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Acetyl-L-carnitine and oxaloacetate in post-treatment against LTP impairment in a rat ischemia model. An *in vitro* electrophysiological study

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Abstract

A high proprotion of research relating to cerebral ischemia focuses on neuroprotection. The application of compounds normally present in the organism is popular, because they do not greatly influence the synaptic activity by receptor modulation, and can be administered without serious side-effects. Oxaloacetate (OxAc) and acetyl-L-carnitine (ALC) are such favorable endogenous molecules. ALC can exert a protective effect by improving the energy state of the neurons under ischemic conditions. A promising neuroprotective strategy is glutamate scavenging, which can be achieved by the intravenous administration of OxAc. This study involved the possible protective effects of ALC and OxAc in different post-treatment protocols against long-term potentiation (LTP) impairment. Ischemia was induced in rats by 2-vessel occlusion, which led to a decreased LTP relative to the control group. High-dose (200 mg/kg) ALC or OxAc post-treatment resulted in a higher potentiation relative to the 2VO group, but it did not reach the control level, whereas low-dose ALC (100 mg/kg) in combination with OxAc completely restored the LTP function. Many previous studies have concluded that ALC can be protective only as pretreatment. The strategy described here reveals that ALC can also be neuroprotective when utilized as post-treatment against ischemia.

Key words: brain ischemia, acetyl-L-carnitine, oxaloacetate, hippocampus, LTP.

Introduction

Stroke is one of the major causes of death: during the past 20 years the number of stroke victims has increased by 26-35% (Lozano et al. 2012). The quest for effective neuroprotective drugs and/or strategies is currently at the focus of much research. Attention recently turned to

compounds normally present in the body, that do not influence synaptic activity by receptor modulation, and that can be administered in high doses without serious side-effects.

Ischemic conditions are associated with the presence of extremely high levels of glutamate (Glu) in the brain interstitial fluid (ISF)/cerebrospinal fluid (CSF) (Castillo et al. 1996), which is one of the major causes of excitotoxicity leading to cell death. During physiological neuronal activity, the Glu transporters (present on both nerve terminals and astrocytes) regulate the extracellular Glu concentration. After release, a high Glu concentration (1 mM) develops in the synaptic cleft, the transporters then taking up Glu and decreasing its level in the ISF (around 1 μ M) (Danbolt 2001). On the other hand, the Glu concentration is also regulated by transporters located on the antiluminal (brain) side of the blood capillaries (O'Kane et al. 1999). It was hypothetized in the early 2000s that a considerable Glu concentration gradient between the ISF/CSF and the blood plasma could provide an increased driving force for the brain-to-blood Glu efflux. This process can be fast and greatly enhanced by intravenous administration of the blood Glu scavengers oxaloacetate (OxAc) and pyruvate (Pyr) (Gottlieb et al. 2003). The failure of the cerebral blood flow triggers the activation of the ischemic cascade. As parts of this process, the failure of ion homeostasis and the increase in calcium concentration result in excess activation of the Glu receptors, initiating Glu excitotoxicity, which develops immediately after the onset of the ischemic event. While Glu excitotoxicity takes from minutes up to a few hours, it has the greatest impact on the final outcome. Excitotoxicity triggers a number of delayed events that further increase the damage (Dirnagl et al., 1999). The aim of Glu scavenging is to decrease the excess Glu level in the brain. As a result of this strategy, the i.v. administered scavenger (e.g. OxAc) decreases the blood Glu level, and the excess Glu can therefore be removed from the brain toward the blood (Gottlieb et al., 2003). Since excitotoxicity appears in the very initial phase of the ischemic

cascade, Glu scavenging must be applied as early as possible after the ischemia. The first evidence of the neuroprotective effect of Glu scavenging was demonstrated in a closed head injury model (Zlotnik et al., 2007). Glu scavenging is nowadays an extensively studied feature of neuroprotective strategies. Its promise is due to the facts that it does not modulate the receptor functions and it exists physiologically in the human body as an element of the Krebs cycle, and it can therefore be administered safely.

