Scavenging of blood glutamate for enhancing brain-to-blood glutamate efflux

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Abstract. The presence of excess glutamate in the brain interstitial fluid characterizes several acute pathological conditions of the brain, including traumatic brain injury and stroke. It has been demonstrated that it is possible to eliminate excess glutamate in the brain by decreasing blood glutamate levels and, accordingly, accelerating the brain-to-blood glutamate efflux. It is feasible to accomplish this process by activating blood resident enzymes in the presence of the respective glutamate cosubstrates. In the present study, several glutamate cosubstrates and cofactors were studied in an attempt to identify the optimal conditions to reduce blood glutamate levels. The administration of a mixture of 1 mM pyruvate and oxaloacetate (Pyr/Oxa) for 1 h decreased blood glutamate levels by ≤50%. The addition of lipoamide to this mixture resulted in a further reduction in blood glutamate levels of >80%. In addition, *in vivo* experiments showed that lipoamide together with Pyr/Oxa is able to decrease blood glutamate levels to a greater extent than Pyr/Oxa alone, and accordingly, this enhances the glutamate efflux from the brain to the blood. These results may outline a novel neuroprotective strategy with increased effectiveness for the removal of excess brain glutamate in various neurodegenerative conditions.

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Introduction

Glutamate, the major excitatory amino acid neurotransmitter in the central nervous system, is involved in the pathological process of excitotoxicity in which neuronal cells are damaged and killed by excess glutamate present in the brain fluid following various acute and chronic neurodegenerative disorders, including stroke, traumatic brain injury, amyotrophic lateral sclerosis, brain tumors, and human immunodeficiency virus (HIV) dementia. Accordingly, strategies to interfere with glutamate activity, including the inhibition of glutamate synthesis, blocking its release from presynaptic terminals, antagonizing its interactions with postsynaptic receptors and accelerating its reuptake from the synaptic cleft, have predominated recent research in the area of neuroprotection. However, all of these strategies have proved to be ineffective (1,2).

The failures of the aforementioned strategies prompted the proposal of an alternative approach to the neutralization of the deleterious effects of glutamate, which is to cause an increased pumping of brain cerebrospinal fluid (CSF) glutamate into the blood in order to accelerate glutamate efflux from the brain into the blood across the blood-brain-barrier. Several examples are available, which show that the decrease of amino acid levels in the blood reduces their concentration in the CSF in parallel. The administration of asparaginase to the blood decreases asparagine levels in plasma and in the CSF (3). To achieve a similar objective for glutamate, it was hypothesized that it may be feasible to boost the brain-to-blood efflux of glutamate by decreasing its levels in the blood.

In previous studies (4,5), we have demonstrated that such a decrease occurs *in vitro* and *in vivo*, upon activation of the blood resident enzymes glutamate-pyruvate transaminase (GPT) and glutamate-oxaloacetate transaminase (GOT), which transform glutamate into α-ketoglutarate in the presence of the respective glutamate cosubstrates, pyruvate (Pyr) and oxaloacetate (Oxa). The activation of GPT by Pyr and

GOT by Oxa causes a decrease in the blood levels of glutamate. This decrease was observed to accelerate the efflux of glutamate from the brain to the blood by increasing the driving force for the brain-to-blood glutamate efflux, and thereby causing a decrease in glutamate levels in the CSF. This strategy was assessed in animal models of closed head injury, stroke, brain glioma and amyotrophic lateral sclerosis, in which it demonstrated highly significant neuroprotective effects (4).

As the ability of Pyr/Oxa to decrease blood glutamate is limited, with the maximum reduction being 50%, the present study investigated the effects of certain GPT- and GOT-related compounds, in regard to their blood glutamate scavenging ability, in order to achieve optimal conditions for further decreasing the blood levels of glutamate.

Materials and methods

Chemicals. All chemicals were purchased from Sigma-Aldrich (Shanghai, China). The procedures of the animal experiments performed in this study were approved by the Animal Care Committee of Ningxia Medical University (Ningxia, China).

