Truncation of the Krebs Cycle During Hypoglycemic Coma

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Abstract: There is a misconception that hypoglycemic nerve cell death occurs easily, and can happen in the absence of coma. In fact, coma is the prerequisite for neuronal death, which occurs *via* metabolic excitatory amino acid release. The focus on nerve cell death does not explain how most brain neurons and all glia survive. Brain metabolism was interrogated in rats during and following recovery from 40 min of profound hypoglycemia using *ex vivo* ^{1}H MR spectroscopy to determine alterations accounting for survival of brain tissue. As previously shown, a time-dependent increase in aspartate was equaled by a reciprocal decrease in glutamate/glutamine. We here show that the kinetics of aspartate formation during the first 30 min (0.36 ± 0.03 μmol g^{-1} min^{-1}) are altered such that glutamate, *via* aspartate aminotransferase, becomes the primary source of carbon when glucose-derived pyruvate is unavailable. Oxaloacetate is produced directly from α-ketoglutarate, so that reactions involving the six-carbon intermediates of the tricarboxylic acid cycle are bypassed. These fundamental observations in basic metabolic pathways in effect redraw the tricarboxylic acid cycle from a *tri*carboxylic to a *di*carboxylic acid cycle during hypoglycemia. The basic neurochemical alterations according to the chemical equilibrium of mass action augments flux through a truncated Krebs cycle that continues to turn during hypoglycemic coma. This explains the partial preservation of energy charge and brain cell survival during periods of glucose deficiency.

INTRODUCTION

Historically, hypoglycemia was used as shock therapy for treating schizophrenia, where 30 minutes was the desired period of coma due to its survivability [1]. But 60 minutes converted reversible coma into irreversible coma, and patients would never regain consciousness. Such "treatment" has long since been abandoned, but the modern rise of diabetes and its intensive treatment have again made hypoglycemia common [2]. Although advances have been made in understanding neuronal *death*, understanding neuronal *survival* without glucose has remained incompletely understood. Glucose deprivation perturbs metabolism, but requires more than 30 minutes of flat EEG to cause significant neuronal death [3] which then appears in a specific distribution [4]. Brain awakening from coma after restitution of normoglycemia becomes impossible due to effective decortication after 60 min resulting from the sheer number of cortical nerve cells that have died. Even then, however, most brain neurons and all glia survive [5, 6].

Hypoglycemic brain damage was once thought to be due to glucose starvation and energy failure. But neuronal death during hypoglycemic coma is now known to occur by a more indirect route: release of excitatory amino acids. Glutamate and especially aspartate are released into the brain extracellular space when the EEG becomes flat [3, 7, 8]. The enormous increase in cellular aspartate occurs as part of a change in the metabolic homeostasis achieved during profound hypoglycemia, which partially preserves the energy charge at 25-30% of normal [9]. This is not enough to prevent cellular leakage of aspartate, which effects neuronal killing by activating excitatory receptors [10, 11]. This manuscript examines neurochemical flux by measuring brain metabolism. We describe fundamental alterations that effectively short circuit metabolites across the Krebs cycle, allowing it to still turn, thereby preserving brain cells during profound hypoglycemia.

Magnetic resonance (MR) spectroscopy is well suited for interrogating metabolism because a large number of metabolites in different pathways can be measured simultaneously with minimal sample preparation.

