Ph.D. Thesis

Development of a new focal ischemic model and investigation of the effects of oxaloacetate on the central nervous system of the rat

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Introduction

Stroke is a major cause of morbidity and mortality worldwide. It accounts for 6 million deaths per year, and an additional 9 million persons suffer from the consequences (cognitive and motor deficits) of stroke. No general treatment is yet available to mitigate the harmful effects of brain ischemia. Only reperfusion therapy (e.g. administration of a tissue plasminogen activator) is given to treat hyperacute ischemic stroke, but only 2-5% of stroke patients receive thrombolytic therapy to restore the blood flow. Further investigations are therefore needed for a deep understanding of the pathomechanisms of stroke and brain ischemia. It is also desirable to develop new stroke treatments to lower the brain damage caused by an ischemic episode and to reduce cellular injury or death. Reliable animal stroke models are a powerful tool with which to evaluate the efficacy and safety of such treatment prior to the findings that emerge being translated into clinical stroke therapy. There are numerous experimental stroke models which give an opportunity to study the processes of ischemia from different aspects. However, some aspects cannot be investigated with these methods; for instance, it is difficult to study the effects of transient ischemic attacks (TIAs), because there is no major tissue damage and no infarcts caused by short-lasting, ischemic periods. Moreover, there is no animal model that can be used to induce focal, well-regulated repetitive ischemia-reperfusion.

During brain ischemia, the reduced level of perfusion induces the heterogeneous chemical processes of the ischemic cascade. In this cascade, the hypoxic and hypoglycemic state leads to cellular bioenergetic failure, membrane depolarization, a high intracellular Ca^{2+} level, excitotoxicity and oxidative stress. These processes finally result in the death of cells and tissue damage. The elevated level of glutamate (Glu), as the main excitatory neurotransmitter in the mammal brain, has an especially important role in the excitotoxicity which is strongly

connected to the secondary tissue damage. A decrese of the Glu level in the interstitial space can therefore be neuroprotective. One of the promising neuroprotective strategies 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. The blood-resident enzyme GOT (Glu-oxaloacetate (OxAc)-transaminase) catalyzes the transformation of Glu and OxAc to 2-α-ketoglutarate (AKG) and aspartate. The peripheral application of OxAc, as GOT cosubstrate, can therefore decrease the blood Glu level, and can be used for Glu scavenging. OxAc has proved to be neuroprotective in numerous studies, but the details of the underlying mechanisms are not yet clearly understood.

Aims

1) Our primary aim was to investigate the reliability and reproducibility of a novel focal ischemia-reperfusion model, in which cortical ischemia is induced by lifting the distal middle cerebral artery (MCA) with a special microsurgery hook. We characterized this method by means of electrophysiological and histological experiments. We also set out to assess the value of this model for the testing of neuroprotective or neuromodulator agents. We investigated the possible protective effects of the administration of OxAc.

2) OxAc has a central role in the energy metabolism, mainly in the citric acid cycle, and also possesses antioxidant properties. These multiple effects may contribute to the neuroprotective action of peripherally applied OxAc. Our aim here was to investigate the effects of OxAc on the neuronal activity in *in vitro* experiments under normal and ischemic conditions.

3) We estimated the effects of OxAc on the somatosensory evoked potentials under normal conditions. We investigated the dose-dependence, and the indirect relationship with the decreasing blood Glu level following by peripheral OxAc administration.

Material and methods

Animals

This study was performed on adult male Wistar rats (200–300 g) maintained under controlled environmental conditions at a temperature of 21±1°C and a 12-h light/dark cycle. The animals were given free access to food and water prior to surgery.

Ischemic model

Experiments were carried out under Nembutal anesthesia. After preparation of the masticatory muscle, the head of the animals was fixed in a stereotaxic head holder. The surface of the left side of the temporal skull was cleaned and the brain was exposed with a high-speed microdrill. The exposed cortical surface involved the trunk and main branches of the MCA. To induce ischemia, the MCA was carefully lifted through 1100-1200 µm with a special microsurgery hook with the aid of a micromanipulator. To terminate the occlusion, the hook was carefully removed, and restoration of the blood flow was confirmed under an operating microscope. Finally, the dura and the temporal muscle were replaced, and the skin was closed with a silk suture. The duration of ischemia was 2 × 15 min (interrupted by a 30-min reperfusion) or 30 min.

