Ph.D thesis

EXAMINATION OF THE NEUROPROTECTIVE EFFECT OF ACETYL-L-CARNITINE ON ISCHEMIC MODELS

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List of abreviations

2VO: 2-vessel occlusion

AcCoA: acetyl coenzyme-A

aCSF: artificial cerebrospinal fluid

ALC: acetyl-L-carnitine

CAT: carnitine-acetyl transferase

CCA: common carotid artery

CoA: coenzyme A

fEPSP: field excitatory postsynaptic potentiation

Glu: glutamate

i.p.: intraperitoneal

LTP: long term potentiation

OGD: oxygen glucose deprivation

PI3K: phosphatidyl inositol 3-kinase

TBS: theta burst stimulation

Introduction

Stroke is one of the leading causes of death worldwide. Among all cases, 87% involve ischemic stroke, which can be caused by thrombosis or embolism resulting in a restriction of the blood flow to the brain. As a consequence, the insufficient oxygen and glucose delivery results in damage to the nervous tissue, giving rise to permanent cognitive and motor dysfunctions in the surviving patients. It is important to reveal the detailed pathomechanisms and processes underlying ischemia in order to develop new diagnostic and therapeutic procedures. The excitotoxicity, acidotoxicity, ionic imbalance, oxidative stress, inflammation and apoptosis caused by the ischemic event have different time-windows. Certain of them appear immediately after the insult, while others do so only some days later. As a result of the ischemia, the normal function of the nervous tissue is disturbed, and the neurons are depolarized, which results in an excessive release of glutamate (Glu). Excitotoxicity accompanied by excessive Glu and Ca^{2+} release develops within a few minutes and causes serious damage to the brain. This is exacerbated by other processes, e.g. oxidative stress or long-lasting inflammation. Despite extensive research on ischemic stroke, the adequate treatment of the patients remains an unsolved problem. The aim of neuroprotective strategies is to prevent or mitigate the neuronal impairments caused by neurodegenerative diseases (e.g. ischemia). Global ischemia, which is of two main types, affects the whole brain. The reduced blood flow to the brain as a consequence of the atherosclerosis of the common carotid arteries (CCAs) is called hypoperfusion, while a cardiac arrest results in the complete interruption of the cerebral blood flow. Agents are often promising in animal models of stroke, but fail completely in clinical studies (because of the ineffectiveness or serious side-effects). There is therefore currently a trend in investigations on neuroprotection to apply compounds physiologically present in the human body; this avoids the harmful side-effects and difficulties involved in the dosing. Such candidates in neuroprotection are antioxidants and metabolic compounds, e.g. acetyl-L-carnitine (ALC). The main function of the carnitines is the transportation of long-chain fatty acids into the mitochondra for β-oxidation. The polar nature of these molecules makes them very mobile throughout the cell, and they can also readily cross the blood-brain barrier. The most common ester of carnitine is ALC, which is important in the transportation of acetyl groups into different regions of the body. In the presence of free coenzyme-A ALC is converted to free carnitine and acetyl coenzyme-A (AcCoA) by carnitine-acetyl transferase in the mitochondria. AcCoA can participate in various biosynthesis pathways or energy-producing processes. ALC has important roles in

diseases accompanied by an impairment of the energy balance (e.g. ischemia-reperfusion injury or Alzheimer disease) since this compound affects the functioning of several metabolic pathways. Carnitines also have neuroprotective, neuromodulatory and neurotrophic functions. ALC has proved to be a very promising agent in studies of various neurodegenerative disorders. Some of its proposed neuroprotective benefits involve an improved mitochondrial function and energetics, an antioxidant effect, the stabilization of membranes, protein and gene expression modulation and the enhancement of cholinergic neurotransmission. The long-term potentiation (LTP) underlying the cellular basis of learning and memory is a sensitive mechanism which is also appropriate for studying the functional impairments caused by ischemia. Global hypoperfusion results in a decay of synaptic efficiacy. Morphological changes can also be detected which are associated with an impaired LTP. 90% of the excitatory synapses are located on the dendritic spines, and changes in the structure of these protrusions therefore basically affects the synaptic communication. In the vulnerable regions of the brain, e.g. in the CA1 subfield of the hippocampus which is extremely sensitive to ischemia, the number of spines decreases as a result of hypoperfusion. Determination of the changes in the LTP function and quantitative analysis of the spine density after ischemia provides a suitable way to measure ischemic impairments and the effects of potential protective agents.

Aims

W eset out to examine the effects of a promising compound which occurs naturally in the human body. Since this is an endogenous molecule, the harmful side-effects and difficulties involved in the dosing are avoided. There were three aspects of our investigations:

1. **Estimation of the potential protective effects of ALC against the global hypoperfusion caused by 2-vessel occlusion (2VO). Electrophysiological and histological measurements were conducted for these experiments.**

2. **Investigation of the effects of ALC against *in vitro* global ischemia, and establishment of whether the application of ALC can result in functional regeneration that enhances stable LTP.**

3. **Investigation of the molecular mechanisms underlying the neuroprotective effects of ALC against global hypoperfusion and global ischemia.**

Materials and methods

Global hypoperfusion model: 2VO surgery

Male Wistar rats (200-250 g) were used in the experiments. 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.). Through a midline neck incision, the CCAs were dissected and exposed. To induce transient forebrain ischemia, the CCAs were clamped for 30 min with non-traumatic clips. The clips were then released from the CCAs and the blood flow was restarted. Electrophysiological and histological measurements were carried out 5 days after the surgery.

