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Neuroprotective effect of oxaloacetate in focal brain ischemic model in the rat

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Abstract

During an ischemic event, the well-regulated glutamate (Glu) homeostasis is disturbed, which gives rise to extremely high levels of this excitatory neurotransmitter in the brain tissues. It was earlier reported that the administration of oxaloacetate (OxAc) as a Glu scavenger reduces the Glu level in the brain by enhancing the brain-to-blood Glu efflux. Here, we studied the neuroprotective effect of OxAc administration in a new focal ischemic model in rats. Occlusion of the middle cerebral artery resulted in immediate reduction of the somatosensory evoked responses (SERs), and the amplitudes remained at the reduced level throughout the whole ischemic period. On reperfusion, the SERs started to increase, but never reached the control level. OxAc proved to be protective, since the amplitudes started to recover even during the ischemia, and finally fully regained the control level. The findings of the histological measurements were in accordance with the electrophysiological data. After Fluoro Jade C staining, significantly fewer labeled cells were detected in the OxAc-treated group relative to the control. These results provide new evidence of the neuroprotective effect of OxAc against ischemic injury, which strengthens the likelihood of its future applicability as a novel neuroprotective agent for the treatment of ischemic stroke patients.

Introduction

Cerebrovascular diseases, stroke and closed head injuries are prominent among the leading causes of death worldwide. In the past two decades, the rank of stroke among the top causes of death has not changed. In 2010, one in four deaths were due to stroke, as compared with one in five in 1990. Thrombolysis is the only possible neuroprotective solution in the clinic. Glutamate (Glu), the main excitatory neurotransmitter in the brain, which is released in excess quantity in the course of an ischemic attack, plays a central role in the secondary damage (Choi, 1988; White et al., 2000; Vesce et al., 2007). Considerable effort has been devoted to reducing the effects or the level of the excess neurotoxic Glu in the brain. Most of the developed methods proved to be effective in animal experiments, with outstanding results from the use of NMDA blockers, for instance, but unfortunately these methods failed in the clinical trials (Ginsberg, 2008). In the past decade, a number of new and promising approaches have emerged to decrease the harmful consequences of brain ischemia. One of the most promising is Glu scavenging, based on the enhanced outflow of excitotoxic Glu from the brain into the blood as a consequence of the decreasing blood Glu level (Gottlieb et al., 2003).

The homeostasis of Glu is a well-regulated process in the brain. Glu is present in widely-ranging concentrations in the different compartments of the brain Glu homeostasis system. Under normal conditions, the Glu level is maintained at ~1 μM in the brain interstitial fluid and cerebrospinal fluid. Its levels are much higher in the neurons (~10 mM) and synaptic vesicles (~100 mM) (O'Kane et al., 1999). During neuronal communication, the Glu transporters of the nerve teminals and perisynaptic astrocytes are responsible for the removal of Glu from the synaptic cleft (Danbolt, 2001). Moreover, the Glu uptake from the interstitial fluid is also regulated by the Na^+-dependent excitatory amino acid transporters (EAATs) present on the brain capillary endothelial cells (Beart and O'Shea, 2007). Under specific physiological and pathological conditions, Glu can pass into the blood stream via the endothelial cells with the aid of EAATs and facilitative Glu transporters on the luminal side (Teichberg et al., 2009). This unidirectional transport can be enhanced by decreasing the blood Glu level. Previous studies have demonstrated the neuroprotective effect of the i.v. administration of the Glu-oxaloacetate transaminase (GOT) in the disappearance of the high level of Glu in the brain under pathological conditions (Zlotnik et al., 2007; Zlotnik et al., 2008; Campos et al., 2011a). This blood-resident enzyme catalyses the transformation of Glu and oxaloacetate (OxAc) to 2-α-ketoglutarate and aspartate. The peripheral application of OxAc, as GOT cosubstrate can also be used for Glu scavenging. The neuroprotective effect of OxAc has previously been

demonstrated in focal (Nagy et al., 2009; Campos et al., 2011a) and global (Marosi et al., 2009) ischemic models. We recently described a new focal ischemic model in the rat (Knapp et al., 2013).

