Brain neuroprotection by scavenging blood glutamate

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Abstract

Excess glutamate in brain fluids characterizes acute brain insults such as traumatic brain injury and stroke. Its removal could prevent the glutamate excitotoxicity that causes long-lasting neurological deficits. As blood glutamate scavenging has been demonstrated to increase the efflux of excess glutamate from brain into blood, we tested the prediction that oxaloacetate-mediated blood glutamate scavenging causes neuroprotection in a pathological situation such as closed head injury (CHI), in which there is a well established deleterious increase of glutamate in brain fluids. We observed highly significant improvements of the neurological status of rats submitted to CHI following an intravenous treatment with 1 mmol oxaloacetate/100 g rat weight which decreases blood glutamate levels by 40%. No detectable therapeutic effect was obtained when rats were treated IV with 1 mmol oxaloacetate together with 1 mmol glutamate/100 g rat. The treatment with 0.005 mmol/100 g rat oxaloacetate was no more effective than saline but when it was combined with the intravenous administration of 0.14 nmol/100 g of recombinant glutamate-oxaloacetate transaminase, recovery was almost complete. Oxaloacetate provided neuroprotection when administered before CHI or at 60 min post CHI but not at 120 min post CHI. Since neurological recovery from CHI was highly correlated with the decrease of blood glutamate levels (r=0.89, P=0.001), we conclude that blood glutamate scavenging affords brain neuroprotection. Blood glutamate scavenging may open new therapeutic options.

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Introduction

It is nowadays well established that abnormally high glutamate (Glu) levels in brain interstitial and cerebrospinal fluids (ISF and CSF, respectively) are the hallmark of several neurodegenerative conditions that result from acute events, such as stroke, traumatic brain injury or bacterial meningitis (Castillo et al., 1996; Johnston et al., 2001; Zauner et al., 1996) or develop chronically in diseases such as glaucoma, amyotrophic lateral sclerosis or HIV dementia (Ferrarese et al., 2001; Shaw et al., 1995; Spranger et al., 1996; Spreux-Varoquaux et al., 2002). Because excess Glu exerts neurotoxic properties, a great deal of efforts have been made in recent years to reach a better understanding of how the brain protects itself from excess Glu, and on ways drugs could provide neuroprotection.

Brain inherent protection is credited mainly to the presence, both on nerve terminals and on astrocytes, of members of a large family of Na+-dependent Glu transporters which bind and take up Glu and guarantee that the very high concentrations of Glu transiently present after synaptic or astrocytic release (Danbolt, 2001) are soon decreased to concentrations at which Glu exerts neither overt excitatory nor excitotoxic activities (Sattler and Tymianski, 2001).

So far, however, little attention has been given to the Glu transporters present on brain blood vessels (Danbolt, 2001) and on their role in the control of brain extracellular Glu. The existence of a brain-to-blood efflux of Glu, though discovered already 45 years (Berl et al., 1961) ago, has also been widely ignored. Nevertheless, in vitro studies (O’Kane et al., 1999) have proposed that the Glu transporters present on the
antiluminal side of the brain capillary endothelial cells could be responsible for the elimination of Glu from brain into blood while in vivo studies (Hosoya et al., 1999) have detailed the efflux of L-aspartate through the blood–brain barrier. Following this lead, we recently established (Gottlieb et al., 2003) that a rapid brain-to-blood Glu efflux indeed takes place since one observes radioactive Glu appearing in blood as soon as it is injected into rat brain lateral ventricles. We further showed that this brain-to-blood Glu efflux can be accelerated by the creation of a larger Glu concentration gradient between the cerebrospinal fluid/capillary endothelial cell and blood plasma. Using two paradigms based on the fate of radiolabeled Glu infused into fluid/capillary endothelial cell and blood plasma. Using two paradigms based on the fate of radiolabeled Glu infused into brain, we observed its increased appearance in blood and enhanced disappearance from brain following a decrease of blood Glu levels (Gottlieb et al., 2003). A decrease of blood Glu levels and an increase of the driving force for the brain-to-blood Glu efflux was achieved by exploiting the Glu scavenging properties of the blood resident enzymes glutamate-pyruvate transaminase (GPT) and glutamate-oxaloacetate transaminase (GOT) which transform Glu into 2-ketoglutarate in the presence of the respective Glu co-substrates, pyruvate and oxaloacetate (Gottlieb et al., 2003).

