An Evaluation of Erythrocytes as Plasma Glutamate Scavengers for Enhanced Brain-to-Blood Glutamate Efflux

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Several acute brain pathological conditions are characterized by the presence of excess glutamate in brain interstitial fluid. We have previously shown that decreasing blood glutamate levels increases the driving force for an enhanced brain-to-blood efflux of glutamate. The present study investigated the glutamate pumping ability of glutamate-depleted erythrocytes both in vitro and in vivo to determine whether the latter could potentially be used in a blood exchange procedure for neuroprotection. We have observed that glutamate is taken up in red blood cells only via a passive diffusive process with a diffusion constant of 0.144/h. When glutamate-depleted blood cells resuspended in 6% hetastarch were injected into recipient rats, using a blood exchange protocol, a decrease of blood glutamate was observed but attributed to plasma dilution. These observations are discussed in light of a novel neuroprotective strategy based on blood glutamate scavenging.

KEY WORDS: Glutamate scavenging; brain-to-blood glutamate efflux; erythrocytes; neuroprotection.

INTRODUCTION

Several intractable acute and chronic degenerative conditions of the brain such as stroke (1), head trauma (2), hemorrhagic shock (3), and AIDS dementia (4), are characterized by the presence of excess levels of the excitatory neurotransmitter glutamate (Glu) in the brain interstitial fluid (ISF) and cerebrospinal fluid (CSF). At such levels, Glu can exert neurotoxic properties and kill neurons; therefore neuroprotective strategies are relentlessly being evaluated with the hope of finding ways to neutralize the deleterious effects of Glu and its ensuing neurological deficits.

In this context, we have made the hypothesis that brain neuroprotection could possibly be achieved by accelerating the still little known process of brain-to-blood Glu efflux. Because this efflux takes place against a ISF/CSF-to-blood Glu concentration gradient, our assumption is that the brain-to-blood Glu efflux should be significantly facilitated by a decrease of Glu concentration in blood.

In a previous paper (5), we demonstrated that such a decrease takes place, both in vitro and in vivo, upon activation of the blood resident enzymes Glu-pyruvate transaminase (GPT) and Glu-oxaloacetate transaminase (GOT) with the Glu cosubstrates pyruvate and oxaloacetate. Repeated additions to blood or intravenous administration of pyruvate and oxaloacetate cause a decrease of Glu both in plasma as well as in the blood cell compartment in which most blood Glu resides. The decrease of blood Glu levels causes an increased Glu efflux from brain CSF to blood. This principle of a blood mediated decrease of a ISF/CSF constituent is thus similar to that underlying the action of intravenous asparaginase that is used to deplete plasma and CSF asparagine in the treatment of acute lymphoblastic leukemia (6).

On the above premises, we reasoned that if the Glu-depleted blood cell compartment would be able to
rapidly pump plasma Glu toward the original cell/plasma Glu concentration ratio of ~6, it could provide a novel blood exchange strategy of blood Glu reduction possibly applicable in emergency cases such as hemorrhagic shock and head trauma in which the observed acute increase of ISF/CSF Glu is thought to initiate the neurodegenerative process.

Previous studies carried out with rat and human blood have emphasized the relative impermeability of erythrocytes to extracellular Glu (7–11), but those were carried out in the presence of an unfavorable Glu concentration gradient, that is, on erythrocytes rich in intracellular Glu. It was thus of interest to investigate the Glu transport properties of Glu-depleted blood cells, determine their possible contribution to the reduction of plasma Glu during blood exchange, and evaluate they potential use for neuroprotection.

**EXPERIMENTAL PROCEDURE**

*Materials.* Glu dehydrogenase was from Roche (Mannheim, Germany); Glu-pyruvate transaminase was from Sigma (Rehovot, Israel). All chemicals were purchased from Sigma unless noted otherwise.

