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Abstract

As ischemic stroke is associated with an excessive release of glutamate into the neuronal extracellular space, a decrease in blood glutamate levels could provide a mechanism to remove it from the brain tissue, by increasing the brain-blood gradient. In this regard, the ability of glutamate oxaloacetate transaminase (GOT) to metabolize glutamate in blood could represent a potential neuroprotective tool for ischemic stroke. This study aimed to determine the neuroprotective effects of GOT in an animal model of cerebral ischemia by means of a middle cerebral arterial occlusion (MCAO) following the Stroke Therapy Academic Industry Roundtable (STAIR) group guidelines. In this animal model, oxaloacetate-mediated GOT activation inhibited the increase of blood and cerebral glutamate after MCAO. This effect is reflected in a reduction of infarct size, smaller edema volume, and lower sensorimotor deficits with respect to controls. Magnetic resonance spectroscopy confirmed that the increase of glutamate levels in the brain parenchyma after MCAO is inhibited after oxaloacetate-mediated GOT activation. These findings show the capacity of the GOT to remove glutamate from the brain by means of blood glutamate degradation, and suggest the applicability of this enzyme as an efficient and novel neuroprotective tool against ischemic stroke.

Introduction

It is well established that during ischemia, glutamate acts as an important mediator of neuronal degeneration, being released in large amounts from neurons and astrocytes, causing cellular overload of calcium, mainly through its action on calcium-permeable NMDA receptors. This overload of calcium leads to necrosis and breakdown of cellular structures including proteins, DNA, and membrane phospholipids (Lipton, 1999). In fact, studies from our group have demonstrated that neurologic deterioration of patients with acute ischemic stroke is associated with higher glutamate levels in blood and cerebrospinal fluid (Castillo et al, 1996, 1997).

Under normal physiological conditions, glutamate release in the synaptic cleft is quickly removed by the excitatory amino-acid transporters (EAATs), located on astrocytes and neurons (Hazell, 2007). Apart from astrocytes and neurons, the presence of EAATs has also been observed in endothelial cells from the brain vasculature, showing an important role of these cells in the maintenance of glutamate levels in the extracellular medium (O'Kane et al, 1999). It has been described that endothelial cells have facilitative carriers for glutamate in the luminal membranes, and EAATs in the abluminal

membrane. This localization allows a unidirectional export of glutamate from the brain to the blood torrent following a gradient of concentration. Therefore, the decrease of blood glutamate levels leads to a larger glutamate gradient between the brain and blood, facilitating the lowering of extracellular levels of brain glutamate (Gottlieb *et al*, 2003; Teichberg *et al*, 2009).

As ischemic stroke is associated with an excessive release of glutamate into the neuronal extracellular space (Castillo *et al*, 1996, 1997), a decrease in the levels of blood glutamate could provide a mechanism to reduce extracellular glutamate at early times, with possible therapeutic implications after an ischemic insult (Gottlieb *et al*, 2003; Hawkins, 2009; O'Kane *et al*, 1999; Teichberg *et al*, 2009).

In this regard, recent studies have reported that glutamate oxaloacetate transaminase (GOT), a blood-resident enzyme, can be used as an important neuroprotective tool for brain diseases associated with elevated levels of glutamate (Gottlieb *et al*, 2003; Teichberg *et al*, 2009; Zlotnik *et al*, 2007, 2008). The potential applicability of GOT is based on the fact that this enzyme, using oxaloacetate as cosubstrate, is able to transform glutamate to 2-ketoglutarate and aspartate, leading to a decrease of glutamate levels in the blood.

On the above premises, we aimed to demonstrate if the reduction of glutamate levels in blood, by means of oxaloacetate-mediated GOT activation, induces a neuroprotective effect in a rat model of cerebral ischemia.

Materials and methods

Animals

Experimental protocols were approved by the local Animal Care Committee according to the European Union (EU) rules (86/609/CEE, 2003/65/CE, and RD 1201/2005). Male Sprague-Dawley rats (Harlan Laboratories, Udine, Italy) with a weight of 300 to 330 g were used. Rats were watered and fed *ad libitum*. Anesthesia was induced by inhalation of 5% sevoflurane in a nitrous oxide/oxygen mixture (70/30). Rectal temperature was maintained at 37.5°C by using a feedback-controlled heating pad. Glucose levels were analyzed before surgery (ranging from 180 to 220 mg/dL).

Surgical Procedures

Transient focal ischemia was induced in rats by using the occlusion of the middle cerebral artery (MCAO). Middle cerebral arterial occlusion model of brain ischemia is considered one of the best models to mimic human ischemic stroke and has been used in numerous studies in rats (Carmichael, 2005). Middle cerebral arterial occlusion was performed as previously described (Longa *et al*, 1989) with some modification. In brief, under operating microscope, the left common, external, and internal carotid arteries were dissected from connective tissue through a midline neck incision. The left external carotid artery and pterygopalatine artery of the internal carotid artery were separated and ligated by 5-0 silk sutures. A 23-mm segment of 3-0 nylon monofilament suture with the tip rounded by heat was inserted into the stump of the left common carotid artery and advanced into the internal carotid artery ~19 to 20 mm from the bifurcation to occlude the origin of the MCA. The suture was removed after 90 minutes of occlusion. A laser-Doppler flow probe (tip diameter 1 mm) attached to a flowmeter

(PeriFlux 5000; Perimed AB, Stockholm, Sweden) was located over the thinned skull in the MCAO territory (4 mm lateral to bregma) to obtain a continuous measure of relative cerebral brain flow during the experiment. Only animals with a cerebral blood flow reduction over 60% and with reperfusion after occlusion were included in the study.

Animal Experimental Procedures

Experimental procedure was performed following five criteria derived from the Stroke Therapy Academic Industry Roundtable (STAIR) group guidelines for preclinical evaluation of stroke therapeutics (Philip *et al*, 2009): (1) cerebral blood flow was measured to confirm the vascular occlusion, as an index of the reliability of the ischemic model; (2) animals were randomly assigned to treatment groups of the study; (3) researchers were blinded to treatment administration; (4) researchers were blinded to treatments during outcome assessment; and (5) temperature was controlled during the ischemic period.