MATERIALS AND METHODS

Hypoglycemic Coma in Rats

All animals used in this study were treated in accordance with guidelines of the Canadian Council on Animal Care and local animal care committees approved all protocols. Male Sprague-Dawley rats weighing 150-170 g (Charles River Laboratories, Wilmington, MA) were intubated and mechanically ventilated with 0.7% halothane and a 2:1 mixture of N_2O/O_2. The femoral artery and vein each were cannulated to permit blood pressure monitoring and sampling of blood gases and glucose. Glucose was measured using a standard blood glucose meter (One Touch, Lifescan Inc, Milpitas, California). The cranium was exposed and wet gauze-covered for later freeze-funnel fixation. Brain temperature was monitored *via* a middle ear probe and maintained at 37.0 ± 0.2°C throughout the experiment by circulating warm or cold water through a water pad underneath the animal, supplemented with a radiant heat lamp. Hypoglycemic coma was induced using an *i.v.* infusion of insulin (Actrapid, 1-1.5 IU/kg in total) and EEG monitored throughout using bipolar electrodes placed in the temporalis muscle on either side of the cranium. At various durations of EEG silence (10, 20, 30 and 40 min, $n = 4$ for each) and periods of recovery (1 hr, 3 hr and 24 hr, $n = 4$ each) cerebral metabolism was arrested using the freeze-funnel fixation technique [12]. For animals allowed to recover, blood glucose was

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normalized following 40 min of EEG silence. Including a control group of four rats, a total of 32 animals were used.

Tissue Extraction and Magnetic Resonance Spectroscopy

Water-soluble metabolites were extracted from frozen neocortex samples (0.20 ± 0.05 g) thrice in 1.0 ml of 0.5 M perchloric acid [13, 14]. Each sample was then reconstituted in 0.70 ml D_2O (Cambridge Isotope Laboratories Inc., Andover, MA) with 3.0 µl of 30 mM sodium 3-(trimethylsilyl) propionate (DSS; Sigma, Oakville, ON) added as a chemical shift reference, pH-adjusted to 6.9-7.1 and transferred to a 5 mm MR tube for ^{1}H MR spectroscopic analysis. All spectroscopy was performed at 298 K on a Bruker AMX 500 MR spectrometer equipped with a commercial cryoprobe operating at a frequency of 500.13 MHz for ^{1}H. ^{1}H MR spectra were acquired using a 2 s low-power saturation pulse to diminish the residual water resonance followed by a 45° excitation pulse, with an interpulse delay of 10 s. Each free-induction decay consisted of 4 K complex data points at least 64 transients were collected.

Analysis of Magnetic Resonance Spectroscopic Data

The ^{1}H MR spectra were analyzed using a commercial MR analysis software package (NUTS, Acorn NMR Software, Livermore, CA). The free-induction decays were zero filled to 16 K complex data points and a 0.3 Hz Lorentzian function was applied prior to Fourier transformation. Metabolite concentrations were determined using integration in the frequency domain and all chemical shifts are in ppm relative to the DSS resonance (defined to be 0 ppm).

Statistics

Statistical comparisons of metabolite concentrations at each time point during and recovery from 40 min hypoglycemic coma were made using the two-tailed Student's *t*-test and the level of significance set at $P < 0.02$.

RESULTS

Control blood glucose levels were 4.0 ± 0.8 mmol/L (mean ± SD), levels at the beginning of coma were 0.9 ± 0.2 mmol/L, at the end of coma, 0.8 ± 0.2 mmol/L, and at one hour recovery had increased to 7 ± 2 mmol/L.

Hypoglycemia altered many metabolite concentrations in the ^{1}H MR spectra (Figs. **1-3**). High-energy phosphates, while decreased, remained visible and spectral quality allowed quantitation of energy metabolites, even those of very low concentration (Fig. **1**). Lactate and alanine, associated with glycolysis, were reduced in concentration (Fig. **2**). Several amino acids associated with the TCA cycle, including glutamate, glutamine and GABA, were significantly decreased with 40 min isoelectric EEG, while aspartate was significantly increased (Fig. **3**).

Over the first 30 min of isoelectric EEG changes in aspartate and glutamate tissue concentration was linear. The rate of increase in aspartate (0.36 ± 0.03 µmol g^{-1} min^{-1}) was significantly faster than the rate of decrease in glutamate (0.21 ± 0.03 µmol g^{-1} min^{-1}; $P = 0.003$), but was equal to the sum of the rates (0.35 ± 0.05 µmol g^{-1} min^{-1}) of disappearance of glutamine (0.13 ± 0.02 µmol g^{-1} min^{-1}) and glutamate (Fig. **4**). The tissue level of succinate, a TCA cycle intermediate, was increased slightly ($P = 0.004$) during hypoglycemic coma (Fig. **4**). Resonances due to the water-soluble fatty acids linoleate and linolenate and the branch-chain amino acid metabolite α-ketoisocaproate were abolished during coma (Figs. **2**, **4**).