Having demonstrated that the latter co-substrates, also called blood Glu scavengers (Gottlieb et al., 2003), cause an increased brain-to-blood Glu efflux, we have tested now the prediction that the intravenous administration of oxaloacetate should provide brain neuroprotection to rats submitted to an insult resulting in the release of excess Glu in brain interstitial and cerebrospinal fluids. In rats as in humans, traumatic brain injury is accompanied by a large increase of Glu in brain fluids (Baker et al., 1993; Bullock et al., 1998; Palmer et al., 1993; Richards et al., 2003; Rose et al., 2002; Vespa et al., 1998) and there is a tight correlation between the elevated levels of Glu and the poor neurological outcome (Koura et al., 1998; Zauner et al., 1996; Zhang et al., 2001). Using a rat model of traumatic brain injury, we confirm here the prediction of the brain neuroprotective properties of the blood resident enzymes glutamate-pyruvate transaminase (GPT) and glutamate-oxaloacetate transaminase (GOT) which transform Glu into 2-ketoglutarate in the presence of the respective Glu co-substrates, pyruvate and oxaloacetate (Gottlieb et al., 2003).

Materials and methods

Materials

pET28 was purchased from Novagen. L-Glutamate, oxaloacetate, NAD, glycine, and hydrazine hydrate were purchased from Sigma. Glutamate dehydrogenase was purchased from Roche. Glutamate-oxaloacetate transaminase (GOT) cDNA was cloned from human hepatoma cells hepG2, expressed in E. coli and purified by Ni-agarose chromatography. GOT assay kit was purchased from Sigma.

Animal anesthesia

The experiments were conducted according to the recommendations of the Declarations of Helsinki and Tokyo and to the Guidelines for the Use of Experimental Animals of the European Community. The experiments were approved by the Animal Care Committees of Ben-Gurion University of the Negev and of the Weizmann Institute. Spontaneously breathing male Sprague Dawley rats weighing 200–300 g were anesthetized with a mixture of isoflurane (initial inspired concentration 2%) in 100% oxygen (1 l/min). The rectal temperature was maintained at 37°C using a heating pad and anesthesia was considered as sufficient for surgery when tail reflex was abolished. Catherization of the tail vein was carried out with a BD Neoflon 24 g catheter for allowing fluid infusion. Catherization of the tail artery was performed to allow blood sampling and determination of blood pressure and heart rate. Blood samples were analyzed for pH, pO2, pCO2, HCO3. Glucose levels were measured with Accu-Chek sensor comfort. After scalp infiltration with bupivacaine 0.5%, it was incised and reflected laterally and a cranial impact of 0.5 J was delivered by a silicone-coated rod which protruded from the center of a free-falling plate as previously described (Shapira et al., 1988). The impact point was 1–2 mm lateral to the midline on the skull’s convexity. Following closed head injury (CHI), the incision was sutured and rats were laid on their left side in order to recover from anesthesia. A neurological severity score (NSS) was evaluated 1 h after CHI.

Neurological severity score

The NSS was determined (Shapira et al., 1988) by a blinded observer. Points are assigned for alterations of motor functions and behavior so that the maximal score of 25 represents severe neurological dysfunction while a score of 0 indicates an intact neurological condition. Specifically, the following were assessed: ability to exit from a circle (3 point scale), gait on a wide surface (3 point scale), gait on a narrow surface (4 point scale), effort to remain on a narrow surface (2 point scale), reflexes (5 point scale), seeking behavior (2 point scale), beam walking (3 point scale), and beam balance (3 point scale).