In vitro blood glutamate scavenging. Blood was collected retroorbitally from specific pathogen-free Sprague Dawley (SD) rats weighing 200-250 g, which were obtained from the Animal Center of Ningxia Medical University (Ningxia, China). The animals were incubated at 37˚C in the presence of injected Pyr, Oxa or other chemicals at different concentrations. Blood aliquots were then taken at different time-points in order to measure the glutamate levels. In order to study the depletion of glutamate from the blood cell pool, the blood was centrifuged at 1,300 x g for 7 min at 4˚C and the cell pellet was resuspended in Ringer-HEPES buffer containing 2.75 mM glucose, 5 mM HEPES, 0.15 M NaCl, 2 mM $CaCl_2$, 0.2 mM $MgCl_2 \cdot 6H_2O$, 5 mM KCl and 6 mM $NaHCO_3$ (pH 7.4). It was then washed three times by centrifugation and resuspension. Glutamate depletion was achieved by supplementing the blood cell pool, maintained at 37˚C, with 1 mM Pyr/Oxa or other chemicals added every 15 min, and the glutamate value was measured at different time-points.

Glutamate analysis. Blood samples were deproteinated by adding an equal volume of ice-cold 1 M perchloric acid (PCA) and then centrifuging the samples at 16,000 x g for 10 min at 4˚C. The pellet was discarded and the supernatant collected, adjusted to pH 7.2 with 2 M K_2CO_3, and, when required, stored at -20˚C for later analysis. The glutamate concentration was measured in the supernatant using the fluorometric method by Graham and Aprison (6). A 20 μl aliquot of the PCA supernatant was added to 480 μl reaction buffer containing 15 units of glutamate dehydrogenase in 0.2 mM nicotinamide adenine dinucleotide (NAD), 0.3 M glycine and 0.25 M hydrazine hydrate adjusted to pH 8.6 with 1 N H_2SO_4. Following incubation for 30-45 min at room temperature, fluorescence was measured at 460 nm with excitation at 350 nm. A glutamate standard curve was established with concentrations ranging between 0 and 6 μM. All determinations were performed at least in duplicate. The results are expressed as the mean ± standard deviation.

Intravenous injections. In accordance with a previously described method (4), female SD rats (250-300 g) were anesthesized by intraperitoneal injection of urethane (0.125 g/0.2 ml per 100 g body weight). Catheterization of the tail vein (for drug injections) and of the femoral vein (for blood aliquot withdrawals) were performed using PE10 polyethylene tubing linked to PE50 polyethylene tubing (Smiths Medical, Barking, UK). All catheters were secured with 5-0 silk thread (Smiths Medical) and flushed with heparin (3-5 μl of 182 U/ml). The body temperature was maintained using a lamp and the rectal temperature was monitored. Intravenous injections of Pyr/Oxa and lipoamide diluted in phosphate-buffered saline (PBS) were performed at a rate of 0.05 ml/min for 30 min with a Pharmacia pump P-1 (Pharmacia, Uppsala, Sweden). During and following the injections, aliquots of 150 μl blood were removed from the femoral vein every 15 min.

Intracerebroventricular injections. A steel cannula made from a 27G needle was implanted into the right lateral ventricle of the rat using the following stereotactic coordinates: 0.8 mm posterior to the bregma; 1.4 mm lateral from the midline; 4 mm below the skull surface and 3.5 mm from the dura. A total volume of 11 μl [^{3}H]glutamate solution in PBS was injected into the lateral ventricle in ~3 min through the implanted cannula using a Hamilton syringe (25 μl) connected to a PE20 tube filled with the solution. For radioactivity determination, 50-μl blood samples, collected from the femoral vein, were diluted in 500 μl H_2O and added to a 16-ml scintillator (PerkinElmer Lifesciences, Waltham, MA, USA). The measured counts per minute (cpm) values were corrected for quenching as determined by comparing the measured cpm of a set volume of [^{3}H] glutamate added to water or to diluted blood.

Statistical analysis. Results are presented as the mean ± standard error of the mean. Data were analyzed by Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Scavenging blood glutamate with Pyr/Oxa in vitro. The optimal concentration and time-point for minimizing blood glutamate levels with Pyr/Oxa were evaluated. Fig. 1 illustrates the changes in blood glutamate levels upon addition of various concentrations of the mixture of Pyr/Oxa, at t = 0, 20, 30, 40, and 60 min. The results show that the blood glutamate levels were reduced by ≤50% by basal levels of the blood resident transaminases GPT and GOT with addition of a mixture of 1 mM Pyr/Oxa for 60 min. Pyr/Oxa act in synergy to decrease glutamate levels but their effects are limited by the build-up of α-ketoglutarate, which facilitates the back reaction of glutamate formation.