Experimental Groups

Two experimental groups were designed: MCAO control group ($n = 10$), treated with saline (0.9% of NaCl) intravenously given as a single-bolus injection, and MCAO-treated group ($n = 10$), with oxaloacetate 3.5 mg/100 g intravenously given as a single-bolus injection. Treatments were administered in both the groups 90 minutes after MCA occlusion (after ischemia). Oxalacetate solution (pH adjusted to 7.4) was prepared in saline solution (0.9% of NaCl) at a final concentration of 10 mg/mL; therefore, the volume of bolus injected in the treated animals was about 1 mL. A volume of 1 mL of saline was injected in control animals.

In both experimental groups, glutamate blood levels, infarct volumes, and edema development analysis were performed. Furthermore, somatosensory tests were performed on day 7. A sham group was also performed to confirm that the surgery did not affect the analyzed variables.

To study the effect of oxalacetate on blood glutamate and aspartate levels in health animals, two doses of oxaloacetate were tested, 1.5 mg/100 g (intravenously) ($n = 3$) and 3.5 mg/100 g (intravenously) ($n = 4$).

Endothelial Cells Culture

Previous studies have established that the close proximity of astrocytes is crucial to the development of endothelial cells in culture models (Cohen-Kashi Malina *et al*, 2009), therefore, an *in vitro* model based on a noncontact coculture of rat endothelial cells with rat brain astrocytes was chosen.

In brief, initial primary astrocyte cultures were prepared from neonatal (P0) Sprague-Dawley rat cortex, as previously described (McCarthy and de Vellis, 1980). For the noncontact coculture, 10^5 astrocytes from the primary culture were seeded on the bottom of poly-D-lysine (PDL) coated six-well plates for 5 to 6 days in Dulbecco's modified Eagle growth medium supplemented with 10% horse serum, 100 U/mL penicillin, and 100 µg/mL streptomycin (all from Gibco-Invitrogen Ltd, Paisley, UK). Then, Dulbecco's modified Eagle medium was removed and 7×10^4 rat endothelial cells (R840-05, Cell Applications Inc, San Diego, CA, USA) were seeded on rat tail collagen

precoated (R35442, BD Biosciences, Madrid, Spain) transwell inserts in the six-well plate. Astrocytes and endothelial cells were grown to confluence for 4 days in EGM MV Microvascular Endothelial Cell Growth Medium (Lonza, Basel, Switzerland).

Immunocytochemistry

To characterize the endothelial cells used, the specific cell marker von Willebrand factor was used (Jaffe, 1977). For this, 10^4 endothelial cells were grown onto culture slides (R354632, BD Biosciences) for 4 days as previously described. Then, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline for 10 minutes and subsequently permeabilized with 0.3% Triton X-100 for 5 minutes. After washing with Tris-buffered saline, they were blocked with 5% bovine serum albumin in phosphate-buffered saline for 30 minutes. Cells were subsequently incubated with a polyclonal antibody raised in rabbit against von Willebrand factor for 1 hour (1:400, Abcam, Cambridge, UK), washed in Tris-buffered saline, and incubated with an anti-rabbit tetramethylrhodamine isothiocyanate (TRITC) conjugated for 1 hour (1:50, Invitrogen). Nuclei were counterstained by incubating with Hoescht 33342 dye (2 µg/mL, Molecular Probes-Invitrogen, Paisley, UK) for 5 minutes and then washed in Tris-buffered saline. All incubations were made at room temperature. After mounting, slides were observed under a fluorescence microscope (Olympus, Hamburg, Germany) and images were acquired with a CCD camera (Nikon, Nikon Precision Europe, Langen, Germany) using the NIS Elements software.

Immunoblotting

Expression of tight junction proteins (Zonula occludens protein-1 (ZO-1) and occludin) and glutamate transporters (EAAT1, EAAT2, and EAAT3) were detected in the endothelial cells by immunoblotting technique. At the end of the culture period, endothelial cells grown on transwell insert were rinsed with phosphate-buffered saline and collected in ice-cold lysis buffer (Invitrogen) with 1% antiprotease cocktail (Sigma-Aldrich Chemie GMBh, Taufkirchen, Germany). A sample of brain cortex (200 mg) from an adult health rat was also lysed to be used as control. The different samples were sonicated for 15 seconds, boiled for 5 minutes, and cooled immediately on ice. Sample extracts were centrifuged at 14,000 g for 5 minutes at 4°C and supernatant protein concentration was quantified using the Bradford method (Bio-Rad Protein Assay Kit, Bio-Rad Laboratories Ltd, Herts, UK). Forty micrograms of protein extract was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis on 8% polyacrylamide gels. Gels were run at 20 mA for 90 minutes. Separated proteins were electroblotted onto polyvinylidene fluoride (PVDF) membranes at a constant current intensity of 15 mA for 15 hours (semidry blotting). Membranes were blocked for 60 minutes at room temperature in blocking buffer containing 5% nonfat dry milk and then the blots were incubated overnight at 4°C with the primary antibody against the different proteins studied. Primary antibodies used were rabbit polyclonal antibody against ZO-1 (1:250, Invitrogen), mouse monoclonal antibody against occludine (1:500, Invitrogen), rabbit polyclonal antibody against EAAT1 (1:1000, Abcam), mouse monoclonal antibody against EAAT2 (1:500, Abcam), mouse monoclonal antibody against EAAT3 (1:500, Abcam), and mouse monoclonal antibody against actin (1:1000, Sigma). Actin was used as loading control. All antibodies used were diluted in Tris-buffered saline-Tween-bovine serum albumin (BSA) buffer (20 mmol/L Tris base, 140 mmol/L NaCl, 0.1% Tween-20). After washing, the blots were incubated for 1hour at

room temperature with the following secondary antibodies: goat anti-mouse Cy3 (1:3000, GE Life Sciences, GE Healthcare Europe GmbH, Munich, Germany) and goat anti-rabbit Cy5 (1:3000, GE Life Sciences), according to the primary antibody used. Western image was acquired with fluorescence scam (Molecular Imager PharosFX Plus System, Bio-Rad).