Another promising approach to neuroprotection is the administration of different carnitine derivates. Carnitine (β-hydroxy-γ-N-trimethylaminobutyric acid) can be obtained from meat and dairy materials, and also by endogenous biosynthesis from L-lysine and L-methionine, which takes place in the liver and kidney (Borum 1983). The main function of carnitine is to transport fatty acids across the inner mitochondria membrane for β-oxidation. These compounds are also scavengers of oxygen free radicals in mammalian tissues (Rani and Panneerselvam 2002). Acetyl-L-carnitine (ALC), which takes part in the carbohydrate, lipid and protein metabolism, has the important favorable property of effectively crossing the blood-brain barrier (Malaguarnera et al. 2011). Glu excitotoxicity is one of the key elements of the ischemic cascade (Dirnagl et al., 1999), and it has also been demonstrated that ALC can reduce the Glu outflow after focal cerebral ischemia (Jalal et al., 2010). An earlier investigation (Kocsis et al. 2014) revealed that the neuroprotective effect of 100 mg/kg ALC on global hypoperfusion was observed only in the case of pretreatment, and not after post-treatment. This is in accordance with the observations of previous *in vivo* and *in vitro* studies (Picconi et al. 2006; Shuaib et al. 1995).

We therefore set out to create a new strategy in which ALC post-treatment can be neuroprotective. In the first part of the present study, a higher ALC dose was used than in the previously reported experiments (Kocsis et al. 2014). In order to use the lowest concentrations

of the compounds possible, treatment with ALC in combination with OxAc was used in the second part of the study.

Materials and methods

Animals and housing conditions

Male Wistar rats (200-250 g) supplied by Charles River Laboratories were used in the experiments (N=31). The animals were housed individually in standard plastic cages with free access to food and water. The animal house was light-controled with 12-h/12-h dark/light cycles and kept under conditions of constant room temperature (23 °C) and humidity. Every effort was made to minimize the number of animals used and their suffering. The principles of animal care (NIH publication No. 85-23), and the protocol for animal care approved by both the Hungarian Health Committee (1998) and the European Communities Council Directive (86/609/EEC) were followed. The rats were randomly divided into 5 groups, as follows: sham-operated controls (N=6); rats subjected to 2-vessel occlusion (2VO) (N=6); 2VO+ALC post-treatment (N=6); 2VO+OxAc post-treatment (N=5) and 2VO+OxAc+ALC post-treatment (N=8).

Modeling of the hypoperfusion

All surgical procedures were carried out under deep anesthesia. Before the induction of transient forebrain ischemia, the rats were anesthetized with sodium pentobarbital (60 mg/ml, i.p.). Their body temperature was measured continuously and maintained at 37±0.5 °C by means of an automatic heat controller (Supertech TMP-5a, Hungary) from the beginning of the operation until the rats had been awakened. The 2VO method used for the modeling of transient global hypoperfusion was as described previously (Marosi et al. 2009). The common

carotid arteries were blunt-dissected free by separation from the vagal nerves and connective tissues, and were clamped with non-traumatic aneurysm clips (Aesculap, B. Braun Medical Ltd, Hungary). After a 30-min period of global hypoperfusion, the blood flow in these arteries was restored by releasing the clips.

Drug administration

The effects of a 100 mg/kg dose of ALC on sham-operated controls, and on 2VO-operated rats were tested in our previous study (Kocsis et al. 2014). In the present study, the rats received 200 mg/kg ALC for 5 additional days after 2VO (once a day). The first treatment was applied 1 h after the 2VO surgery. ALC was dissolved in 0.9% saline (total volume 1 ml) and was administered i.p. In the OxAc+ALC-treated group, the OxAc was administered i.v. (20 mg/100 g/bw) immediately after the reperfusion and the low-dose ALC (100 mg/kg) post-treatment was then applied for 5 additional days. OxAc was dissolved in distilled water and the pH was then adjusted to 7.4; the total volume was 1 ml. The effect of OxAc administered alone was also measured. Figure 1 indicates the time points of the treatments, 2VO surgery and electrophysiological measurements.