Experimental design

The 1-h assessment of NSS was followed 15 min later by a 30-min-long intravenous administration at a rate of 30 μl/min/100 g of a solution containing either saline, or the drugs to be administered. Following treatment, the animals were returned to their cages and given free access to food and water. At 24 h and 48 h, the assessment of NSS was repeated.

Determination of blood/plasma Glu

Aliquots of deproteinized whole blood/plasma (200 μl aliquot) were used for the determination of Glu concentration by the fluorometric method of Graham and Aprison (1966). All determinations were done at least in duplicates. The results are expressed as mean±SD.

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Brain water content

Brain hemispheres were removed 120 min after CHI in some groups while in others, brain tissue samples of approximately 50 mg were excised at 24 h post CHI from a location immediately adjacent to the area of macroscopic damage in the left hemisphere and from a corresponding area in the right hemisphere. These tissue samples were used for determination of water content. Water content was determined from the difference between wet weight (WW) and dry weight (DW). Specifically, after WW of fresh brain tissue samples was obtained, samples were dried in a desiccating oven at 120°C for 24 h and weighed again to obtain DW. Tissue water content (%) was calculated as \((\text{WW} - \text{DW}) \times 100 / \text{WW}\).

Statistical analysis

The a priori hypothesis was that the Glu concentrations in blood samples would differ for treatment groups versus controls. Accordingly, this comparison was made with a \(t\)-test (differences were considered as significant when \(P<0.05\)). The significance of comparisons of the NSS between different groups was assessed using analysis of variance (ANOVA) with Bonferroni post hoc testing. The minimal level of significance accepted was \(P<0.05\). Data are presented as means±SD or SEM when \(n\geq8\). Differences were considered as significant when \(P<0.05\).

Results

Intravenous OxAc improves recovery from CHI

We examined whether OxAc-mediated blood Glu scavenging (Gottlieb et al., 2003) causes neuroprotection in a pathological situation, such as closed head injury (CHI), in which there is a well-established deleterious increase of Glu in brain fluids (Baker et al., 1993; Bullock et al., 1998; Palmer et al., 1993; Richards et al., 2003; Rose et al., 2002; Vespa et al., 1998). We therefore submitted rats to CHI and treated them either with intravenous OxAc, Glu or saline, as described in Fig. 1. We also investigated the effects of an intravenous treatment with OxAc+Glu, as the presence of elevated Glu in blood is expected to neutralize the OxAc-mediated decrease of blood Glu levels. Fig. 1 shows that at both 24 and 48 h post CHI, animals treated with OxAc recovered best from CHI while those treated with Glu recovered the least. Animals treated with OxAc+Glu had a similar recovery as those treated with saline. It is of interest to note that in comparison to the NSS measured 1 h post CHI, all rats showed a very significant recovery after 48 h.

To correlate the therapeutic effect of OxAc with its blood Glu scavenging activity, we investigated the blood Glu levels before and after CHI, as well as the outcome of the subsequent 30-min-long treatments with either OxAc or saline. Fig. 2 shows that CHI caused by itself a 20% reduction (Student’s \(t\)-test \(P=5 \times 10^{-4}\)) of the blood Glu levels measured after 60 min, and those were further reduced to 40% by the treatment with intravenous OxAc, but not with saline. The OxAc-induced decrease of blood Glu is transient and is soon followed by a suggested compensatory influx into blood of Glu originating from peripheral organs that sense the momentous decrease in the blood Glu levels (Gottlieb et al., 2003).

Intravenous OxAc fastens recovery from CHI

To compare the time course of the spontaneous recovery from CHI of saline- versus OxAc-treated rats, we monitored their NSS values over 24 days. Fig. 3 shows that an extensive recovery from the neurological deficits inflicted by CHI occurs over this time period. However, the rate of recovery in the OxAc-treated rats is significantly faster than that of the saline-treated rats, and the latter remain at 25 days with an overt neurological handicap.