Impact of cofactors on glutamate depletion. Blood contains resident enzymes, such as GPT (substrate, Pyr), GOT (substrate, Oxa) and glutamate dehydrogenase (substrate, GDH). In the presence of their substrates, glutamate is transformed into α-ketoglutarate by these transaminases (Fig. 2). The products and cofactors of these reactions may affect the efficiency of the glutamate conversion.
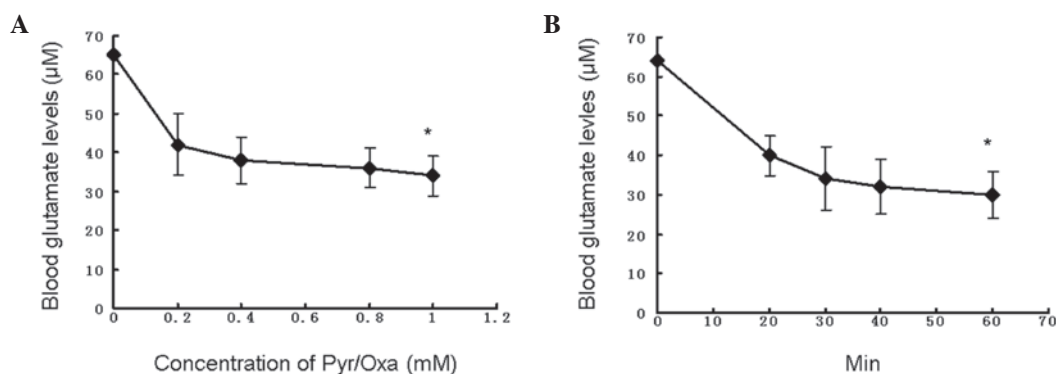
Figure 1. *In vitro* scavenging of blood glutamate upon supplementation with various concentrations of pyruvate and oxaloacetate (Pyr/Oxa) over (A) 60 min or (B) at different time-points. Each point represents the average of eight experiments ± standard error of the mean. *$P<0.05$ compared with basal glutamate level.

In the presence of Pyr, blood glutamate is converted to α-ketoglutarate by GPT, and Pyr to alanine (Fig. 2). However, this process is reversible as the enzyme operates equally well in both directions. As shown in Fig. 3, the addition of alanine diminished the ability of Pyr to decrease the glutamate concentration. As the concentration of alanine was increased, the glutamate conversion into α-ketoglutarate was reduced.

In regard to the fact that glutamate is converted into α-ketoglutarate and that the latter is able to drive the GPT- and GOT-catalyzed back reactions, thereby limiting the conversion of glutamate, the present study investigated whether α-ketoglutarate, the common coproduct of GPT and GOT, was able to serve as a substrate of α-ketoglutarate dehydrogenase, which converts α-ketoglutarate into succinyl coenzyme A (CoA) in the presence of CoA and NAD (Fig. 2). Therefore, the consequences of the addition of CoA and NAD on glutamate conversion were assessed. In addition, the effects of the addition of pyridoxal phosphate, which is the cofactor of GPT/GOT, and of cocarboxylase and lipoamide, which are cofactors of α-ketoglutarate dehydrogenase (Fig. 2), were examined. As shown in Fig. 4, the extent of glutamate scavenging in the blood increases with increasing concentration of Pyr/Oxa. Pyridoxal phosphate exerted a marginal effect, while cocarboxylase decreased the glutamate conversion. However, lipoamide increased the conversion from a value of 55% in the control (1 mM Pyr/Oxa) to 83%.

Since, as observed in Fig. 4, lipoamide appeared to contribute to the scavenging of blood glutamate, the dose-efficiency correlation was investigated by applying various concentrations of lipoamide to blood. Fig. 5 shows that a maximal extent of glutamate conversion was obtained with 100 μM lipoamide.