Treatments with ALC

ALC was dissolved in 0.9% saline (total volume 1 ml) and was administered i.p. The animals received the treatment once a day for 5 days either before (pretreatment) or after (post-treatment) the 2VO operation. The animals in the pretreated group received the last ALC injection 1 day before the 2VO operation. The first ALC treatment was applied 1 h after the surgical intervention in the post-treated group. We measured the effects of 100 mg/kg ALC in the pre-treated, and of 100 mg/kg and 200 mg/kg ALC in the post-treated group.

In vitro electrophysiology

For the electrophysiological measurements, 350-μm hippocampal slices were used. A bipolar concentric stainless steel electrode was placed in the stratum radiatum between the CA1 and CA2 regions of the hippocampal slices, and 0.2-ms pulses were delivered at 0.033 Hz to evoke field excitatory postsynaptic potentials (fEPSPs). The amplitudes of the fEPSPs were recorded with a 2-3-MΩ resistance glass micropipette filled with artificial cerebrospinal fluid (aCSF). In the global hypoperfusion, experimental groups after a 10-min control period, high-frequency stimulation (theta burst stimulation, TBS) was delivered for the induction of the LTP. Changes in fEPSP amplitudes were recorded for a further 60 min. In the global ischemic experimental groups, the 10-min control period was followed by 15-min oxygen-glucose deprivation (OGD), and the changes in the fEPSPs were then recorded for a further 40 min. After this regeneration period, the LTP was induced by TBS, and this was followed by a 35-min registration period.

Golgi-Cox impregnation

In order to determine the number of apical dendritic spines of the CA1 pyramidal cells, the Golgi-Cox staining method was used. The numbers of dendritic spines were measured in the control, 2VO, ALC-pretreated and ALC-post-treated groups (4 animals from each group). For the spine number analysis, a specified, 100-μm-long apical dendritic section of 15 randomly selected pyramidal cells was examined in the hippocampal CA1 subfield.

In vitro ischemia: OGD

The aCSF perfused onto the brain slices was replaced by a modified, OGD aCSF during the ischemic period. In this solution, the glucose was replaced by sucrose (and it was gassed with N_2 instead of O_2). OGD results in global ischemia, since the cells receive neither glucose or oxygen, just like the human brain during an ischemic stroke. After OGD, the slices were perfused with normal aCSF again until the end of the following period. In the first part of these OGD experiments, we determined the detailed parameters of the protocol, and primarily the period of the ischemic insult. For this, 5, 8, 10, 15, 16 or 17-min OGD was applied. In these experiments, the amplitude and slope of the fEPSPs were recorded.

Application of ALC in the OGD experiments

After the determination of the suitable parameters of the OGD protocol, the potential protective effect of ALC at different concentrations (125 μM, 250 μM and 500 μM) was measured. ALC was dissolved in aCSF or in OGD aCSF, and was perfused onto the slices during the 10-min control and the 15-min OGD period, respectively. After OGD, the slices were perfused with normal aCSF again until the end of the following period.

Investigation of the underlying mechanisms of ALC

For the investigation of the underlying mechanisms of ALC, a specific phosphatidyle inositol 3-kinase (PI3K) inhibitor (LY294002) was used. It was perfused at 50 μM onto the slices during the 10-min control and the 15-min OGD, together with the most effective, 500 μM ALC.

Statistical analysis

In LTP measurements on the global hypoperfusion experimental groups, the fEPSP amplitudes were expressed as a percentage of the 10-min baseline value before the TBS. For statistical analysis of the LTP data, the Mann-Whitney U-test was used. For analysis of the

spine density data, one-way ANOVA with the Tukey *post hoc* test was utilized. In the OGD experiments to express the potentiation level of the fEPSPs after the LTP induction, the last 10 min before the TBS was determined as baseline (100%). The post-TBS amplitude or slope values were expressed as a percentage of this baseline period. For the statistical analysis of the LTP data, the Mann-Whitney U-test was used, with Origin Pro 8 software (OriginLab Corporation, Northampton, USA). In each analysis, a p value of <0.05, <0.01 or <0.001 was considered significant.