The aim of the present work, was to investigate the possible protective effect of the administration of OxAc in this new focal ischemic model by electrophysiological and histological methods.

Materials and methods

Animals and surgery

Male adult Wistar (Charles River) rats weighing 250-300 g (N = 5 in each group) were used in the experiments. The animals were given free access to food and water prior to surgery. All procedures were approved by the Animal Care Committee of the University and were conducted according to the recommendations of the Declaration of Helsinki and Tokyo. Every effort was made to minimize animal suffering and to reduce the number of animals used in this study. Experiments were carried out under Nembutal anesthesia (65 mg/kg and 40 mg/kg/30 min). Body temperature was maintained at 37 ± 0.5 ºC through the use of a self-regulating heating pad (TMP 5-b, Supertech, Budapest, Hungary) and a rectal probe. The surgical procedure, the experimental protocol applied including the induction of focal ischemia, the electrophysiological recording and the histological procedures were similar to those in our previous study (Knapp et al., 2013). Briefly, craniotomy was performed over the primary somatosensory cortex, and on the left side the temporal skull was opened for preparation of the trunk of the middle cerebral artery (MCA). To induce ischemia, the MCA was lifted with a special micromanipulating hook moved by a micromanipulator. The duration of ischemia was adjusted to 2 x 15 min (interrupted by a 30-min reperfusion). The survival rate was 91% (10/11 animals).

Experimental groups and treatments

OxAc (Sigma–Aldrich, Munich, Germany) or saline was administered into the right tail vein during the first 15-min ischemic period (volume: 1 ml, duration: 15 min, speed: 66.6 µ l/min) with the aid of a microinjection pump (CMA/100, CMA Microdialysis AB, Kista, Sweden). On the basis of previous studies (Nagy et al., 2010; Campos et al., 2013), the dose of OxAc was 3.5 mg/100 g. OxAc was solved in phosphate buffer; the pH was set to 7.4.

Electrophysiology

Somatosensory evoked responses (SERs) were induced as described previously (Toldi et al., 1994) and were

recorded (in 5 animals per group) before, during and after the ischemic period (120 min). Briefly, the trigeminal nerve was stimulated by electrical stimulation of the whisker pad (4 V, 0.2 ms, 0.1 Hz) through a bipolar needle electrode (Toldi et al., 1994; Farkas et al., 2000). The recordings were made on the surface of the dura with the aid of a silver electrode. The punctum maximum of the SERs was identified; it was generally localized 3.5 mm behind the bregma and 5 mm laterally. The amplified responses were processed and averaged with Experimetria Intrasys software (Experimetria Ltd., Budapest, Hungary). The 30-min control period was followed by 2 x 15-min ischemic episodes interrupted by a 30-min reperfusion.

Histology

For histological assessment after a 1-day survival period (following surgery), the animals (N = 10) received an overdose of urethane, and were perfused transcardially with 0.1 M ice-cold phosphate-buffered saline (pH 7.4), followed by 4% buffered paraformaldehyde. The brains were removed, and postfixed overnight in paraformaldehyde at 4 °C. Coronal sections (20 μ m) were obtained from -2.0 to -6.0 mm behind the bregma with a vibratome (Leica VT1000 S, Leica Microsystems, Wetzlar, Germany). In this work, every tenth 20-μm slice was analyzed in the length of 4 mm (Fig. 2A) from the area supplied by the MCA. The early changes in neural viability induced by the ischemia and reperfusion were visualized by means of Fluoro Jade C (FJC) staining. Cortical FJC-positive cells were counted in the quadrant (0.25 mm^2) of slices in the same position. The slices were mounted on gelatine-coated slides, then coverslipped with Fluoromount. Fluorescence photomicrographs were obtained with an Olympus BX51 microscope fitted with a DP70 digital imaging system. Solvents were obtained from Sigma-Aldrich Co (Munich, Germany).

Statistical analysis

Electrophysiology

Repeated measurements of SER amplitudes of the control and OxAc treated groups were compared separately with the aid of the non-parametric Related-Samples Friedman's Two-Way Analysis of Variance by Ranks.