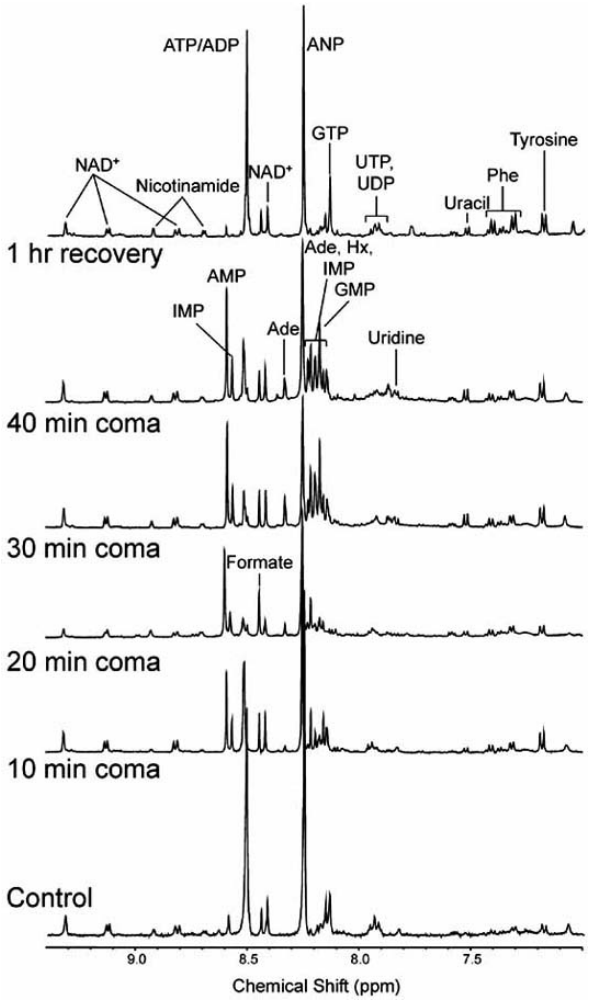

Fig. (1). Magnetic resonance spectra showing changes in high-energy phosphates during hypoglycemia. 500 MHz ^{1}H MR spectra of perchloric acid extracts of neocortex tissue from rats at various times during 40 min isoelectric EEG and 1 hr following normalization of blood glucose. The spectral region contains resonances from purine and pyrimidine phosphates, demonstrating their depletion and degradation. Changes in purines are rapidly reversed with glucose administration, while pyrimidines (lacking an inherent recovery mechanism) require longer periods for recovery.

Recovery of metabolite levels from hypoglycemic coma was varied. While energy charge and metabolic breakdown products of ATP and GTP (adenosine, guanosine, hypoxanthine, AMP and IMP) were recovered by 1 hr, total purine phosphates were lower than pre-coma level (Figs. **1**, **4**). Uracil, a breakdown product of UTP, remained elevated at 1 hr (Figs. **1**, **4**). Amino acid levels recovered rapidly to pre-coma values, but compounds associated with other cellular metabolic pathways required longer periods for full recovery