The recovery from CHI correlates with the decrease of blood Glu levels

In order to demonstrate that the NSS changes are correlated with the changes of blood Glu levels, we plotted in Fig. 4 the % NSS improvement of individual rats assessed 24 h after CHI versus the %decrease of their blood Glu levels measured 90 min after CHI. A strong correlation (\(r=0.89\)) with a high statistical significance (\(P=0.001\)) was observed revealing that a 40% decrease of blood Glu levels affords an almost optimal improvement of the NSS. Thus, low blood Glu levels that facilitate an increased brain-to-blood Glu efflux appear to exert a brain neuroprotective effect while high blood Glu levels are deleterious. This conclusion is in line with the clinical
observations that establish a linear correlation between CSF and plasma Glu concentrations (Castillo et al., 1997), and a very significant association between the high blood Glu levels and the neurological deterioration and outcome after stroke (Castillo et al., 1997; Serena et al., 2001) and intracerebral hemorrhage (Castillo et al., 2002).

**OxAc decreases brain edema after CHI**

To assess whether the treatment with OxAc improves, in addition to the NSS, other parameters that reflect the severity of the inflicted CHI, we also measured, 120 min and 24 h after CHI, the extent of brain edema in OxAc-treated versus saline-treated animals. Brain edema is partly due the presence of excess Glu since it is decreased by glutamate receptor antagonists (Furukawa et al., 2003; Shapira et al., 1992; Westergren and Johansson, 1992; Westergren and Johansson, 1993). The efficient removal of brain excess Glu is thus expected to decrease the edema. Fig. 5 shows that the OxAc treatment caused a significant reduction of brain water content at 24 h.

The beneficial effect of OxAc was not observed in the other groups for which the treatment includes the intravenous administration of Glu (data not shown). Since OxAc could act on brain edema as an osmotic agent, we measured both the blood osmolality and Na content both before and after the 30-min-long treatments with OxAc or saline. There was no change in the saline group (303±3.5 mosM (pretreatment) versus 299±3 mosM (post-treatment); Na: 140±1 meq/l versus 141±0.8 meq/l) but a very significant increase in the OxAc-group: (301±5 mosM versus 338±8 mosM; Na: 139±1 meq/l versus 164±3.4 meq/l). The hypernatriema and hyperosmolarity in the OxAc-treated rats can be accounted by the fact that OxAc is administered together with 2 Na equivalents. To determine that the NSS improvement and edema reduction in the OxAc-treated rats were not due to an osmotic "therapy," we submitted rats after CHI to a hypertonic (3% NaCl) saline treatment. As the respective NSS values obtained at 60 min and

**Fig. 4. Correlation between the decrease of blood Glu levels and the improvement of NSS.** The %blood Glu decrease and the %NSS decrease of individual rats were calculated respectively as follows: (Glu<sub>t=0</sub>−Glu<sub>t=90 min</sub>)/Glu<sub>t=0</sub>, (NSS<sub>t=1 h</sub>−NSS<sub>t=24 h</sub>)/(NSS<sub>t=1 h</sub>−NSS<sub>t=0</sub>−NSS<sub>t=90 min</sub>−NSS<sub>t=120 min</sub>). The %blood Glu decrease and the %NSS decrease of individual rats were calculated respectively as follows: (Glu<sub>t=0</sub>−Glu<sub>t=90 min</sub>)/Glu<sub>t=0</sub>, (NSS<sub>t=1 h</sub>−NSS<sub>t=24 h</sub>)/(NSS<sub>t=1 h</sub>−NSS<sub>t=0</sub>−NSS<sub>t=90 min</sub>−NSS<sub>t=120 min</sub>).
24 h post CHI were 15.6±3.6 and 12.4±5.3 (n=7; P=0.16), we conclude that the beneficial effects of OxAc are not the result of an osmotic therapy.

