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ORIGINAL PAPER

Pyruvate Carboxylation in Different Model Systems Studied by ^{13}C MRS

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Abstract Pyruvate carboxylation is of great importance in the brain since it is responsible for adding net carbons to the tricarboxylic acid cycle following removal of carbon backbone for synthesis of the two most abundant neurotransmitters, glutamate and GABA. Despite having such a pivotal role, there is still much uncertainty in the exact metabolic details about where and how this carbon is returned. Pyruvate carboxylation has been studied in various model systems of the brain and ^{13}C magnetic resonance spectroscopy is an excellent tool for doing this. This review will focus on results dealing with the extent and cellular location of pyruvate carboxylation and its role in pathophysiology and concludes that pyruvate carboxylation is an extraordinarily important predominantly astrocytic pathway which plays a pivotal part in a number of diseases.

Keywords Pyruvate carboxylation · Glucose · Magnetic resonance spectroscopy · Acetate · Glutamate · Glutamine

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Introduction

There are several enzymes involved in carboxylation in the brain (Fig. 1); pyruvate carboxylase (PC), phosphoenolpyruvate carboxykinase (PEPCK), pyruvate kinase and malic enzyme (ME) [1]. Some of these enzymes have a cell specific localization. Only astrocytes have PC and cytosolic ME whereas mitochondrial ME and PEPCK have been found in both neurons and astrocytes [2–9]. The importance of these enzymes is apparent from the severity of disease associated with genetic modifications of these systems. Mitochondrial malic enzyme (ME2) was found to be missing in partially complementing genotypes between lethal deletion alleles at the albino locus in Chromosome 7 of the mouse. Since such partial complementers survive to adulthood, the absence of normal mitochondrial malic enzyme is compatible with life [10]. However, it has been shown that genetic variation in or near the ME2 gene is associated with both psychotic and manic disorders, including schizophrenia and bipolar disorder [11].

Pyruvate carboxylase is a nuclear encoded homotetramer found in most eukaryotic and in many prokaryotic tissues and is a member of the family of biotin-dependent carboxylases [12]. It is also ATP-dependent and catalyses the carboxylation of pyruvate to form oxaloacetate [12]. In mammalian tissues, PC participates in various anabolic pathways including lipogenesis and the synthesis of neurotransmitter substances [12]. Five alternative forms of rat pyruvate carboxylase cDNAs have been identified and these are expressed in a tissue-specific manner in liver, kidney, brain, and adipose tissue [13]. Defects in the expression or biotinylation of pyruvate carboxylase in humans is a rare autosomal recessively inherited disorder of pyruvate metabolism presenting with lactic academia and neurological involvement and almost invariably results

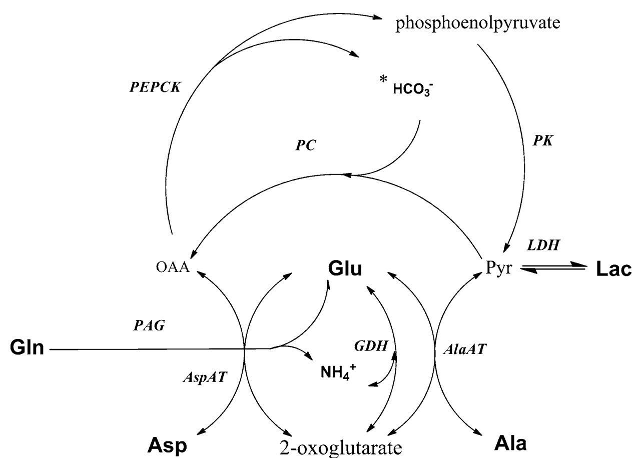

Fig. 1 Schematic presentation of reactions pertinent to pyruvate carboxylation. This scheme illustrates how the concentrations of pyruvate, oxaloacetate and 2-oxoglutarate, which are normally relatively low, are important in regulating pyruvate carboxylation and how these reactions can restore balance in these compounds (and hence in the TCA cycle). For simplicity, malic enzyme is not included. It can carboxylate malate to pyruvate and can theoretically carry out the reverse reaction. * The bicarbonate pool is fed by all decarboxylation reactions from the tricarboxylic acid cycle. *AspAT* aspartate amino transferase, *AlaAT* alanine amino transferase, *GDH* glutamate dehydrogenase, *Lac* lactate, *LDH* lactate dehydrogenase, *OAA* oxaloacetate, *PAG* phosphate activated phosphatase, *PC* pyruvate carboxylase, *PEPCK* phosphoenolpyruvate carboxykinase, *PK* pyruvate kinase

in early death or at best a severely debilitating psychomotor retardation [12]. The above clearly reflects the vital role PC plays in intermediary metabolism in many tissues including the brain [14].