**Blood Glutamate Scavenging.** Blood was collected retroorbitally from 200–250 g Sprague-Dawley rats anesthesized intraperitoneally with 40 mg ketamine and 5 mg xylazine/kg body weight. It was incubated at 37°C in the presence of pyruvate, oxaloacetate, or a combination of both, 40 mg ketamine and 5 mg xylazine/kg body weight. It was incubated at 37°C with oxaloacetate and pyruvate, added every 15 min, to obtain a final concentration of 1 mM (from 40 mg ketamine and 5 mg xylazine/kg body weight). It was incubated at 37°C with oxaloacetate and pyruvate, added every 15 min, to obtain a final concentration of 1 mM (from freshly prepared 100 mM stock solutions). When depletion of Glu from the blood cell pool was studied, blood was centrifuged at 1300 × g for 7 min at 4°C and the cell pellet (erythrocytes, granulocytes, leukocytes, platelets) was resuspended in Ringer-HEPES buffer containing 2.75 mM glucose and washed three times by centrifugation and resuspension. Glu depletion was obtained by supplementing the blood cell pool, maintained at 37°C, with a 1 mM final concentration of pyruvate and oxaloacetate added every 15 min. When Glu incorporation was studied in Glu-depleted cells, the latter were washed and resuspended in Ringer-HEPES buffer containing 2.75 mM glucose and various Glu concentrations from 0.1 to 1 mM. When blood compartments were analyzed, the blood cell compartment was defined as the blood cells present in the pellet obtained by centrifugation of blood at 9000 × g for 10 min and the plasma withdrawn. The supernatant was measured and its ratio was defined as the hematocrit.

**Glutamate Analysis.** For Glu analysis, blood aliquots of 150 μl were removed at each time point and centrifuged at 1300 × g for 7 min. The volume of supernatant (plasma) was measured and an identical volume of 1 M PCA (perchloric acid) was added to precipitate proteins. The cell pellet was resuspended in double distilled water up to a final volume of 1 M PCA (perchloric acid) was added to precipitate proteins. The cell pellet was resuspended in double distilled water up to a final volume of 150 μl, and an identical volume of 1 M PCA was added. Both plasma and cell PCA-precipitated fractions were centrifuged at 16,000 × g for 10 min and the pellet discarded. Glu concentration was measured in the supernatant using the fluorometric method of Graham and Aprison (10). A 20-μl aliquot from PCA supernatant was added to 480 μl HG buffer containing 15 U of Glu dehydrogenase in 0.2 mM NAD, 1 M glycine, 2.4% hydrazine hydrate adjusted to pH 8.6 with 1 M HSO₄. After incubation for 30–45 min at room temperature, the fluorescence was measured at 460 nm after excitation at 350 nm. A Glu standard curve was established with concentrations ranging from 0–6 μM. All determinations were done at least in duplicates. The results are expressed as mean ± SD. When the number of measurements exceeds a n = 10, the results are expressed as mean ± SEM.

**Blood Exchange.** The blood transfusion experiments were carried out with SPD rats (250–300 g). The blood donor rat was anesthetized with 60 mg/kg pentobarbital; its chest was opened and blood was withdrawn from the heart and collected into a tube containing heparin (0.8 mg/ml of collected blood). The blood was incubated at 37°C with oxaloacetate and pyruvate, added every 10 min, to a final concentration 1 mM. After 40 min, the blood was centrifuged at 4000 rpm/10 min and the plasma withdrawn. The pellet was resuspended to the original blood volume into a 6% hetastarch solution in 0.9% NaCl. Blood exchange into an anesthetized recipient rat was performed by placing a polyethylene cannula (PE 10) in the femoral vein for blood infusion and a polyethylene cannula (PE 10) in the femoral artery for blood withdrawal. Blood was transfused at a rate 0.75 ml/min using a peristaltic pump while arterial blood was withdrawn at the same rate of 0.75 ml/min with another peristaltic pump. Glu levels in the donor rat blood were monitored after blood withdrawal and during the in vitro incubation with oxaloacetate and pyruvate. Glu levels in the recipient rat were monitored by removal of 200-μl blood aliquots from each milliliter of blood removed. The remaining 900 μl was centrifuged at 4000 rpm/10 min, and the respective volumes of pellet and supernatant were measured and their ratio was defined as the hematocrit.

**RESULTS**

Figure 1 illustrates the effect of repetitive additions of pyruvate together with oxaloacetate on Glu levels in blood and in its separated cellular and plasma compart-