Serum Glutamate and Aspartate Analysis

Glutamate and aspartate serum levels were determined by high performance liquid chromatography analysis, following a previously described method (White et al, 1986). The experiments with oxalacetate administration with health animals, glutamate and aspartate levels were measured before the treatment (basal) and 1, 2, and 3 hours after the treatment injection. In experiments with animal model of ischemia, the blood samples were acquired before surgery (basal sample) and 90 minutes (before treatment injection), 4 hours, 24 hours, 3 days, and 7 days after the occlusion.

Magnetic Resonance Imaging Protocol

Infarct size and edema extension were assessed by means of magnetic resonance imaging. Magnetic resonance imaging studies were conducted on a 9.4-T horizontal bore magnet (Bruker BioSpin, Ettligen, Germany) with 20 cm wide actively shielded gradient coils (440 mT/m). Radiofrequency transmission was achieved with a birdcage volume redsonator; signal was detected using a fourelement surface coil, positioned over the head of the animal, which was fixed with a teeth bar, earplugs, and adhesive tape. Transmission and reception coils were actively decoupled from each other. Gradient-echo pilot scans were performed at the beginning of each imaging session for accurate positioning of the animal inside the magnet bore. Apparent diffusion coefficient maps were acquired using a spin-echo echo-planar imaging sequence with the following acquisition parameters: field-of-view 19.2×19.2 mm², image matrix 128×128 (in-plane resolution 0.15 mm/pixel $\times$ 0.15 mm/pixel), 14 consecutive slices of 1 mm thickness, repetition time = 4 seconds, four segments, echo time = 30 ms, spectral bandwidth 2,50,000 Hz, and seven diffusion b values: 0, 100, 300, 600, 800, 1000, and 1400. T2-weighted images were acquired using a multislice multiecho spin-echo sequence with the following acquisition parameters: field-of-view 19.2×19.2 mm², image matrix 192×192 (isotropic in-plane resolution of 0.1 mm/pixel $\times$ 0.1 mm/pixel), 14 consecutive slices of 1 mm thickness, repetition time = 3 seconds, and 16 echoes with echo time = 9 ms. Diffusion-weighted images were acquired immediately after removing the suture from the animal (90 minutes after the occlusion). T2-weighted image was acquired 24 hours, 3 days, and 7 days after the onset of ischemia. All images were processed and maps were constructed with ImageJ (Rasband WS, ImageJ, US National Institutes of Health, Bethesda, MD, USA, http://rsb.info.nih.gov/ij/, 1997–2009).

In vivo Magnetic Resonance Spectroscopy

Magnetic resonance spectra were acquired as previously described (Higuchi et al, 1997; Tkac et al, 1999). Local shimming was performed by manual adjustment of first- and second-order shim coil currents using a Press-waterline sequence. The field homogeneity in a 3-mm³ voxel typically resulted in signal line widths of 11 to 20 Hz for water. Water signal was suppressed by variable power RF pulses with optimized

relaxation delays. *in vivo*[1]H magnetic resonance spectra of the rat brain were acquired by using a proton stimulated-echo acquisition mode (STEAM-1H) sequence with echo time = 3 ms, mixing time (TM) = 5 ms, repetition time = 2,500 ms, 176 averages, cubic voxel = $3 \times 3 \times 3$ mm^3 (27 µL). Spectra were processed using TOPSPIN 2.0 PV (Bruker BioSpin). For the quantitative analysis, glutamate signals were normalized to creatine peak areas for each single spectrum. Magnetic resonance spectra were acquired during 1 hour before surgery, during the occlusion (90 minutes), and during 4 hours after reperfusion and treatment/saline injection. Middle cerebral arterial occlusion control group ($n = 3$) was treated with saline and MCAO-treated group ($n = 4$) was treated with oxaloacetate 3.5 mg/100 g, following the protocol described previously.

Image Analysis

All images were processed using ImageJ (Rasband WS, ImageJ, NIH, http://rsb.info.nih.gov/ij). Infarct volumes were determined from quantitative diffusion and T2 maps by manually selected areas of elevated apparent diffusion coefficient/T2 values by a researcher blinded to animal handling. Edema was estimated by measuring the volumes of the affected (V_{Les}) and contralateral (V_c) hemispheres and using the formula: edema (%) = $100 \times (V_{Les} - V_c)/V_c$.

Somatosensory Test

Sensorimotor deficits were assessed as previously described (Reglodi *et al*, 2003). All sensorimotor tests were performed during the light cycle of animal housing, with environmental conditions consistently maintained across examinations. Briefly, the rodent's walking pattern was observed during a 1-minute period by a researcher blinded to the animal grouping. A sensorimotor score on a scale of 0 to 3 was assigned to rats on the basis of their gait behavior. 0, Straight walking; 1, walking toward the contralateral side; 2, alternate circling and walking straight; 3, circling and/or other gait disturbance (backing, crawling, walking on digits).

Statistical Analysis

Results are expressed as percentages for categorical variables and as mean (s.d.) or median (quartiles) for the continuous variables, depending on the normal or not normal distribution of data. Proportions were compared using the χ^2 test, and the Student's *t*-test or the Mann-Whitney test to compare continuous variables between groups. Spearman or Pearson analysis was used for bivariate correlations.