^{13}C magnetic resonance spectroscopy (MRS) is an excellent tool for the study of metabolic pathways of glucose and other substrates. Using this method and ^{13}C labeled glucose it could be shown that pyruvate carboxylation occurs in humans, rats and mice in vivo [15–17]. In experiments where slices were superfused with ^{13}C labeled glucose the evidence from MRS analysis of glutamate suggested that pyruvate carboxylation in slices is minimal [18–20]. However, using ^{13}C and ^{15}N labeled alanine it could be shown that carboxylation was taking place in astrocytes in brain slices [21]. Most cell culture studies also showed that carboxylation was a glial pathway [22].

Discussion

It has been shown that there is a moderate efflux of glutamine from the human brain [23, 24] and thus there must be an anaplerotic process that compensates for this since it is essentially a loss of α-ketoglutarate (via glutamate) from the TCA cycle and would lead to a stop of this cycle unless another intermediate is channeled into the cycle. This intermediate is typically oxaloacetate produced by pyruvate carboxylation [1]. To measure the anaplerotic contribution, Mason et al. [15] measured ^{13}C incorporation into glutamate and glutamine in the occipital-parietal region of awake humans while infusing [2-^{13}C]glucose, which labels the C2 and C3 positions of glutamine and glutamate via pyruvate carboxylase (and the pentose phosphate shunt). Metabolic modeling of the labeling data indicated that pyruvate carboxylase flux is used for replacing glutamate lost due to glutamine efflux and therefore can be considered to support neurotransmitter trafficking. Oz et al. [26] have reported a high pyruvate carboxylase rate of approximately 0.14–0.18 μmol/gm/min contributing to the glial TCA cycle flux.

Is Pyruvate Carboxylation an Astrocyte Specific Pathway?

Even though it has been shown that pyruvate carboxylation in the brain is carried out primarily by the glial specific enzyme PC and only to a minor extent by ME and PEPCK [1] there is some debate about the cellular location of pyruvate carboxylation. ^{13}C MRS studies in rats injected with ^{13}C labeled lactate, predominantly a neuronal substrate [27], showed that lactate was metabolized in a compartment without measurable pyruvate carboxylation [28, 29]. This can be interpreted as indicating that carboxylation is taking place in astrocytes. However, Hassel and Brathe [30] concluded from experiments were large amounts of lactate were infused that pyruvate was carboxylated in neurons. The reason for this discrepancy is not known.

In brain tissue slices carboxylation was also detected in astrocytes [31, 32]. Guinea pig cortical tissue slices were incubated with glucose and alanine which was labeled in the C-2 position with ^{13}C and the amino nitrogen was ^{15}N [21]. The ^{15}N coupling could be seen in the ^{13}C MR spectrum [21]. The authors observed [2-^{13}C, ^{15}N]aspartate, but no [2-^{13}C]aspartate was detected [21]. There was very little dilution of the ^{15}N which would have had to be passed to a glutamate intermediate in order to move from alanine to aspartate. There was no detection of [3-^{13}C]aspartate above natural abundance, which would have been found 50/50 with [2-^{13}C]aspartate if the ^{13}C had been incorporated into aspartate via pyruvate deghydrogenase [21]. These data and those of others suggest that alanine is taken up into an astrocytic compartment [21, 33–35]. Furthermore, it is clear from the results with [2-^{13}C, ^{15}N]alanine that aminotransferase and aspartate aminotransferase are in very tight association with a pyruvate carboxylating enzyme (Fig. 1).

Using primary mono cultures of neurons and astrocytes it is possible to obtain information about the cellular

location and magnitude of pyruvate carboxylation. Using [1-^{13}C]glucose or [2-^{13}C]acetate as substrates in cultures of cortical astrocytes and GABAergic neurons Sonnewald et al. [36] showed pyruvate carboxylation in astrocytes but not in neurons However, it should be noted that the interpretation of results obtained in these studies is complicated since oxaloacetate can be converted to citrate directly or via several steps to fumarate and subsequent oxaloacetate and citrate (back cycling) [37]. The latter pathway will lead to scrambling of label due to the symmetrical fumarate molecule. Merle et al. [37] suggested incomplete back cycling for astrocytes and complete back cycling for cerebellar neurons. If back cycling in neurons is indeed complete, the study above, utilizing [1-^{13}C]glucose, was not capable of showing carboxylation in neurons. Waagepetersen et al. [22] have performed a study using [U-^{13}C]glucose plus [U-^{13}C]lactate and 3-nitropropionic acid (3-NPA) to specifically block the TCA cycle at the succinate dehydrogenase step [38–40]. In this way multiple cycling of TCA cycle constituents was avoided and carboxylation would be clearly detected in labeling of the C-3 position, regardless of back cycling. Analyzing the labeling patterns in amino acids, pyruvate carboxylation was detected in astrocytes but not in neurons [22]. The authors stated: "Based on the present results it may be safely concluded that neuronal pyruvate carboxylation is unlikely to play an important quantitative role. Therefore it is also unlikely to be able to sustain release of transmitter glutamate in cerebellar granule neurons, a process which is quantitatively demanding."