In vitro electrophysiology

The electrophysiological experiments were conducted 5 days after the ischemic insult. The animals were killed by decapitation and coronal slices (350 μm) were prepared from the middle part of the hippocampi with a vibratome (Campden Instruments, UK) in an ice-cold artificial cerebrospinal solution (aCSF) composed of (in mM): 130 NaCl, 3.5 KCl, 1 NaH_2PO_4, 24 $NaHCO_3$, 1 $CaCl_2$, 3 $MgSO_4$ and 10 D-glucose (all from Sigma, Germany), gassed with 95% O_2 + 5% CO_2. The slices then were transferred to a Haas recording chamber

for recording. The recording solution differed only in that it contained 3 mM $CaCl_2$ and 1.5 mM $MgSO_4$ (the flow rate of the perfusion was 1.5-2 ml/min). For the electrophysiological recordings, 1-2 slices were used from each animal. A bipolar concentric stainless steel electrode (Neuronelektrod Ltd, Hungary) was placed in the stratum radiatum between the CA1 and CA2 regions of the hippocampal slices. The stimulus intensity was adjusted to between 30 and 70 μA (constant current, 0.2 ms pulses delivered at 0.05 Hz) to evoke the half-maximum response. Field excitatory postsynaptic potentials (fEPSPs) were recorded with a 1.5-2 MΩ resistance glass micropipette filled with aCSF. The recordings were amplified with a neutralized, high input-impedance preamplifier and filtered (1 Hz-3 kHz). The fEPSPs were digitized (AIF-03, Experimetria Ltd., Hungary), acquired at a sampling rate of 10 kHz, saved to a PC and analyzed off-line with Origin Pro 8 software (OriginLab Corporation, Northampton, USA). The fEPSPs were monitored for at least 50 min until the amplitudes were generally stable, and their mean value was determined for the 10-min baseline. For the induction of long-term potentiation (LTP), theta burst stimulation (TBS) was used (bursts of 4 impulses at 100 Hz, with a train duration of 40 ms and a burst interval of 350 ms). Changes in fEPSP amplitudes were recorded for a further 60 min.

Statistical analysis

The fEPSP amplitudes were expressed as a percentage of the 10-min baseline value before the TBS. The Mann-Whitney U-test was chosen for the statistical analysis of the LTP data. Origin Pro 8 software (OriginLab Corporation, Northampton, USA) was utilized. In each analysis, a p value of ≤ 0.05 was considered significant.

Results

The effects of ALC and OxAc on the 2VO-induced LTP impairment were tested on hippocampal slices. After stabilization of the baseline, a 10-min control period was recorded, and TBS was then delivered for LTP induction. In the control group, TBS resulted in a notable fEPSP amplitude increase (145.37±0.25%), which remained stable during the further 60-min. The value of the fEPSP amplitude increasing was 145.79±0.74% at the end of the recording period. In the 2VO group, a significantly lower potentiation was observed (117.82±0.49%); the amplitudes showed a constant decay over time and had almost reached the pre-TBS level by the end of the registrations (113.10±1.65%). These data are in accordance with our previous findings (Kocsis et al. 2014). Figure 2 shows representative fEPSPs from the control and 2VO groups. As a result of the damage caused by the ischemia, there were no pathological responses to the stimulation. The recorded neuronal activity was normal, and the waveform of the fEPSPs recorded in the 2VO group was the same as that for the control group. In the present study, a high-dose ALC (200 mg/kg) was used as post-treatment after the transient global hypoperfusion. The LTP was 127.05±0.30% and underwent a slight decrease up to the end of the recordings. The level of the potentiation in the last 10 min of the registrations was 124.07±1.17%. Even this elevated concentration did not restore the LTP function to the control level (similarly to our previous findings with the low-dose (100 mg/kg) ALC). When low-dose ALC was applied in combination with OxAc as post-treatment, however, the fEPSP amplitude increase returned completely to the control level (144.78±0.17%) and it remained stable over time (145.79±0.88%). There was a significant difference between the 2VO and OxAc+ALC groups. As a result of the scavenging effect of OxAc on the 2VO-induced LTP impairment, the induced LTP was 123.94±0.38%, and this potentiation was stable until the end of the recording period (126.03±1.14%) (Fig. 3). The result of this treatment was in accordance with our previous findings, since the LTP did not regain the control level (unpublished data). OxAc treatment alone partly prevent the decay