Results

Characterization of Excitatory Amino-Acid Transporters on Endothelial Cells

A noncontact coculture of rat endothelial cells with rat brain astrocytes was chosen to check the expression of EAATs. This model allows the removal of endothelial cells from the culture without contamination from astrocytes (Figure 1A). Our cells were positive for von Willebrand factor, a specific marker for endothelial cells (Figure 1B). To check that cultured endothelial cells had functional properties close to those

observed *in vivo*, the expression of tight junction's proteins was assessed by Western blot analysis (Figure 1C). We used a sample of rat brain as a positive control.
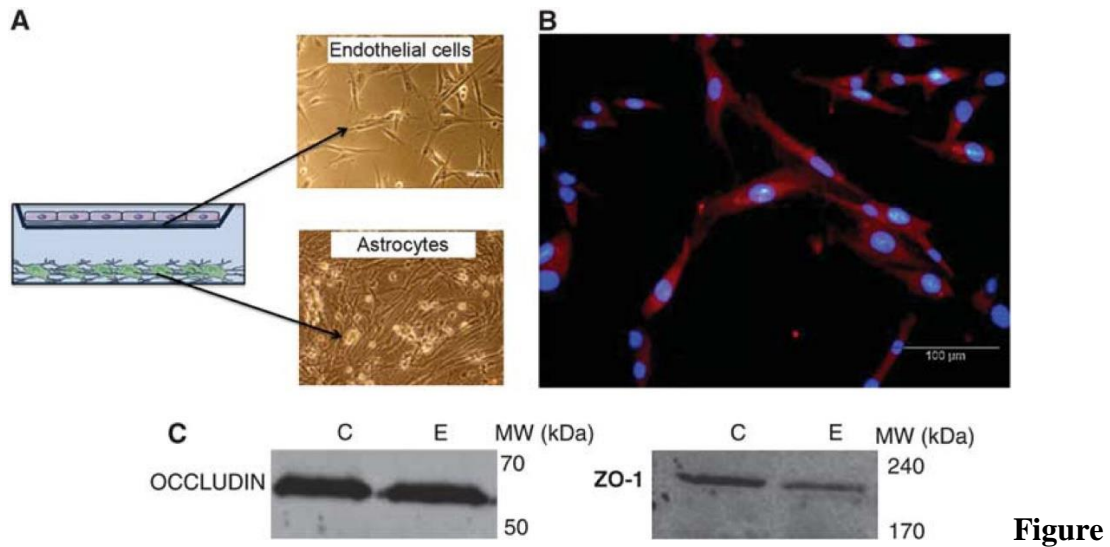
Figure 1 Coculture of astrocytes and endothelial cells used in the study. (**A**) Schematic drawing of the noncontact coculture of rat endothelial cells with rat astrocytes. Astrocytes were grown on the top of the well and the endothelial cells in transwell. (**B**) Immunocytochemistry of endothelial cells stained with von Willebrand factor. Nuclei were counterstained with Hoechst. (**C**) Western blot analysis of the expression of occludin and ZO-1 proteins, in the endothelial cells (**E**), and rat brain cortex (C). Rat brain cortex was used as positive control. ZO-1, Zonula occludens protein-1

Once endothelial cells culture was characterized, EAATs expression was analyzed. Figure 2 shows the expression of EAAT1 and EAAT3 in endothelial cells. We did not detect the presence of EAAT2 under our experimental conditions. The expression of EAAT1, EAAT2, and EAAT3 in a brain tissue sample, used as positive control, confirmed that bands observed in the Western blots effectively corresponded to the analyzed proteins.
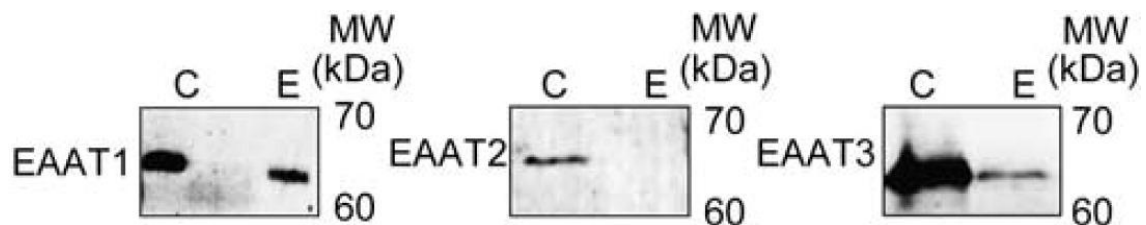

Figure 2 Excitatory amino-acid transporters (EAATs) protein expresion in rat endothelial cells. Western blot analysis shows the expression of EAAT1 and EAAT3,

but not EAAT2, in endothelial cells (E). Tissue from the brain cortex (C) was used as

positive control of EAATS.

Effect of Oxaloacetate-Mediated Glutamate Oxaloacetate Transaminase Activation on Glutamate Blood Levels