What Factors Affect Pyruvate Carboxylation?

Depolarization, depresses transmission in glutamatergic neurons, activates astrocytes but has less effect on GABAergic neurons [41–44]. Using brain slices it was shown that depolarization did not affect carboxylation [25]. This indicates that a mostly astrocytic workload doesn't involve increased pyruvate carboxylation. Furthermore, the presence of $GABA_B$ or $GABA_C$ ligands had no effect on pyruvate carboxylation in brain slices [45, 46].

Increased Carboxylation

Zwingman [47] showed that increased ammonia concentration increased pyruvate carboxylation both in astrocytes in culture and in animal models. This carboxylation might be coupled to the increased glutamine formation which is a hallmark of ammonia detoxification [47]. Glutamine formation is the only efficient method of ammonia fixation in brain.

The effect of ketone bodies on pyruvate carboxylation by rat brain mitochondria has been investigated [1]. Melo et al. [48] showed that pyruvate carboxylation increased after rats were fed a ketogenic diet. This increase in carboxylation was coupled to an increased pyruvate recycling.

Activation of the glutamatergic AMPA receptor induces large post-synaptic action potentials [49] and should therefore stimulate release of neurotransmitter glutamate. In experiments with brain tissue slices metabolizing [1-^{13}C]glucose it was possible to show evidence of pyruvate carboxylation in aspartate [50]. Increased carboxylation was detected when the slices were incubated with AMPA or exogenous glutamate [50] suggesting that increased neuronal work increases carboxylation. These results contrast with the above mentioned results using K^+ depolarization where no increased carboxylation was detected and where the workload is greater in astrocytes. Interestingly, inhibition of the monocarboxylate transporters MCT1 and MCT2 using AR-C122982 at 10 nM increased carboxylation significantly but increasing the inhibition further by using 100 nM AR-C122982 reduced carboxylation [50].

Decreased Carboxylation

Decreased carboxylation has been reported after pentylenetetrazole (PTZ) injection in rats [51]. PTZ administration is generally used as a seizure model, but when low doses are used it can cause anxiety [51]. Interestingly, the antiepileptic drug carbamazepine (CBZ) decreased biotin concentration in both humans and rats and decreased pyruvate carboxylase activity in rat brain [52]. These results support the use of biotin supplementation as a concurrent strategy during CBZ administration to help maintain pyruvate carboxylation and other important metabolic pathways.

Not surprisingly, ischemia has a profound effect on carboxylation, a pathway dependent on ATP concentration. In rats subjected to 120 min of middle cerebral artery occlusion followed by 120 min of reperfusion it could be shown that use of astrocytic precursors originating from the pyruvate carboxylase pathway was markedly reduced compared to the pyruvate dehydrogenase pathway in glutamate, and completely stopped in GABA in the ischemic core area [53]. Furthermore, in the reperfused penumbra glutamatergic and GABAergic neurons used relatively more astrocytic metabolites derived from the pyruvate carboxylase pathway than in the ischemic core [53]. The pyrrolopyrimidine lipid peroxidation inhibitor, U-101033E was administered to rats after middle cerebral artery occlusion and was shown to specifically decrease mitochondrial pyruvate metabolism via both pyruvate dehydrogenase and pyruvate carboxylase pathways [54]. In the canine cardiac arrest model of global ischemia carboxylation was decreased in the hippocampus in animals that had

undergone hyperoxic resuscitation [55]. Scafidi et al. [56], showed that labeling via PC was significantly decreased after traumatic brain injury in immature rats (a model of pediatric traumatic brain injury).

Qu et al. [57] showed that exogenous glutamate had an inhibiting effect on pyruvate carboxylation in astrocytes, presumably by formation of oxaloacetate from 2-oxoglutarate derived from glutamate. Inhibition of pyruvate carboxylation was also observed when glutamine was added to astrocytes [58]. When metabolism of ^{13}C labeled glucose and lactate was investigated in cultured mouse cerebellar astrocytes, it could be shown that carboxylation of pyruvate using glucose as the precursor was more important for biosynthesis of releasable glutamine and citrate, compared with their intracellular pools [59].

Incubation of brain slices with increasing amounts of the solvent dimethylsulphoxide (DMSO) which induces a workload on the mitochondria via an unknown mechanism showed decreased carboxylation of aspartate with increasing amounts of DMSO [60]. This was the case despite increasing flux through the TCA cycle and increased incorporation of label into glutamine via pyruvate dehydrogenase [60]. Thus, where the increased activity was not due to glutamatergic load, there was no increase (indeed there was a decrease) in pyruvate carboxylation.

Conclusion

Taken together, these data support the assumption that anaplerotic activity is increased due to glutamatergic activity but that loss of four carbon molecules due to GABAergic, mitochondrial or astrocytic activity is minimal, due to the reuptake of GABA into the synaptic terminal. It can be concluded that pyruvate carboxylation is an extraordinarily important predominantly astrocytic pathway.

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