of the potentiation relative to the 2VO group, and the fEPSPs stabilized at a lower level, but did not reach that of the controls.

Discussion

Although cerebral ischemia has remained the leading cause of death in the past 20 years, there are some drugs which can be used as clinical intervention of ischemic stroke, however one of the most effective treatment is the thrombolysis. Intensive research into neuroprotective strategies against ischemic injury is therefore ongoing.

One of the most promising methods developed during the last 10 years is Glu scavenging. This is based on enhancement of the brain-to-blood Glu efflux, which removes the excess Glu caused by the ischemia/reperfusion injury (Gottlieb et al. 2003). It involves the intravenous administration of a Glu scavenger, e.g. OxAc or Pyr, as a substrate for a bidirectional process catalyzed by the blood-resident enzymes Glu-OxAc transaminase (GOT) or Glu-Pyr transaminase (GPT), respectively. In the presence of the respective Glu co-substrates, OxAc or Pyr, GOT or GPT transaminates Glu into 2-α-ketoglutarate in the blood. This process reduces the Glu concentration in the blood, resulting in an enhanced Glu concentration gradient between the brain and the blood, which eliminates the excess Glu from the ischemic brain (Teichberg et al. 2009). The time-window for the application of Glu scavengers is very narrow, since Glu excitotoxicity appears immediately after the ischemic insult. This harmful process can not only cause damage to the nervous tissue, but also triggers series of other, delayed events, which worsen the outcome. These processes include the peri-infarct depolarization, edema, damage to the membranes and the DNA, inflammation and apoptosis (Dirnagl et al., 1999).

Another direction of the investigations on neuroprotection is the application of compounds that are physiologically present in the human body; this avoids the harmful side-effects and difficulties involved in the dosing. One of the most promising agents is ALC, a naturally occurring substance that participates in the β-oxidation of fatty acids (Jones et al. 2010). Pretreatment with 100 mg/kg ALC in our previous study proved to restore the 2VO-impaired LTP, whereas the post-treatment did not. Our present aim was to find a method in which ALC can be neuroprotective as post-treatment, which is of much greater clinical relevance than pretreatment. We tested the effects of the treatments in one of the most vulnerable regions of the brain. LTP measurements were made on hippocampal slices in the sham-operated controls, in animals subjected to 2VO, and in 2VO groups post-treated with high-dose ALC (200 mg/kg), OxAc scavenging or with the combination of OxAc and low-dose ALC (100 mg/kg). The results in the controls and the 2VO group were similar to our previous findings (Kocsis et al. 2014). The 30-min global hypoperfusion resulted in an impaired LTP, where the fEPSP amplitudes were significantly lower relative to those in the control group. In the first part of the present experiments, we tried to reach a much more noteworthy neuroprotective effect (relative to our previous study) by only elevating the concentration of ALC. A 200 mg/kg dose of ALC was slightly more effective than 100 mg/kg (applied previously), but it did not restore the LTP function to the control level either. It was earlier demonstrated that particularly pretreatment with ALC can be neuroprotective (Picconi et al. 2006; Shuaib et al. 1995). One possible explanations for the ineffectiveness of ALC post-treatment that is the excess Glu appearing immediately after the ischemia results in irreversible, harmful effects and leading to cell death (Choi 1988). In our present experiments, Glu scavenging was combined with ALC post-treatment. The scavenger OxAc was administered immediately after the reperfusion, which caused a decrease in the Glu level in the brain. As a result, ALC post-treatment could be much more effective (even in a lower dose). In the OxAc+ALC group, the