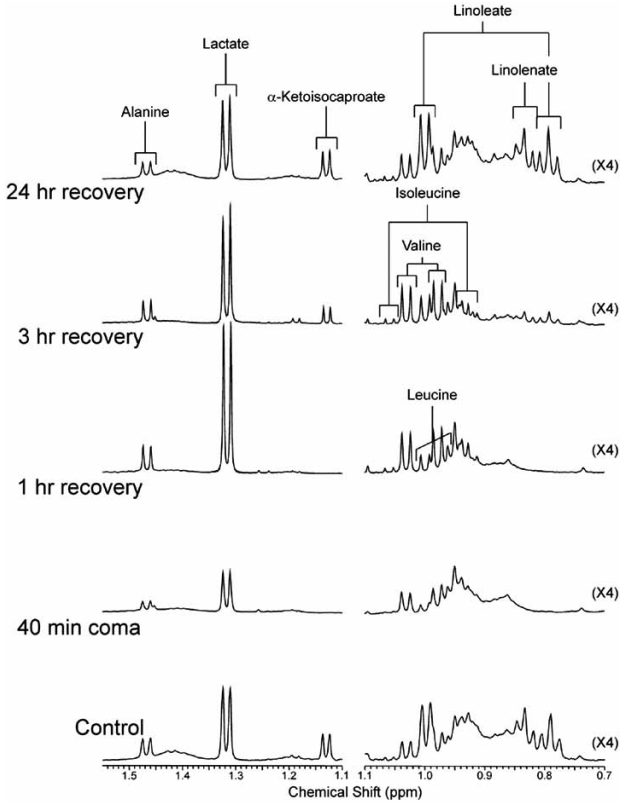

Fig. (2). Magnetic resonance spectra showing changes in various metabolites during hypoglycemia. 500 MHz ^{1}H MR spectra of perchloric acid extracts of neocortex tissue from rats at the end of 40 min isoelectric EEG and at various times following normalization of blood glucose. The spectral region contains resonances from lactate and alanine (left), and fatty acids, branch-chain amino acids and their metabolites (right, X4). Note the delay in recovery of α-ketoisocaproate and linoleic and linolenic acids.

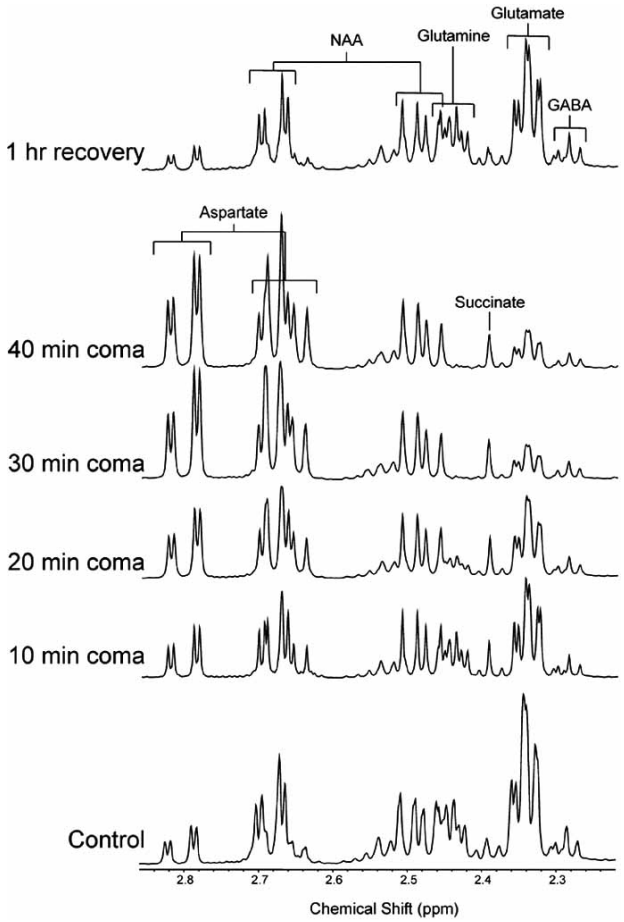

Fig. (3). Magnetic resonance spectra showing changes in amino acids during hypoglycemia. 500 MHz ^{1}H MR spectra of perchloric acid extracts of neocortex tissue from rats at various times during 40 min isoelectric EEG and 1 hr following normalization of blood glucose. The spectral region contains resonances from various amino acids and succinate, demonstrating the conversion of glutamate to aspartate and the depletion of glutamine during severe hypoglycemia, with rapid recovery with glucose administration.