A 1.5-mg/100 g dose of oxaloacetate was administered by intravenous injection to healthy animals, observing no changes on blood glutamate levels within the first 3 hours after administration (Figure 3A). As GOT metabolizes glutamate to 2-ketoglutarate and aspartate, we also analyzed aspartate levels on blood to confirm that the dose of oxaloacetate used was not enough to activate GOT. No changes on aspartate levels were observed (Figure 3B). When a higher dose of oxaloacetate of 3.5 mg/100 g (intravenously) was used, a significant decrease of glutamate levels was observed at 1 hour after treatment, and maintained within the first 3 hours after administration. The reduction of glutamate was about 60% with respect to basal levels ($P < 0.0001$) (Figure 3A). A concomitant increase on aspartate levels (Figure 3B) seems to prove that the effect of oxaloacetate on glutamate was actually mediated by GOT activation ($P < 0.0001$). Glutamate and aspartate blood levels were stable in control group treated with saline (results not shown).
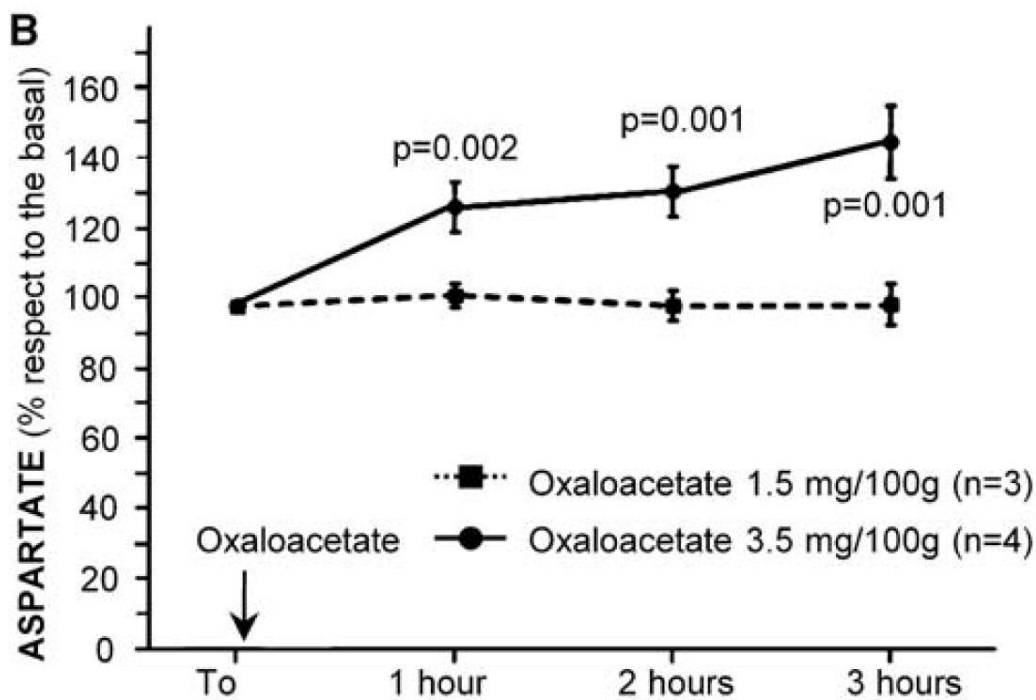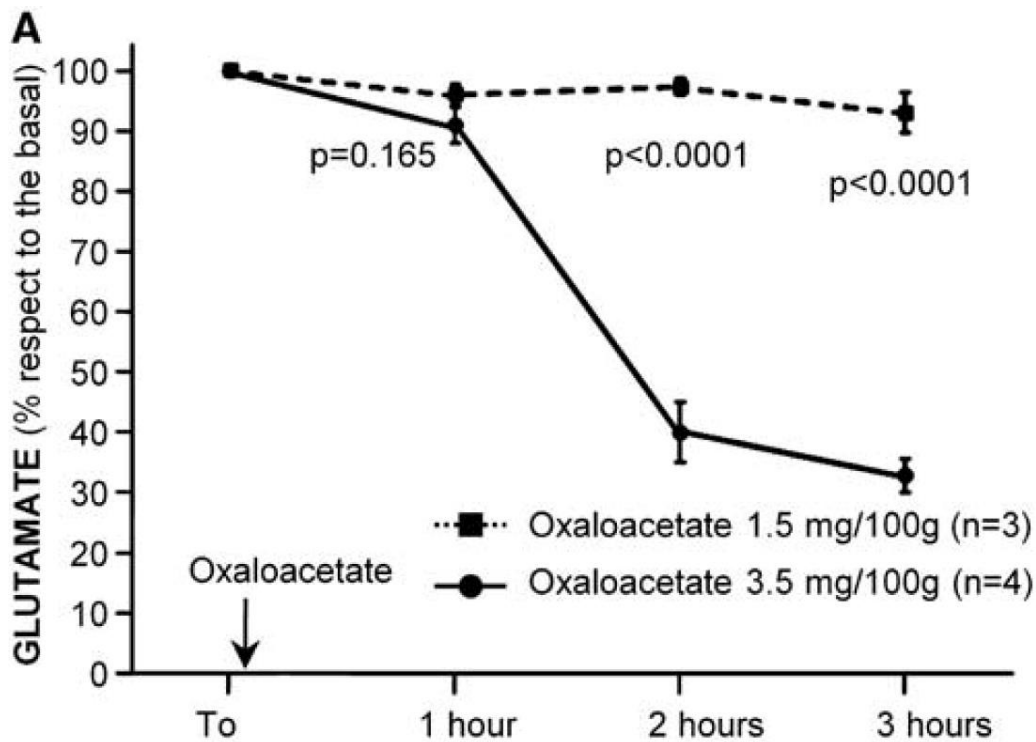
Figure 3 Doses-reponse effect of oxaloacetate on blood glutamate (**A**) and aspartate (**B**) levels. The treatment with oxaloacetate 3.5 mg/100 g (intravenously) ($n = 4$) induces a greater reduction in blood glutamate levels and increase in aspartate than the dose of 1.5 mg/100 g (intravenously) ($n = 3$). Glutamate and aspartate data are shown as mean ± s.e.m. of percentage with respect to basal levels (considered as 100%).

Neuroprotective Effect of Oxaloacetate-Mediated Glutamate Oxaloacetate Transaminase Activation on Middle Cerebral Arterial Occlusion Animal Models