As blood contains, in addition to GPT and GOT, other enzymes, such as GDH, the possible contribution of GDH to the scavenging of glutamate was investigated. GDH is a multimeric enzyme that uses NAD or nicotinamide adenine dinucleotide phosphate (NADP) as cofactors to transform glutamate into α-ketoglutarate and ammonia. It is allosterically activated by leucine and ADP (Fig. 2). Therefore, the glutamate scavenging effects of NAD, ADP and leucine (all at 1 mM) were assessed. Fig. 6 illustrates the effects of the repeated addition of 1 mM NAD to blood on glutamate levels in the cell and plasma compartments: While a decrease in glutamate levels was found to occur in the blood cells, an increase in glutamate
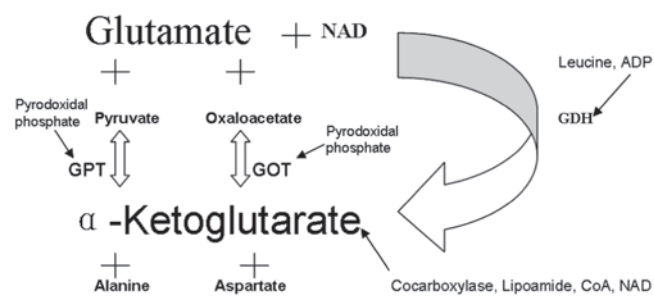

Figure 2. Schematic of the chemical reactions leading to glutamate depletion. GPT, glutamate-pyruvate transaminase; GOT, glutamate-oxaloacetate transaminase; NAD, nicotinamide adenine dinucleotide; GDH, glutamate dehydrogenase; CoA, coenzyme A.
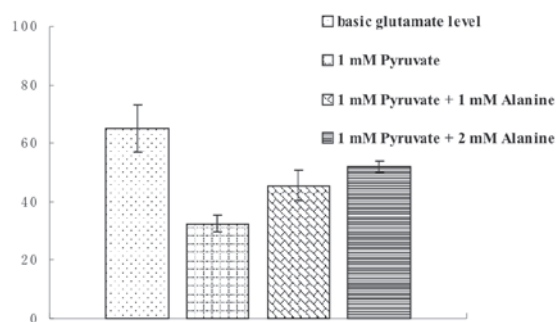

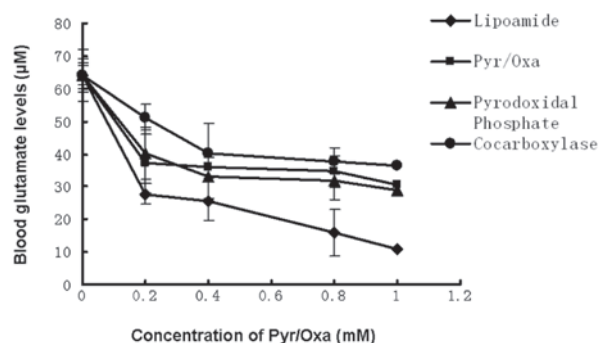Figure 3. Impact of alanine on glutamate transamination.

Figure 4. Impact of cofactors together with various concentrations of pyruvate and oxaloacetate (Pyr/Oxa) on glutamate scavenging in blood. Data for one representative experiment out of five performed are shown.
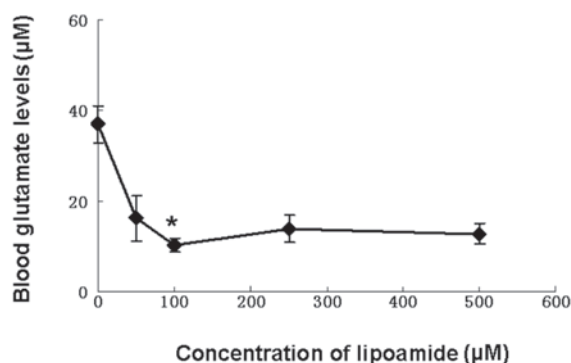
Figure 5. Blood glutamate scavenged by 1 mM pyruvate and oxaloacetate (Pyr/Oxa) together with different concentrations of lipoamide. *P<0.05 compared with 1 mM Pyr/Oxa alone.

