating that glutamine, or endogenously formed glutamate, can be a preferred substrate for metabolism in astrocytes.

Overall, the data from the present study provide evidence that glutamate, glutamine, lactate, glucose and 3-hydroxybutyrate can be used as energy substrates for astrocytes. The earlier report that 50% of the oxidative metabolism of lactate and 35% of oxidative glucose metabolism *in vivo* takes place in astrocytes [51] supports the concept that these data may indeed reflect metabolism in brain.

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Figure 1.

Schematic diagram indicating the location of $^{14}\text{CO}_2$ release by the different substrates used in this study. Note that $^{14}\text{CO}_2$ release occurs in one to three reactions for some substrates (e.g. glutamate, lactate and glutamine); whereas, the release of labeled CO_2 for $[6-^{14}\text{C}]$ glucose occurs far downstream from the initial metabolism and in the 3rd turn of the TCA cycle. The complete oxidation of some substrates including glutamate, glutamine, requires metabolism via the pyruvate recycling pathway as noted in the text. (Abbreviations: Glc, glucose; Lac, lactate, 3-HOB 3-hydroxybutyrate; Glu, glutamate, Gln, glutamine; Mal, malate; OAA, oxaloacetate; ME, malic enzyme; KGDH, alpha-ketoglutarate dehydrogenase; PC, pyruvate carboxylase; PDH, pyruvate dehydrogenase; PAG, phosphate activated glutaminase).

2a. Effect of added unlabeled substrates on glutamate oxidation by astrocytes **2b. Effect of added unlabeled substrates on glutamine oxidation by astrocytes**

2c. Effect of added unlabeled substrates on glucose oxidation by astrocytes

2d. Effect of added unlabeled substrates on lactate oxidation by astrocytes

2e. Effect of added unlabeled substrates on malate oxidation by astrocytes

2f. Effect of added unlabeled substrates on 3-HOB oxidation by astrocytes

Figure 2.

Effect of added unlabeled substrates on the oxidation of each of the ^{14}C -labeled substrates by cultured rat brain astrocytes. Values are means $\pm$ SEM for 4–8 experiments and are expressed as percent of oxidation of each ^{14}C -labeled substrate indicated with no unlabeled substrate added. Control rates are given in Table 1. Data were analyzed by using ANOVA and Dunnett's post hoc test comparing the rates of $^{14}\text{CO}_2$ production from the metabolism of the labeled substrates *with* each added unlabeled substrate to the control rate of $^{14}\text{CO}_2$ production from each labeled substrate *without* any added unlabeled substrates. Asterisks indicate values which are significantly different from the control value for that substrate: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Table 1

Rates of production of $^{14}\text{CO}_2$ from ^{14}C -labeled substrates by primary cultures of rat brain astrocytes in the absence of any other substrates

<u>Labeled substrate</u>	<u>n</u>	<u>nmol $^{14}\text{CO}_2$ produced/hr/mg protein</u>
L-[U- ^{14}C]Glutamate	6	85.30 $\pm$ 12.25
L-[U- ^{14}C]Glutamine	8	28.04 $\pm$ 2.84
D-[6- ^{14}C]Glucose	9	1.17 $\pm$ 0.19
D-3-Hydroxy[3- ^{14}C]butyrate	7	13.55 $\pm$ 4.56
L-[U- ^{14}C]Lactate	6	14.84 $\pm$ 2.40
L-[U- ^{14}C]Malate	6	5.20 $\pm$ 0.50

Values are means $\pm$ SEM for the number of experiments indicated in parentheses. Astrocytes were cultured and $^{14}\text{CO}_2$ production measured with 4–5 replicates per experiment in 6–9 different primary cultures of rat brain astrocytes as described in the Methods section.