EEG

The EEG was recorded on the surface of the skull with a silver electrode (2 mm lateral to the sutura sagittalis and 3 mm behind the bregma), before and after the ischemic period. EEG power analysis was performed with the EEGLab toolbox and custom-written MATLAB 7.1 software. The range of frequency of interest was 2–20 Hz and analysis was performed within this range.

Somatosensory-evoked responses

The anesthetized animals were secured in a stereotaxic head holder. Craniotomy was performed on the left hemisphere over the primary somatosensory cortex. Somatosensory-

evoked responses (SERs) were induced by electrical stimulation of the whisker pad (4 V, 0.2 ms, 0.1 Hz) through a bipolar needle electrode. The SERs were recorded via a silver electrode before, during and after the ischemic period. The recordings were made where the punctum maximum of the SERs was identified; it was generally localized 3.5 mm behind the bregma and 5 mm laterally.

OxAc or saline was administered into the right tail vein (volume: 1 ml, duration: 15 min, speed: 66.6 µl/min) with the aid of a microinjection pump. The dose of OxAc applied during ischemia was 3.5 mg/100 g, while OxAc doses of 1.25; 2.5 and 5 mg/100 g were used in *in vivo* experiments under normal (non-ischemic) conditions. OxAc was dissolved in phosphate buffer and the pH was set at 7.4.

In vitro electrophysiology

The anesthetized animals were decapitated and the middle parts of the hippocampi were placed in ice-cold artificial cerebrospinal fluid (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, saturated with 95% O_2 + 5% CO_2. Coronal hippocampal slices (350 µm) were prepared with a vibratome, transferred to a Haas recording chamber and incubated at room temperature for 1 h to allow the slices to recover in the solution used for recording (differing only in that it contained 3 mM $CaCl_2$ and 1.5 mM $MgSO_4$). The flow rate of the recording solution was 1.5– 2 ml/min and the experiments were performed at a controlled chamber temperature of 34 °C. For the electrophysiological experiments, a bipolar concentric stainless steel electrode 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 60 µA (constant current, 0.2-ms pulses delivered at 0.05 Hz) to evoke the half-maximum response. The amplitudes and slope of the field excitatory postsynaptic potentials (fEPSPs) were recorded with a 1.5–2-MΩ resistance glass micropipette filled with aCSF (pH 7.4).

In vitro ischemic model

During oxygen-glucose deprivation (OGD), the aCSF otherwise 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 a global ischemia, since the cells receive neither glucose or oxygen. After OGD, the slices were perfused with normal aCSF again until the end of the following period. The OxAc dose of 0.1 or 1 mM was dissolved in aCSF (or OGD-aCSF).

Histology

For histological assessment after a 1- or 5-day survival period following surgery, the animals received an overdose of nembutal, and were perfused transcardially with 0.1 M ice-cold phosphate-buffered saline (pH 7.4)(PBS), followed by 4% buffered paraformaldehyde. The brains were removed, and postfixed overnight in paraformaldehyde at 4 °C. Coronal sections (20 μm) were obtained with a vibratome. The early changes in neural viability induced by the ischemia and reperfusion were visualized by means of 1.5% 2,3,5-triphenyltetrazolium chloride (TTC) and Fluoro Jade C (FJC) staining in 14 animals. After 5 days, the delayed consequences of the 30-min ischemic attack were also demonstrated with FJC, and were assessed with the conventional free-floating immunohistochemistry of reactive astrocytes (S100) and activated microglia (CD11b). Three adjacent slices were obtained at 500 μm from bregma 1 to 4.5. Briefly, the slices were washed in PBS, then incubated in a blocking solution containing 10% normal donkey serum, and next incubated in primary antibody overnight at 4 °C and in secondary antibody for 2 h at room temperature. The antibodies were diluted in a solution containing 0.1% PBS, 0.4% Triton X-100, 2% NDS and 0.01% sodium azide. The slices were coverslipped with Fluoromount

For quantitative comparisons, every tenth 20-μm slice was analyzed in a length of 4 mm from the area supplied by the MCA. The early changes in neural viability induced by the ischemia

and reperfusion were visualized by means of FJC staining. Cortical FJC-positive cells were counted in the quadrant (0.25 mm^2) of slices in the same position.