**Therapeutic dose range of OxAc**

The pharmacological effects described above were all obtained following the administration of 1 mmol OxAc/100 g rat weight. This dose produces an almost complete recovery of rat submitted to CHI. To determine the effective dose range of OxAc, we repeated CHI experiments while decreasing the OxAc dose administrated down to 0.01 mmol/100 g rat weight. Fig. 6 shows that the therapeutic effects of OxAc are dose-dependent with an EC50 value around 0.01 mmol/g rat weight.

**The therapeutic effect of OxAc depends on the presence of GOT**

As the smaller extent of neurological recovery obtained with the low doses of OxAc (<1 mmol/100 g rat weight—Fig. 6) could be due to slower rates of activation of blood resident GOT, we investigated whether the low doses of OxAc (i.e., of the GOT co-substrate concentration) could be compensated by increasing blood GOT concentrations, as expected from the Michaelis–Menten enzyme rate equation. Fig. 7 shows that it is indeed the case since an almost complete neurological recovery could be obtained when rats were treated IV with 0.005 mmol OxAc/100 g (a dose which by itself has no detectable therapeutic effect) administered together with 0.14 nmol recombinant GOT/100 g rat. This GOT dose increases by about 30-fold the basal amount of blood GOT.

**Therapeutic time window of OxAc**

Fig. 8 illustrates the fact that OxAc exerts a neuroprotective action when administered just before submitting rats to CHI or when the treatment is delayed to 60 min post CHI. It is however no more effective when it is delayed by 2 h post CHI. These particular time points were selected because of the well-established duration of the excess Glu in rat brain fluids following CHI (Faden et al., 1989; Palmer et al., 1993; Rose et al., 2002; Stoffel et al., 2002) or stroke (Guyot et al., 2001; Margaill et al., 1996; Phillis et al., 1996). In all cases, following the brain insult, there is a rapid and transient elevation of excess Glu in brain fluids that does not last more than 2 h. Thus, one can predict that OxAc will be effective in removing excess brain Glu only when administered before the brain injury or during the time window of elevated Glu but not after. The results presented in Fig. 8 are in line with this prediction.
Discussion

In the present work, we tested the prediction that blood Glu scavenging should produce brain neuroprotection in a pathological situation characterized by the presence of excess Glu in brain fluids. CHI was chosen as a test system because the occurrence of elevated Glu levels in brain fluids upon brain injury has been extensively documented (Baker et al., 1993; Bullock et al., 1998; Palmer et al., 1993; Richards et al., 2003; Rose et al., 2002; Vespa et al., 1998). Moreover, there is a tight correlation between the elevated levels of Glu and the poor neurological outcome (Koura et al., 1998; Zauner et al., 1996; Zhang et al., 2001) suggesting that the removal of excess Glu may possibly improve the neurological outcome after CHI. OxAc was chosen as a blood Glu scavenger because it is more effective than pyruvate (Gottlieb et al., 2003).

As predicted from its blood Glu scavenging activity and ability to increase the efflux of excess Glu from brain into blood (Gottlieb et al., 2003), OxAc was found here to exert a potent brain neuroprotection manifested by the enhanced neurological recovery after CHI and brain edema reduction.

It could be argued that the neuroprotective effects of OxAc are not due to its blood Glu scavenging activity but rather to a therapeutic activity exerted within the brain. Two major neuroprotective mechanisms could be here involved: (a) OxAc can contribute to an improvement of NAD-linked mitochondrial energetics via an enhancement of the malate-aspartate shuttle. (b) Being a ketoacid, OxAc has the ability to scavenge hydrogen peroxide ($H_2O_2$), one of the main reactive oxygen species generated by traumatic brain injury (Desagher et al., 1997).