(Figs. **3**, **4**). Branched-chain amino acid and fatty acid metabolism, indicated by respectively α-ketoisocaproate and linoleate/linolenate, remained severely compromised at 1 hr and was only just beginning to recover at 3 hr following glucose administration (Figs. **2**, **4**).

DISCUSSION

The results account for why the majority of neurons and glia survive relatively prolonged episodes of hypoglycemic coma. While there is a significant decline in brain energy metabolism the TCA cycle continues to turn through formation of α-ketoglutarate from oxaloacetate *via* aspartate aminotransferase. The reaction results in the formation of aspartate from glutamate and accounts for the known time-dependent increase in aspartate with a reciprocal decrease in glutamate/glutamine.

High-Energy Phosphate Metabolism

Unlike ischemia, energy metabolism does not collapse completely during hypoglycemia, falling to only 25-30% of normal [8]. Decreases in high-energy phosphates, particularly ATP, result in a decrease in the total adenine nucleotide pool and increases in nucleoside phosphates [15]. Release of these into the extracellular space may be a cause of disrupted synaptic transmission [16, 17]. Hydrolysis of ATP to AMP and GTP to GMP during hypoglycemic coma results in a diversionary cascade reducing the availability of purines upon recovery with glucose. AMP is converted to adenosine, which forms inosine, or IMP, which can in turn be converted into a number of other compounds such as inosine, XMP and succinyladenosine. Inosine forms hypoxanthine, and thence xanthine, finally leading to the dead-end products urate and, in lower mammals such as the rat, urea. Similarly, GMP can be converted to guanosine and guanine, which can then form xanthine and thereby urate. We could not quantify urate, however, since with no protons in its chemical structure it is invisible to ^{1}H MR spectroscopy. Pyrimidines follow a different pathway. While no thymidine was detected, uridine (formed through hydrolysis of UTP, UDP and UDPG) forms uracil.

While energy charge and metabolic breakdown products of ATP and GTP (adenosine, guanosine, hypoxanthine, AMP