Middle cerebral arterial occlusion induced an increase of 40% on blood glutamate levels at 4 hours after ischemia onset (Figure 4), which returned to normal levels 24 hours later. The increasing effect of cerebral ischemia on glutamate levels was inhibited when oxaloacetate-mediated GOT activation (3.5 mg/100 g) was induced ($P < 0.0001$).
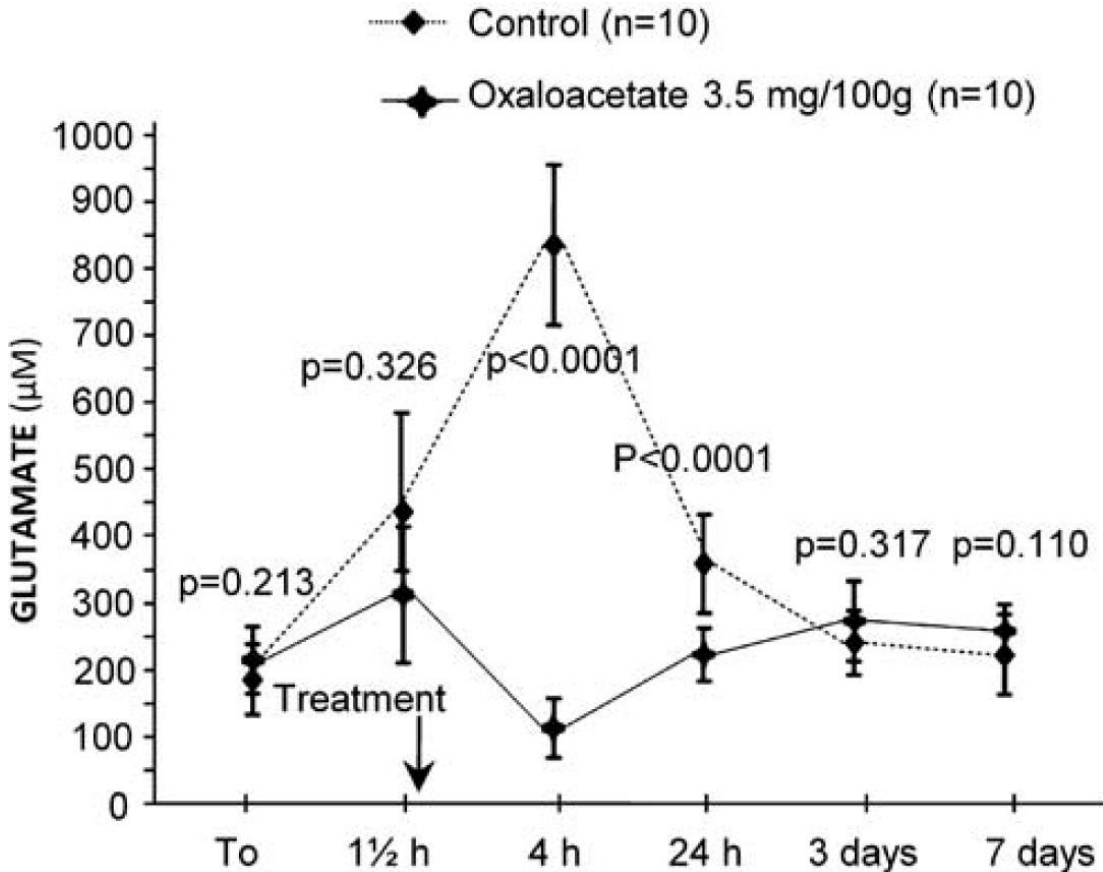

Figure 4 Time course of blood glutamate levels in control ($n = 10$) and treated ($n = 10$)

middle cerebral arterial occlusion (MCAO) animals. The treatment with oxaloacetate

3.5 mg/100 g (intravenously) induces a reduction in blood glutamate levels. Glutamate

levels are presented as mean ± s.e.m.

To demonstrate that the reduction on blood glutamate levels observed after GOT activation led to a neuroprotective effect on our animal model of cerebral ischemia, the infarct volumes of treated versus control animals were compared. As shown in Figures 5A and 5B, oxaloacetate-mediated GOT activation decreased lesion sizes about 80%. At day 7, infarct volumes of treated group were significantly lower than those for control group ($P < 0.0001$). Diffusion-weighted images show that infarct volumes were similar for both experimental groups before treatment. Regarding edema development, control group showed higher edema than treated animals (10% versus 2%, $P < 0.0001$) (Figure

. Edema resolves at day 7 for all animals. This effect is typical for this experimental model.
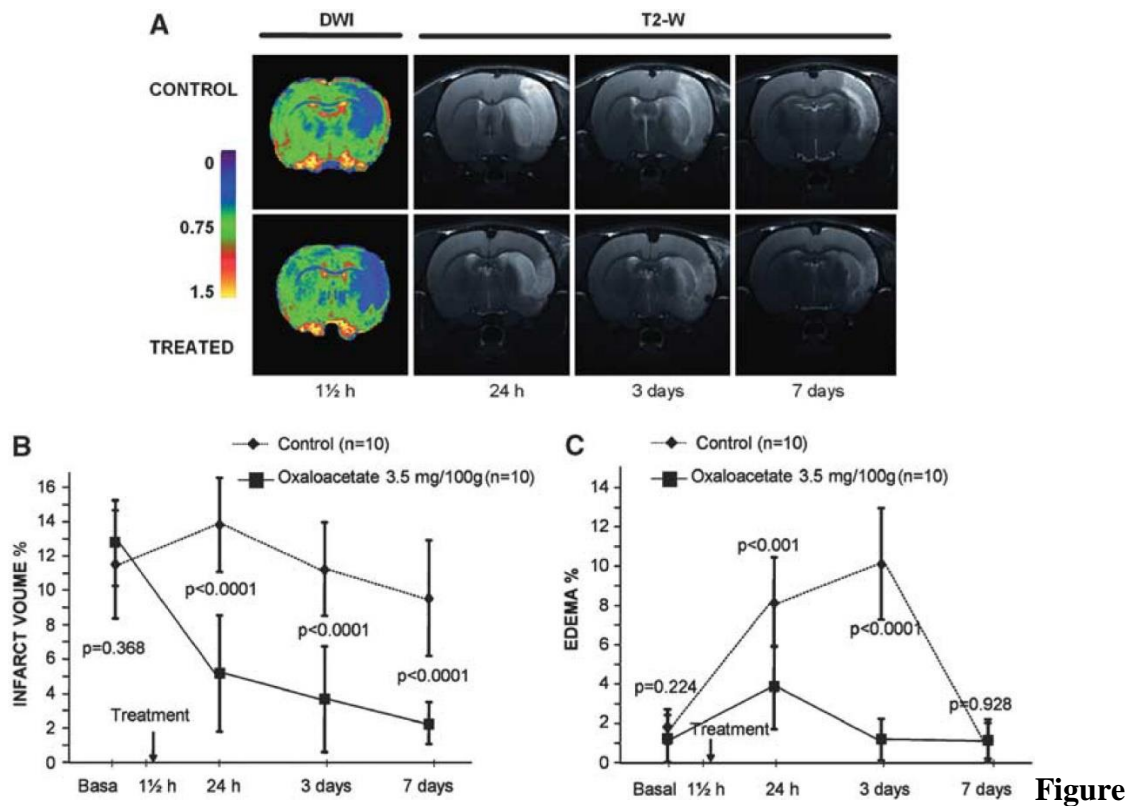
Figure 5 Infarct size and edema development were assessed by means of magnetic resonance image (MRI). Diffusion-weighted images (DWI) were acquired immediately after removing the suture from the animal (90 minutes after the occlusion); T2-weighted (T2-W) images were acquired at 24 hours, 3 days, and 7 days after ischemia onset (**A**). (**B**) The time course of infarct volumes. Treated middle cerebral arterial occlusion (MCAO) animals ($n =10$) had smaller infarct volumes than control animals ($n = 10$). (**C**) The time course of the edema volumes decreases in control and treated groups after MCAO. Edema volume decrease in the MCAO-treated animals during the first 7 days. Infarct volumes (% of ipsilesional hemispheric volume) and edema (% ipsilesional versus contralesional hemispheric volumes) are shown as mean ± s.e.m.