Several rather compelling arguments, however, can be raised to support the contention that OxAc exerts its neuroprotective effects mainly via blood Glu scavenging.

(1) The OxAc-induced neurological recovery after CHI is not observed in rats treated with OxAc and glutamate. This finding is in line with the view that the presence of excess Glu in blood prevents OxAc to effectively exert its blood Glu scavenging action which is necessary for a therapeutic enhanced efflux of excess Glu from brain into blood. Would OxAc exert its therapeutic effect in brain, it is not clear why this action would be prevented by intravenous excess Glu since the entrance of OxAc first from blood into the brain and then further into neurons takes place via dicarboxylate transporters such as the NaDC-3 which are not inhibited by Glu (Burckhardt et al., 2005; Pajor et al., 2001). One can further argue that the Glu-induced neutralization of the therapeutic effect of OxAc must be exerted within the blood and not within the brain since Glu does not significantly penetrate from blood into brain neither in normal physiological conditions (Smith, 2000) nor after the breakdown of the blood–brain barrier (Ronne Engstrom et al., 2005).

(2) The OxAc-induced neurological recovery after CHI takes place following the administration of OxAc within the time window during which excess Glu is present in brain i.e., up to 2 h post CHI. Though we did not measure the kinetics of brain Glu elevation after CHI, numerous studies demonstrate that the excess Glu in rat brain fluid does not last more than 2 h following either CHI or stroke (Faden et al., 1989; Farooque et al., 1996; Guyot et al., 2001; Phillis et al., 1996; Rose et al., 2002) but see (Alves et al., 2005). Assuming that OxAc therapeutic action is unrelated to blood Glu scavenging but rather to an improvement of mitochondrial energetics, the observation that OxAc has no therapeutic effect when administered 2 h after CHI cannot be easily reconciled with the observation that mitochondrial dysfunction is observed for several hours after CHI (Singh et al., 2006; Takamatsu et al., 1998; Xiong et al., 2005).

(3) The observation that the neurological recovery after CHI is highly correlated with the decrease of blood Glu levels (Fig. 4) is strongly in line with the concept that blood Glu scavenging enhances the efflux of excess Glu from brain into blood and provides thereby neuroprotection. This strong correlation cannot be explained by an intraparenchymal site of action of OxAc.

Though we do not rule out the possibility that OxAc exerts some neuroprotective effects within the brain, we believe that the major therapeutic action of OxAc is decisively linked to its blood Glu scavenging activity.

It is of interest to note that saline-treated rats recover from TBI to a significant extent, though neither as fast nor as much as the OxAc-treated rats. It is a tantalizing hypothesis that the spontaneous recovery of rats after TBI would rely on the same mechanism as that amplified by OxAc.

OxAc is here shown to have a relatively short time window that appears to be tightly linked to the limited duration of excess Glu in the rat brain after CHI (Faden et al., 1989; Farooque et al., 1996; Guyot et al., 2001; Phillis et al., 1996; Rose et al., 2002). Since, excess Glu in human brain can be observed after CHI for hours and even days (Baker et al., 1993; Bullock et al., 1998; Palmer et al., 1993; Richards et al., 2003; Rose et al., 2002; Vespa et al., 1998), and is in correlation with the poor neurological outcome (Koura et al., 1998; Zauner et al., 1996; Zhang et al., 2001), one might possibly expect for...
OxAc a much longer therapeutic time window in human patients than in rats. One might further expect that the administration of this blood Glu scavenger may display in man a therapeutic effect which has so far not been observed for glutamate receptor antagonists. One possible benefit is that the enhanced brain-to-blood Glu efflux produced by blood Glu scavenging is self-limiting since it slows down in parallel with the decrease of brain Glu. Thus, it will eliminate excess Glu without preventing Glu from exerting a role in neurorepair (Biegon et al., 2004), a factor that has been suggested (Ikonomidou and Turski, 2002) to account for the failure of glutamate receptor antagonists in human clinical studies.

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