Figure 8. Glutamate levels in blood following repeated addition of 1 mM pyruvate (Pyr), oxaloacetate (Oxa) or nicotinamide adenine dinucleotide (NAD) or their combinations every 15 min for 60 min.
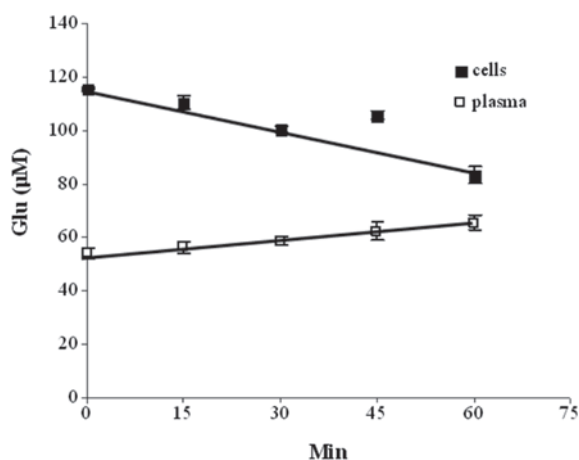

Figure 6. *In vitro* glutamate evolution in blood plasma and blood cells upon repeated additions of 1 mM nicotinamide adenine dinucleotide (NAD) every 15 min for 1 h. Glu, glutamic acid.
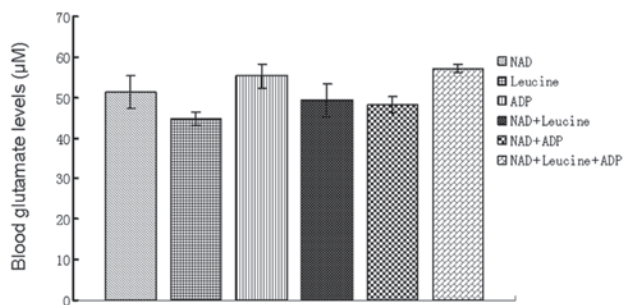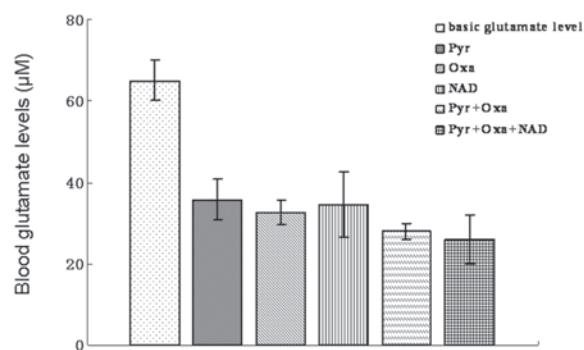

Figure 7. Glutamate levels in blood following repeated addition of 1 mM nicotinamide adenine dinucleotide (NAD), leucine, adenine dinucleotide phosphate (ADP) or their combinations every 15 min for a total duration of 60 min.

levels occured in the plasma. These results may be interpreted as NAD possibly activating GDH in the cellular compartment, with the α-ketoglutarate produced being released from the cells and converted back into glutamate via the GPT or GOT present in the plasma. In all instances of blood being incubated with NAD, leucine or ADP either separately or in combination, increases in plasma glutamate levels occurred (Fig. 7). However, systemic decreases in the cellular compartment were

observed when NAD was present alone or in combination with leucine and ADP, while the latter did not significantly affect glutamate levels alone (data not shown). When blood was incubated with a mixture of NAD, Pyr and Oxa, glutamate levels decreased to a marginally greater extent than with Pyr or Oxa alone (Fig. 8).

Scavenging of blood glutamate by Pyr/Oxa and lipoamide in vivo. Previously, we reported that upon continuous intravenous administration of Pyr/Oxa, blood, as well as CSF/interstitial fluid glutamate levels were reduced to ≤50% (4). Since the present *in vitro* study demonstrated that by administration of lipoamide together with Pyr/Oxa, it was possible to further reduce blood glutamate levels by 83% compared with 50% for Pyr/Oxa alone, an *in vivo* study was performed to examine whether Pyr/Oxa together with lipoamide was able to further reduce blood glutamate levels compared with the effect of Pyr/Oxa alone. Fig. 9 illustrates the effect of Pyr/Oxa alone or together with lipoamide administered continuously through an intravenous catheter at a rate of 50 μmol/min for Pyr/Oxa and 100 μmol/min for lipoamide for a duration of 30 min. A marked decrease (55% for Pyr/Oxa and 70% for Pyr/Oxa/lipoamide) in blood glutamate occurred after 15 min. This is consistent with the *in vitro* results. However, as soon as the administration of Pyr/Oxa and lipoamide was ceased, the blood glutamate levels increased. When the levels of glutamate were monitored for ~200 min following the completion of the injection of Pyr/Oxa and lipoamide, glutamate levels clearly exceeded basal blood levels, suggesting that the increase in glutamate levels is not only due to the back reactions as a result of the build-up of the enzymatic products, α-ketoglutarate, alanine and aspartate, but possibly to additional compensatory processes causing various organs to release their glutamate content into the plasma to adjust to the novel conditions.