Hystology

Numbers of FJC+ cells were compared with the Generalized Linear Mixed Model. During the data analysis Poisson distribution of the data was considered. The effects of the different rats were used as nested random

effects and the different treatments were used as fixed effects in the mixed effect linear model (IBM SPSS Statistics version 20). A p-value of 0.05 was considered significant.

Results

Electrophysiology

There was an obvious effect of the focal ischemia on the SERs (Fig. 1). In the control group, the amplitudes of the SERs decreased immediately at the beginning of the occlusion of the MCA and remained at the reduced level throughout the ischemic period. At the beginning of reperfusion, the amplitudes started to increase, but never regained the control level. This result was quite similar to that in our previous work (Knapp et al., 2013). In the other group, where OxAc was administered during the first ischemic event, the result was significantly different. The decreased amplitudes began to recover even during the ischemia, and finally reached the control level (Fig. 1). The increase in the SER amplitudes was markedly faster than that in the controls. In the OxAc-treated group, the recovery of the amplitudes was even faster during the second ischemic event than during the first one. The increase was already observed within 2 min after the beginning of the ischemic period.

Histology

As a result of the occlusion of the MCA with a 1-day survival period, well-outlined FJC-positive cells emerged throughout the ipsilateral somatosensory cortices supplied by the MCA. Staining was prominent in the cell membrane and the cytoplasm (Fig. 2B). The FJC-positive cells were easily counted since the FJC dye did not penetrate into the damaged brain parenchyma during the survival period. In the control group, the average number of labeled cells in the slices on the given rectangle obtained at from -2 to -6 mm from the bregma was different from those of treated group. The OxAc treatment proved to be protective against the focal cerebral ischemia, since it led to fewer labeled cells in the same area. Significant difference was evinced between the control and the treated groups. The data of cell counts are showed in Box diagrams (Fig. 2C), the median of the cell numbers was 38 in the control and 6 in the OxAc treated group.

Discussion

Despite an intensive search for different neuroprotective strategies and agents against ischemic brain injury, the only effective treatment is thrombolysis (Ginsberg, 2008). From the early 2000s, attention turned to Glu scavenging (Gottlieb et al., 2003). This new trend in the field of neuroprotection had the aim of reducing the excess Glu after ischemia. The procedure is based on decreasing the blood Glu level, which enhances the brain-

to-blood Glu efflux via the Na^+-dependent EAATs and the facilitative Glu transporters present on the abluminal and luminal sides of the endothelial cells, respectively (Teichberg et al., 2009). An important factor which supports this process is the huge number and surface area of the brain capillaries. The human brain contains approximately 10^8 capillaries, which have a surface area of ~12 m^2 (Bickel et al., 2001). In the compartment of the blood-brain barrier, the average distance between a capillary and a neuron is ~8-20 μ m, and it may therefore be stated that virtually every neuron has its own capillary in the brain (Pawlik et al., 1981).

The blood-resident GOT is the key enzyme that influences the blood Glu level. Like in case of Glu–pyruvate transaminase (GPT) and pyruvate, GOT converts glu into 2-ketoglutarate in presence of oxaloacetate, serving as the co-substrate for this reaction. The enzymatic activity of GOT has been reported to be higher than that of GPT both in rats and in humans (Teichberg, 2011). Boyko et al. (2012) first described the pharmacokinetic and pharmacodynamic properties of GOT and GPT. They found that these two enzymes are largely distributed in the central circulation and their interactions with Glu also take place there. The explanation of this phenomenon is the low permeation of GOT and GPT to the peripheral organs. These enzymes require a several-hour delay before they can affect the pool of serum Glu. The Glu-lowering property of GOT proved to be more effective in a higher dose, which is due to the low OxAc level in the blood. They therefore concluded that a noteworthy neuroprotective effect against ischemia may be achieved through the administration of GOT in a higher dose or through coadministration of the co-substrate OxAc.

The neurological outcome after stroke has been demonstrated to depend on the blood level of GOT. Campos et al. (2011b) concluded that GOT itself can act as a protective factor against Glu-mediated pathophysiological processes: a high GOT level in the blood resulted in a better neurological outcome relative to that at a low GOT level. It has also been reported, that migraine patients exhibit a lower blood GOT activity and a higher blood Glu level than those in healthy control subjects (Campos et al., 2013). In one interesting study, the long-lasting neuroprotective effects of Glu scavengers against brain-implanted gliomas were described. Chronic OxAc treatment proved to reduce the proliferation and invasiveness of the glioma cells, decreasing the size of the gliomas and prolonging the survival of rats and mice. The excess Glu in the peritumoral space of a glioma enhance its invasiveness. The released Glu kills the neighboring cells, thereby providing an increased space for the occupancy in the brain (Ruban et al., 2012).