Results

Effects of ALC against global hypoperfusion

In the first part of the experiments, we measured the effect of ALC against global hypoperfusion. 2VO resulted in significant functional damage, manifested in a lower LTP relative to the control group. This potentiation was unstable, and constantly decayed in time. As a result, the amplitudes of the fEPSPs almost reached the level before LTP induction. As concerns, the relevance of the treatment of stroke patients in clinical practice, we tested the effects of ALC (100 mg/kg) administered after the ischemic insult. There was a higher potentiation after the LTP induction relative to the 2VO group, but these amplitudes were stable only in the first 15 min, and then slightly decayed and stabilized at a lower level. As increase of the dose (200 mg/kg) did not repair the impaired LTP function, we examined the effects of ALC pretreatment (100 mg/kg). The protective effect was clear, since the potentiation was the same as that of the control, and this LTP was stable until the end of the recording period. To exclude the modulating effect of ALC on the synaptic transmission and plasticity, we administered the compound to sham-operated animals. There was no significant difference between the LTP of the control and the ALC-treated sham-operated group. The results of the histological measurements were in accordance with the electrophysiological ones; in the 2VO group, the number of dendritic spines was significantly lower relative to the control. The post-treatment was not effective against this decrease, but the level of the spines in the pretreated group was the same as in the control. These histological results parallel the LTP functions measured during the electrophysiological recordings. The results of the pre- and post-treatment are in accordance with the findings of other research groups that ALC can be protective only when it is applied as pretreatment. The Glu excitotoxicity or oxidative

stress presumably caused irreversible damage, which could not be prevented by ALC post-treatment given 1 h after the ischemic insult.

Effects of ALC against global ischemia

Despite the differences between *in vitro* and *in vivo* ischemia models, there are many similarities which make *in vitro* models suitable for the measuring of ischemia and the testing of potential protective agents. *In vitro* models provide well-controlled, simple systems, where the cellular responses against ischemic injury can be detected and characterized. To test the effects of ALC in an OGD model, we first determined a suitable time for the ischemic insult in our system. The preliminary studies revealed that, after 15-min OGD, the fEPSPs did not appear. In this case, it was presumed that a potentially neuroprotective drug could restore the fEPSPs after the ischemic insult. W eset out to identify the lowest effective concentration which could restore the fEPSPs to the control level and also provide the possibility of induction and the maintenance of stable LTP. In the 125 µM ALC-treated group, half of the cases displayed the definite elimination of the fEPSPs; thus, the treatment did not prevent the damage and cell death caused by ischemia. In the other half of this experimental group, the fEPSPs appeared after OGD, but the increases in amplitude and slope were low and saturated below the control level. LTP was induced in only two recordings, but was not stable, steadily decaying. The application of 250 µM ALC was effective, since the fEPSPs returned to the control level after OGD. On the other hand, this concentration was not sufficiently high to induce stable LTP. In the 500 µM ALC-treated group, the amplitudes and slopes of the fEPSPs reached the control level, and after the high-frequency stimulation there was higher potentiation than in the 125 and 250 µM ALC-treated groups. Furthermore, this LTP was stable until the end of the recordings.

Mechanisms underlying the protective effects of ALC

After we had found this effective concentration, we set out to identify the mechanisms underlying the protective effect of ALC. A phosphatidyl inositol 3-kinase (PI3K) inhibitor (LY294002) was applied in these experiments. The PI3K/Akt pathway is one of the main intracellular signalizations in the regulation of the different processes in the cells (e.g. the survival and antiapoptotic processes). In the presence of this inhibitor, the effective ALC concentration (500 µM) did not prevent the loss of the fEPSPs after OGD. LY294002 presumably blocked the antiapoptotic mechanisms through the inhibition of the Akt protein,

and thus ALC could not be protective. Our results are in accordance with those of other research groups which revealed that ALC can be neuroprotective through activation of the PI3K/Akt pathway. However, there are numerous other mechanisms underlying the neuroprotective effec of ALC, including the induction of heat-shock proteins and hemoxygenase-1, reduction of the level of tumor necrosis factor-α, increase of the level of nerve growth factors, regeneration of the nerve fibers, improvement of the brain energetics, provision of an alternative AcCoA source, preservation of the integrity of the cell and the mitochondrial membrane, or modulation of the gene expression. It is possible that several of these processes can simultaneously underly the protective effect of ALC revealed in our experiments. Because of these roles and effects of ALC, it has not only been used in animal studies, but also tested in preclinical trials, and has proved very promising. The number of studies relating to the neuroprotective effects of various natural substances is steadily increasing. These compounds can be applied as dietary supplements and may can act as neuroprotectants in different neurodegenerative diseases.

Summary

Our assessment of the potential neuroprotective effects of ALC in different ischemic models revealed that ALC pretreatment prevents the functional and morphological damage caused by global hypoperfusion, preventing the impairment of the LTP and the decrease in the number of dendritic spines in the hippocampal CA1 subfield. In contrast, ALC was not effective when applied as post-treatment. In *in vitro* experiments, ALC dose-dependently prevented the decay of the synaptic transmission caused by OGD. 15-min OGD abolished the fEPSPs, but 500 μM ALC completely prevented this and resulted in stable LTP. These experiments demonstrated that the PI3K/Akt pathway has a key role in the mechanisms underlying the neuroprotective effect of ALC.

Publications directly related to the PhD thesis

Journal of Neural Transmission (Epub ahead of print) (DOI 10.1007/s00702-014-1343-7)
IF: 2,871

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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IF: 3,327

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