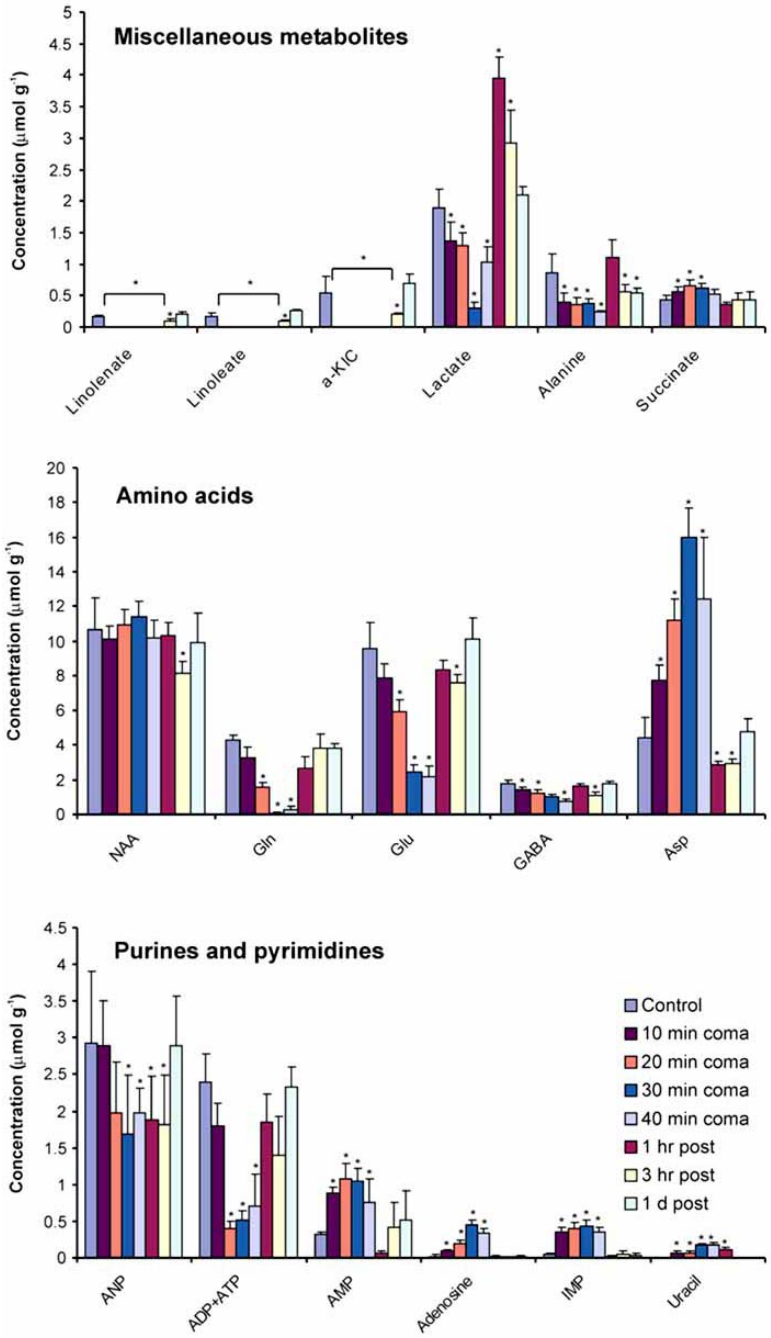

Fig. (4). Hypoglycemia-induced alterations in metabolite concentrations. Changes in cerebral tissue concentrations (means ± s.d.) of purines and pyrimidines (bottom), amino acids (middle) and various metabolites (top) with insulin-induced EEG silence and their recovery following return to normoglycemia. Asterisk indicates statistical differences from the naïve state ($P < 0.02$).

and IMP) recover by 1 hr, total purine phosphates are lower than pre-coma level for up to 24 hr. Uracil, a breakdown product of UTP, remains elevated for at least several hours. The difference in recovery times of purines and pyrimidines is likely due to differences in the rates of the purine and pyrimidine recovery mechanisms. AMP is recovered from IMP *via* an intermediate (adenylosuccinate) rather than through direct conversion, a pathway known as the purine nucleotide cycle [18]. The aspartate required for this process is initially available in high concentration upon glucose administration, facilitating recovery of adenine and guanidine nucleotide phosphates. Cerebral tissue is thus well suited to rapidly recover purine phosphates after hypoglycemia. In contrast, replenishment of pyrimidines is slower, occurring from uracil metabolism through uridine, with ribose-1-phosphate as the ribose donor [19].

Fatty Acids, Branched-Chain Amino Acids and Lactate Metabolism

Linoleate and linolenate are rapidly depleted during EEG silence. Fatty acids become a rich source of energy, producing ~9 kcal g^{-1} in comparison to ~4 kcal g^{-1} for carbohydrates and proteins. Their oxidation produces $FADH_2$ and NADH as well as a source of carbon for entry into the TCA cycle as acetate. α-Ketoisocaproate can also serve as a source of carbon for the TCA cycle, being converted to acetyl CoA and acetoacetate, the latter also capable of producing acetate for the TCA cycle.

These observations confirm that tissue makes use of any and all potential sources of carbon for entry into the TCA cycle under conditions of hypoglycemia. The delay in recovery of fatty acids and α–ketoisocaproate may be due to time necessary for leucine and fatty acids to cross the blood brain barrier. Linolenic and linoleic acids serve as precursors for the synthesis of longer chain polyunsaturated fatty acids such as docosahexaenoic acid, necessary for the formation of cell membranes. The delay here found in their availability, may have important implications for membrane repair in the recovery period. Also observed are coma-induced decreases in lactate and alanine, metabolites associated with glycolysis. Following glucose administration, lactate is elevated for at least 3 hr, matching post-coma decreases in pyruvate dehydrogenase activity.