On the other hand, a lower sensorimotor deficit was observed in treated animals compared with controls (1 [1,2] versus 2 [2,3]; $P = 0.011$).

Magnetic Resonance Spectroscopic Analysis of Cerebral Glutamate Levels in Middle Cerebral Arterial Occlusion Animal Models

Magnetic resonance spectroscopic (Figures 6A and 6B) analysis was used to study the time course of cerebral glutamate levels in ischemic animals treated with (3.5 mg/100 g) oxaloacetate and with saline. Quantitative analysis of magnetic resonance spectra revealed a persistent increase of glutamate levels after the occlusion onset for control group. Four hours after occlusion, the increase of extracellular glutamate levels was about 300% with respect to basal levels. A significant decrease of glutamate levels in the brain parenchyma was observed in the treated group with respect to the control animals ($P < 0.0001$) (Figure 6C).
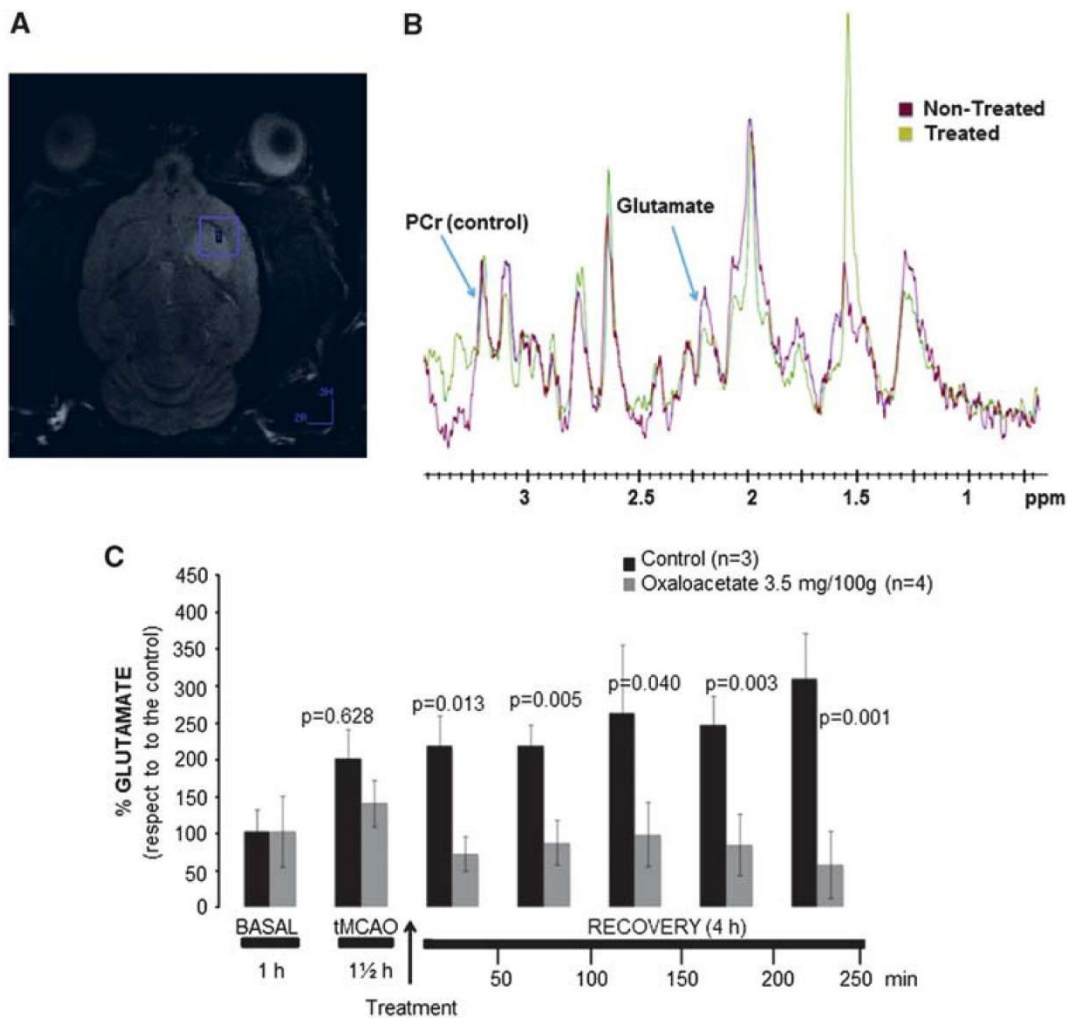

Figure 6 Magnetic resonance spectroscopy (MRS) studies. (**A**) The brain region where the cubic voxel of $3 \times 3 \times 3$ mm³ (27 µL) was located. (**B**) The spectra that corresponded to control and treated animals at 3 hours after occlusion. The quantitative analysis of glutamate signals were normalized to creatine peaks for each single expectrum. Brain glutamate levels were lower in treated middle cerebral arterial

occlusion (MCAO) animals. (**C**) The time course of brain glutamate levels in control

($n = 10$) and treated ($n = 10$) MCAO animals. Treated animals showed lower brain

glutamate levels. Glutamate is shown as mean ± s.e.m. of percentage with respect to

basal levels (considered as 100%).