combination of the two agents and the two strategies resulted in a completely restored LTP. A recent review described many aspects of the protective effect of ALC (Jones et al. 2010). Among the possible actions of neuroprotection, and one of the main studied mechanisms, is the stimulation of the aerobic energy metabolism in the brain (Zanelli et al. 2005). After short-term cerebral ischemia, the extent of glucose oxidation decreases significantly within the first hour of reperfusion and remains at the reduced level for many hours (Sims 1995). This is most likely due to the decreased activity of the Pyr dehydrogenase complex (Fukuchi et al. 1998), which serves as the critical link between the anaerobic and aerobic metabolisms by catalyzing the oxidative decarboxylation of Pyr to acetyl CoA (Reed 1981). Under metabolically compromised circumstances (when the function of the complex is damaged), ALC can serve as an exogenous, alternative fuel in the presence of a reduced conversion of Pyr to acetyl CoA (Martin et al. 2005). Furthermore, OxAc (as a member of the Krebs cycle) can also possibly exert its effect by enhancing the energy metabolism of the cells.

The present study has revealed an approach for the application of ALC in combined treatment. This is the first demonstration of the neuroprotective effect of ALC post-treatment resulting in complete restoration of the LTP function. It is also important that this effect was achieved through low-dose ALC post-treatment. Here, we have provided a speculative explanation which may underly these results, but the exact mechanisms of the effects of OxAc+ALC treatment require further investigations.

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Conflict of interest

The authors declare that they have no conflict of interest.

Legends to Figures

Fig. 1:

A schematic graph indicating the time points of the 2VO surgery, the ALC and OxAc treatment, and the electrophysiological experiment. In the case of OxAc treatment, the rats received the i.v. administration immediately after the reperfusion. In the ALC post-treated groups, the first ALC treatment (i.p.) was applied 1 h after the 2VO surgery, and the animals received four additional ALC injections.

Fig. 2:

Representative fEPSPs recorded from the control and the 2VO groups. As a result of the damage caused by the ischemia there were no pathological responses to the stimulation. The recorded neuronal activity was normal, and the waveform of the fEPSPs recorded in the 2VO group was the same as that for the control group.

Fig. 3:

LTP inducibility in the control, 2VO, 2VO+ALC post-treatment, 2VO+OxAc and 2VO+OxAc+ALC post-treatment groups (numbers of electrophysiological recordings: N=11 in the sham-operated control group, N=10 in the 2VO group, N=9 in the 2VO+ALC post-

treatment group, N=8 in the 2VO+OxAc group and N=10 in the 2VO+OxAc+ALC post-treatment group). As a result of the ischemia, the potentiation of the fEPSPs was significantly lower relative to the control level (4). The high-dose ALC post-treatment resulted in a higher and more stable LTP as compared with the 2VO group (2), but the fEPSP amplitudes did not reach the control level (1). As a result of the scavenging effect of OxAc, there was a similar potentiation as that in the high-dose ALC-treated group. The LTP was significantly weaker than the control (5), but it also was stable relative to the ischemic group and the amplitudes remained at a constant level during time (6). The increase in the fEPSPs after TBS in the 2VO+OxAc+ALC post-treatment group was the same as that in the control group, and remained stable throughout the recording period. The LTP of this group was significantly higher as compared with the 2VO group (3). There was a significant difference between the 2VO+ALC and 2VO+OxAc+ALC groups and also between the 2VO+OxAc and 2VO+OxAc+ALC groups. Furthermore, there was no sigficant difference between the 2VO+ALC and 2VO+OxAc groups. Data points are means ± S.E.M. of the normalized amplitudes of the fEPSPs. Asterisks denote significant differences from the control, and $^{\#}$ denotes significant differences from the data for the 2VO group (**$p<0.01$, ***$p<0.001$, $^{\#}p<0.05$, $^{\#\#\#}p<0.001$; Mann-Whitney U-test).

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