Amino Acid Metabolism

The most profound changes in the ^1H MR spectrum are associated with amino acid tissue metabolism. Unlike ischemia, where glutamate, glutamine and aspartate remain unchanged due to total arrest of the TCA cycle, sustained metabolism of these amino acids, continues during hypoglycemia. Significant decreases in glutamate, glutamine and GABA and an increase in aspartate, associated with the TCA cycle, are known to occur within 5 min of the onset of a flat EEG [20]. During the first 30 min of flat EEG, changes in concentration of aspartate and glutamine+glutamate were linear, demonstrating zero order kinetics over this interval. The rate of aspartate formation closely matched the sum of the rates of disappearance of glutamate and glutamine, consistent with the actions of glutamine synthetase and aspartate aminotransferase. This implies that an effective truncation of the Krebs cycle is part of the metabolic homeostasis achieved during hypoglycemia. Glutaminase, located in mitochondria [21], rather than the cytosolic glutamine synthetase, converts glutamine into glutamate and would account for the ammonia release accompanying hypoglycemia [22].

The main source of carbon for the TCA cycle is normally glucose. The status quo achieved by the brain can be generally characterized as the tissue replenishing its TCA cycle intermediates by scavenging compounds associated with the TCA cycle. As the source of acetate is severely limited, maintenance of citrate concentration through the condensation of oxaloacetate and acetate is no longer possible. A potential source of carbon is the large glutamate pool and, through its deamination, the glutamine pool. There are two routes by which glutamate may be used to replenish α-ketoglutarate: *via* the direct route (glutamate dehydrogenase) or through the action of various aminotransferases. Under physiological conditions, aspartate aminotransferase is the most important [23]. The rate of formation of aspartate observed here is equal to the sum of the rates of decrease in glutamate and glutamine, indicating that aspartate aminotransferase is the major source of the alteration of their concentrations during severe hypoglycemia as well. If glutamate were instead metabolized directly to α-ketoglutarate *via* glutamate dehydrogenase, the tissue would gain one more NADH (or NADPH) for every glutamate utilized, but the reaction would end at oxaloacetate rather than turning a truncated Krebs cycle. The neurochemical explanation for the increased aspartate in hypoglycemia that has been generally accepted, is the law of mass action from increased oxaloacetate. No accumulation of oxaloacetate was observed, indicating that a re-thinking of this chemical mechanism is necessary.

Adaptation of Cerebral Metabolism to Severe Hypoglycemia

While limited availability of glucose is the cause of the large alterations in metabolite concentrations, the event signaling the onset of these changes is EEG silence [3]. Conversion of glutamate to aspartate occurs only under conditions of severe hypoglycemia accompanied by a flat EEG [24]. The other substrate necessary for this reaction in the formation of aspartate from glutamate is another TCA cycle intermediate, oxaloacetate, so there is no net increase in these intermediates over a full turn of the cycle. However, several reactions in the TCA cycle produce the reducing molecules NADH and $FADH_2$ in the path from α-ketoglutarate to oxaloacetate, which staves off energy failure while the conversion of glutamate to aspartate remains significant. Thus under these conditions the TCA cycle is truncated (Fig. **5**), removing reactions involving six carbon intermediates. If the action of aspartate aminotransferase is the primary source of α-ketoglutarate then the rate of production of aspartate is also the rate of this smaller cycle. While the truncated version of the TCA cycle we describe here during hypoglycemia turns more slowly than under physiological conditions (about 0.5-0.7 μmol g^{-1} min^{-1} in rat brain [25-27], the rate is still higher than the rate of the glial TCA cycle (0.14 μmol g^{-1} min^{-1} in human brain [28]). Combined with the low availability of glucose-derived pyruvate, it is clear that under these conditions the large metabolic (not synaptic) glutamate pool becomes the major energy source.

Upon restitution of plasma glucose levels, recovery of glutamate, and hence glutamine *via* glutamate-glutamine cycling, remains limited for a significant period [29]. While glucose is the major source of carbon for glutamate and glutamine, a significant fraction of amide for glutamate is supplied by branched-chain amino acids. Leucine alone serves as a significant source of nitrogen (25-30% of total glutamate nitrogen [30]). It is likely that the delay in glutamate recovery is at least partly due to limited availability of leucine and other branched chain amino acids, and would also account for the delay in recovery of α-ketoisocaproate concentration.