The scavenging reaction detailed above can be achieved through the i.v. administration of OxAc after an ischemic event. This promising four-carbon molecule, takes part in many metabolic and energy-producing

pathways in the body (e.g. the citric acid cycle, gluconeogenesis and the urea cycle). It is well known that, as a component of the citric acid cycle, OxAc plays a pivotal role in ATP production (Campos et al., 2012). Numerous studies have described the neuroprotective effect of OxAc. It is protective as a Glu scavenger against focal cerebral ischemia (Campos et al., 2011a) or global hypoperfusion (Marosi et al., 2009), and also as an antioxidant against excitotoxic damage (Yamamoto and Mohanan, 2003).

The present study revealed the effects of Glu scavenging on the cortical evoked potentials in our newly-developed focal ischemia model. In the control group, the SER amplitudes decreased during the ischemic event, and were not fully restored after it, reaching ~60% of the control level. As a result of the i.v. injection of OxAc, the reduced amplitudes started to increase even during the ischemic period and finally regained the control level. This indicates the rapid changes caused by Glu scavenging. The cortical function maintained its state following the ischemia. After a 1-day survival period, a marked difference was detected between the two groups on the use of FJC dye. In the treated group, significantly fewer FJC-positive neurons were counted than in the controls.

In these experiments our aim was to investigate the effect of OxAc treatment in our model. Therefore, in order to reach the most remarkable effectiveness, the treatment was performed during the ischemic period. Nevertheless, in the clinical trials the neuroprotective effect of Glu scavenging also can be achieved during the hours of occuring Glu excitotoxicity. Our results clearly demonstrate the neuroprotective effect of OxAc as a Glu scavenger, and the value of this ischemic model for the investigation of different neuroactive pharmacological agents.

Several promising studies have suggesed the advantageous effects of Glu scavengers, and especially OxAc. The most beneficial properties of this molecule are that its effects are not mediated through receptor modulation, while the process is self-contolled (Teichberg et al., 2009) and occurs naturally in the human body, so that OxAc can be administered without serious side-effects. Our results additionally provide new evidence of the neuroprotective effect of OxAc, which strengthens the view that it may potentially be applied as a novel neuroprotective agent for the treatment of ischemic stroke patients in the future.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Legends to figures

Fig. 1. Changes in SER amplitudes during the short-lasting (15-min) ischemic periods. In the control group (■) saline was injected intravenously during the first ischemic event. The maximum amplitudes in both reperfusion periods were significantly lower than those in the control period (see the labeled data range). For the statistical analysis non-parametric Related-Samples Friedman's Two-Way Analysis of Variance by Ranks model

was used. (*** $p < 0.001$; Chi-Square: 81,61; df: 2; N = 5, SER amplitudes are expressed as the mean ± S.E.M.) In the treated group (○), 3.5 mg/100 g OxAc was administered analogously to the saline treatment of the controls. The ischemia-reduced amplitudes finally resumed the control level, the last 10 min of the reperfusion periods showed no significant (n.s.) difference from the control period. (p=0,592; Chi-Square:

1,05; df: 2; N = 5; mean ± S.E.M.)

Fig. 2. Histological studies. (A) Illustration of the location of the analyzed sections (parallel lines) between -2 and -6 mm behind the bregma (Br.). The filled black circle indicates the position of electrophysiological recording. (B) Representative photomicrograph of the FJC labeling after a 1-day survival. The insert shows FJCpositive cells at higher magnification. (C) Box-diagrams of the count of FJC-positive cell in 0.25 mm^2 quadrants of the cortex. Significant difference was detected between the groups with the aid of Generalized Linear Mixed Model. (** $p < 0.01$; F: 21,18; df: 1; medians: 38; 6)

Fig 1.

Fig 2.