Impact of blood glutamate scavenging on the brain-to-blood glutamate efflux. To monitor the fate of excess glutamate in the CSF, a bolus injection of 10 μCi [^{3}H]Glu (230 pmol glutamate/10 μl) was administered to the lateral ventricles of rats and the appearance of radioactivity in the blood was monitored in relation to time. Fig. 10 shows the changes in radioactivity levels in the blood upon intracerebroventricular injection of 10 μCi [^{3}H]glutamate followed by blood glutamate scavenging by Pyr/Oxa and lipoamide. As soon as a constant level of
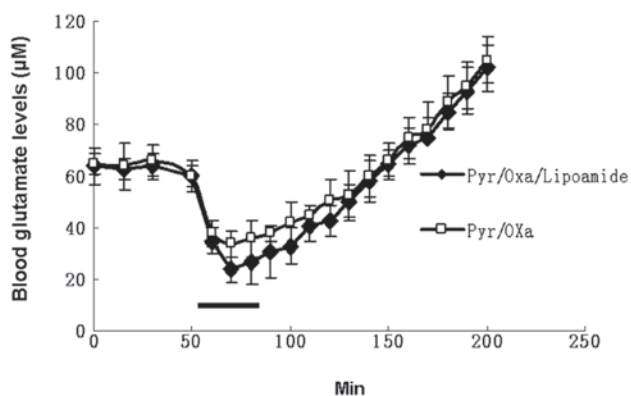
Figure 9. Evolution of blood glutamate levels *in vivo* upon intravenous administration for a duration of 30 min (black bar) of pyruvate and oxaloacetate (Pyr/Oxa) alone or with lipoamide.
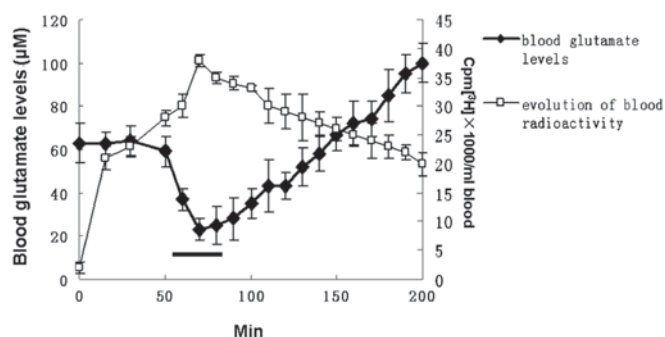

Figure 10. Impact of blood glutamate scavenging by pyruvate/oxaloacetate/lipoamide on the brain-to-blood [^{3}H]glutamate efflux upon intravenous administration for a duration of 30 min (black bar).

radioactivity was reached in the blood, blood glutamate levels were transiently decreased by the intravenous administration of Pyr/Oxa and lipoamide. The changes in blood radioactivity levels originating from the brain mirrored those of blood glutamate. While the latter decreased by ~50% during the administration of Pyr/Oxa with lipoamide and then increased, the blood radioactivity increased by ~40% and then decreased.