The TCA cycle is the final common pathway for oxidization of molecular fuels, where entry of carbohydrates occurs *via* acetyl CoA. During severe hypoglycemia, glucose is lim-

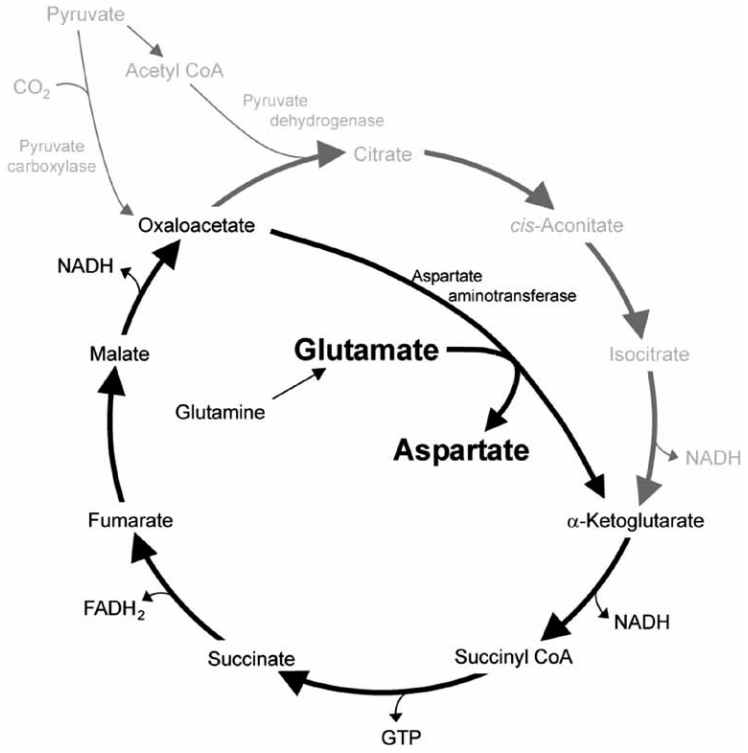

Fig. (5). Model describing adaption of cerebral metabolism during severe hypoglycemia. Under normoglycemic conditions, glucose is metabolized to pyruvate and can enter the tricarboxylic acid (TCA) cycle through the action of either pyruvate dehydrogenase or (in the case of glia) pyruvate carboxylase. Under conditions of severe hypoglycemia, the availability of pyruvate is limited. Cerebral tissue is able to maintain a limited ability for oxidative phosphorylation by the production of reducing compounds (NADH and $FADH_2$) by metabolizing glutamate (and glutamine) using enzymes from part of the TCA cycle. This model removes reactions involving the six-carbon intermediates (citrate, *cis*-aconitate and isocitrate) with limited entry of carbon from pyruvate, but can only partially sustain metabolism so long as a source of glutamate is available.

ited as the main source of energy, and maintenance of the Krebs cycle occurs to a surprising extent from other sources. While fatty acids supply some energy, the brain draws upon its large glutamate pool to replenish Krebs cycle intermediates for as long as this carbon source is available. But the entry point for carbon is no longer acetyl CoA and with glucose availability limited, formation of six-carbon tricarboxylic acid intermediates cannot be maintained. The aspartate aminotransferase reaction obviates the need for reactions involving six-carbon intermediates, at the expense of only a 1/3 reduction in NADH production. This steady state enables the brain to still produce reducing compounds for a limited level of oxidative phosphorylation and serves to maintain energy metabolism. This metabolic homeostasis during hypoglycemic coma is achieved by a truncation of the TCA cycle from a tri- to a *di*carboxylic acid cycle.

This mechanistically explains how the mammalian brain is able to survive without a glucose-derived source of carbon for the TCA cycle.

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