Discussion

The brain is an organ with a high content of glutamate, the most important excitatory neurotransmitter. Although its presence is essential for the correct function of neuronal tissue, its excitatory properties become dangerous for neurons when its concentration in the extracellular fluid rises under pathological conditions like ischemic stroke.

During ischemic stroke, glutamate is highly released to the extracellular space, mediating a marked increase in intracellular calcium, followed by the activation of intracellular enzymes, which provoke neuronal death. The central role glutamate in the ischemic cascade, make this neurotransmitter a good target in the search for neuroprotective agents in ischemic stroke.

In agreement with the previous findings (Zlotnik *et al*, 2007, 2008), in the present manuscript, we demonstrated that in ischemic stroke, a decrease in blood glutamate levels by means GOT, provides a neuroprotective mechanism to remove this neurotransmitter from the brain tissue to the blood through the blood-brain barrier.

The expression of EAATs on brain capillary endothelial cells has been described to be essential for the diffusion of glutamate from the brain parenchyma to blood. Prior to study the effects of GOT and given that we used rats in our experiments, the first step of our research was to demonstrate the presence of EAATs on rat endothelial cells, where, to the best of our knowledge, they have never been described before. We focused our studies on EAAT1, EAAT2, and EAAT3 due to their ubiquitous location and because they have been previously described in porcine and bovine brain endothelial cells (O'Kane *et al*, 1999; Teichberg *et al*, 2009). We detected EAAT1 and EAAT3, but not EAAT2 expression in rat endothelial cells. The expression of at least two types of EAATs on this cell type in rats seems to demonstrate the permeability for glutamate from the brain parenchyma into the blood torrent through the blood-brain barrier.

A high number of neuroprotective agents found in experimental research have failed in stroke clinical trials, most of the cases due to the narrow therapeutic window (Castellanos *et al*, 2006). Another important reason has been the low quality and adequacy of animal testing for neuroprotective agents for stroke (Philip *et al*, 2009). For these reasons, oxaloacetate was administered to demonstrate the properties of GOT activation as a neuroprotective tool within a wide therapeutic window and following the STAIR guidelines for preclinical evaluation of stroke therapeutics (Philip *et al*, 2009). Besides, our primary end point in the experimental study was infarct volume. The infarct volume analysis represents an objective and quantitative tool to estimate ischemic brain injury and it is commonly used to determine the efficacy of neuroprotective agents in preclinical studies (Lin *et al*, 1993).

It has been described that GOT activation with oxaloacetate 1.2 mg/100 g (intravenously) reduces blood glutamate levels (Gottlieb *et al*, 2003; Nagy *et al*, 2009).

In disagreement with these results, we observed no effects with a dose of 1.5 mg/100 g dose within the first 4 hours after treatment under our experimental conditions. However, when oxaloacetate 3.5 mg/100 g (intravenously) was tested, a significantly maintained decrease on blood glutamate levels was observed within the first hours. Therefore, this last dose was chosen to test the neuroprotective effect of oxaloacetate-mediated GOT activation.

The increase of blood glutamate levels observed after MCAO disappeared when GOT was activated in the treated group with oxaloacetate. This effect on the reduction of blood glutamate levels was associated with a decrease of infarct sizes and edema development, as well as to a reduction on motor deficits.

Preclinical testing criteria for neuroprotective agents establishes that they have to reduce the ischemic damage at least by 50% or even better to 80% before being considered for clinical trials (Fisher *et al*, 2007; Rother, 2008). Considering that GOT activation reduces the ischemic damage about 80%, an optimal neuroprotective capacity is expected for this enzyme.

High levels of glutamate in brain parenchyma cause an overstimulation of AMPA and NMDA receptors. Overstimulation of both receptors induces an influx of calcium and sodium, leading to an electrolytic disturbance in brain tissue and the subsequent formation of cytotoxic edema. The increase in tissue water content and brain swelling that contribute to the ischemic injury worsen (Lin *et al*, 1993; Moldes *et al*, 2008). Therefore, the development of brain edema represents another good marker to determine the neuroprotective efficacy of GOT activation. In line with this, our results show that oxaloacetate-mediated GOT activation reduced the edema development.

It could be argued that observed effects could be actually caused by neuroprotective properties of oxaloacetate and not to a real effect of GOT on brain glutamate levels. To clarify this point, we performed a magnetic resonance spectroscopy analysis of glutamate levels in brain parenchyma. In agreement with previous *in vivo* microdialysis studies (Berger *et al*, 2008) and with the pathogenesis of ischemia (Castillo *et al*, 1996, 1997) our *in vivo* spectroscopic analysis showed an increase of glutamate levels in the brain after MCAO, which is inhibited after oxaloacetate-mediated GOT activation. These results reinforce the proposed neuroprotective mechanism, revealing the efficacy of GOT to remove brain glutamate.

As it is shown in Figures 4 and 6, the decrease of blood and brain glutamate levels takes place simultaneously after oxaloacetate treatment, reflecting a rapid diffusion of glutamate from the brain into the blood torrent. In this regard, kinetic studies by means of radiolabeled glutamate injection into rat lateral ventricles have also shown a fast (within 1 minute) diffusion of glutamate from the brain parenchyma to blood (Gottlieb *et al*, 2003). Therefore, the efficacy of GOT to remove brain glutamate depends on its ability to metabolize blood glutamate to increase the concentration gradient between the brain parenchyma and blood torrent.

Although clinical studies are necessary to perform and validate these results, in conclusion, the capacity of the GOT to remove glutamate from the brain by means of blood glutamate degradation show that this enzyme could be considered as an efficient and novel neuroprotective tool against ischemic stroke.

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