Discussion

The neuroprotective properties of pyruvate have been thoroughly studied (7-12); however, they have not been used in the clinic. Our previous studies revealed that it is possible to regulate blood glutamate levels by activation of the blood resident enzymes GPT and GOT with their respective substrates Pyr and Oxa (4,5). This blood glutamate decrease was observed to accelerate the efflux of glutamate from the brain to the blood, thereby causing a decrease in glutamate levels in the CSF by increasing the driving force of the brain-to-blood glutamate efflux. Due to the ability of Pyr/Oxa to decrease blood and CSF glutamate levels, these natural GPT and GOT cosubstrates are potentially suitable for therapeutic applications in the context of the large number of neurodegenerative conditions, in which excess CSF glutamate has been indicated to exert a crucial excitotoxicity. In this study, the optimal conditions for Pyr/Oxa to reduce blood glutamate levels were investigated,

and numerous cofactors of these reactions for glutamate degeneration were assessed in an attempt to further reduce glutamate levels in blood concomitant with the decrease in glutamate levels in CSF.

The results show that the blood glutamate levels were reduced by ≤50% with 1 mM Pyr/Oxa administered over 60 min. The GTP-catalyzed conversion reaction of glutamate and pyruvate to α-ketoglutarate and alanine is reversible. With the accumulation of α-ketoglutarate and alanine, the reaction is inhibited. Consistent with this, the results of the present study demonstrate that the addition of alanine inhibits the ability of pyruvate to reduce glutamate levels. α-ketoglutarate is the most common product of GPT and GOT. The reduction or removal of α-ketoglutarate shifts the reaction towards the conversion of glutamate. α-ketoglutarate dehydrogenase is another resident enzyme in the blood, which converts α-ketoglutarate into succinyl CoA in the presence of CoA and NAD. It was shown that CoA and NAD have no marked effects on the glutamate conversion by Pyr/Oxa, and cocarboxylase, a cofactor of α-ketoglutarate dehydrogenase, decreases glutamate conversion. However, lipoamide, the other cofactor of α-ketoglutarate dehydrogenase, exerts a significant effect on blood glutamate levels and decreases them by >80% compared with 50% for Pyr/Oxa alone. In the *in vivo* experiments, blood glutamate levels were reduced to 30% of the basal level and the efflux of glutamate from the brain to the blood was enhanced upon intravenous administration of Pyr/Oxa and lipoamide.

Lipoic acid is an essential cofactor for numerous enzyme complexes. One of the most visible roles of lipoic acid is as a cofactor in aerobic metabolism, specifically in the pyruvate dehydrogenase complex. Lipoamide is the functional form of lipoic acid in which the carboxyl group is attached to protein by an amide linkage to a lysine amino group. Lipoate was initially termed the pyruvate oxidation factor (POF) by Irwin C. Gunsalus (13). The structure was determined by Gunsalus *et al*, and the synthetic compound designated as α-lipoic acid proved to be the correct molecule (13). Lipoic acid has a number of uses. It has been consumed as a dietary supplement since the early 1990s and has been successfully used for the treatment of chronic liver disease (viral hepatitis, autoimmune hepatitis) (14). A chronic toxicity/carcinogenicity study in rats revealed that racemic lipoic acid was noncarcinogenic and there was no evidence of target organ toxicity (15). In addition, due to the ability of lipoic acid to modify gene expression by stabilizing the transcription factor nuclear factor-κB, Berkson (16) used it for the treatment of various cancers for which no effective treatments existed. Lipoic acid was also suggested to be an effective antioxidant when its ability of preventing the symptoms of vitamin C and vitamin E deficiency was revealed. Lipoic acid has been shown to have an important neuroprotective role in numerous brain injury conditions (17-25). The present study shows that that lipoamide is able to further decrease blood glutamate levels together with Pyr/Oxa *in vivo* and *in vitro*. The results provide further evidence for the neuroprotective role of lipoic acid.

The validity of the neuroprotective strategy of using Pyr/Oxa to reduce blood and CSF glutamate levels has been tested by Dr V.I. Teichberg in several animal models of neurodegenerative conditions, including head trauma, stroke, glioma and exposure to neuronal agents (26). These studies obtained

promising results, which established the proof of concept that blood glutamate scavenging provides highly effective neuroprotection against acute and chronic neurodegenerative conditions. Lipoamide appears to enhance the effects of Pyr/Oxa; however, the validity of this finding requires further investigation.

The present study further demonstrated that blood glutamate scavenging enhances glutamate efflux from brain to blood and is therefore likely to provide highly effective neuroprotection against acute and chronic neurodegenerative conditions.

Acknowledgements

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