Statistical analysis

Statistical analysis of the EEG data was performed with the General Linear Model/Repeated Measures. EEG power spectra filtered at 2–20 Hz were decomposed at 1-Hz intervals. The EEG power of a given frequency was considered as an individual case.

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

In *in vitro* measurements, fEPSP amplitudes and slopes were expressed as a percentage of the 10-min baseline value before OGD or drug application. One-way ANOVA with the Tukey *post hoc* test was chosen for the statistical analysis of the data.

Results

In our first series of experiments, we tested the novel focal ischemic model based on MCA occlusion (MCAO). The functional changes were readily detected in the cortical fields supplied by the MCA: the power spectra of the recorded EEG samples and the amplitudes of the cortical SERs from the control and ischemic periods differed significantly. In the first group, we applied 2 × 15-min ischemic episodes interrupted by a 30-min reperfusion. The amplitudes of the SERs decreased immediately at the beginning of the ischemic period and soon disappeared. Reperfusion resulted in the appearance of and a gradual increase in the amplitudes of the SERs, although the amplitudes never regained the control level, attaining only around 60% of it. As a result of the 2 × 15-min MCAO, after a survival time of 1 day, there was no detectable tissue damage on the use of TTC, but well-outlined FJC-positive degenerating neurons were seen throughout the ipsilateral somatosensory cortex. In another

series of experiments with a 5-day survival period, we examined the changes following 30-min MCAO. FJC-positive labeling was observed in half of the animals, and S100+ and CD11b+ immunohistochemically labeled reactive astrocytes and activated microglia cells were also found in these animals. The FJC staining pattern closely followed the microglia distribution.

In a 2 x 15-min experimental paradigm, OxAc was applied in the first ischemic period. During the administration (and ischemia), the decreased amplitudes of the SERs began to recover, and finally reached the control level. After a survival time of 1 day, the OxAc treatment proved to be protective against the focal cerebral ischemia, since it led to fewer FJC-labeled cells in the same area as compared with the ischemic control group.

In *in vitro* experiments, we investigated the possible metabolic and antioxidant effects of OxAc on hippocampal fEPSPs under normal and ischemic conditions. The OxAc doses of 0.1 and 1 mM in aCSF caused no changes in the fEPSPs during the recording period. The fEPSP amplitudes showed a slight increase, but then started to decrease and finally disappeared during and after OGD. The application of 0.1 mM OxAc resulted in a short transient increase in the amplitudes of the fEPSPs, but they had disappeared again at the end of the recording period. 1 mM OxAc, however, increased the slope and amplitudes of the fEPSPs, which finally reached the control level.

In our final experiments, we examined the possible effects of OxAc on the SERs under normal (non-ischemic) conditions. The applied doses of OxAc (1.25, 2.5 and 5 mg/100 g) earlir proved to be already neuroprotective in other *in vivo* studies. In contrast with our expectation, OxAc administration (i.v.) resulted in an increase in the amplitudes of the SERs. This phenomenon showed a definite time and dose dependences. OxAc at 1.25 mg/100 g had no effect on the cortical evoked responses, whereas the highest, 5 mg/100 g dose of OxAc increased the amplitudes up to 180–200% of the control level. In the next step, AKG was used

i.v.; this compound has an opposite effect on the blood Glu level (a decreasing effect), but its metabolic and antioxidant properties are similar. Equimolar AKG, and also a 1:1 OxAc-AKG mixture administration resulted in increases in the SER amplitudes, in a similar manner as in the OxAc group.

Discussion

This simple model involving lift of the distal MCA is highly reproducible for the induction of short-lasting, focal transient cortical ischemia in the rat. The early functional and later structural consequences of the brain ischaemia following MCAO can be readily observed and examined. OxAc proved to be neuroprotective. These results clearly demonstrate the effect of OxAc as a Glu scavenger, and the value of this ischemic model for the investigation of different neuroactive pharmacological agents. This new method offers an easy way to study the effects of TIAs with precise regulation of the blood flow, and to set the required level of perfusion. In our *in vitro* experiments, 0.1 and 1 mM OxAc had no effect on the evoked hippocamnpal fEPSPs under normal conditions, whereas 1 mM OxAc was neuroprotective against the harmful effect of 15-min OGD via its metabolic and antioxidant effects. In our last *in vivo* experiments, OxAc and AKG increased the amplitudes of the SERs. This result suggests that this phenomenon is not based on the changes in blood Glu level. At present, we cannot specify the underlying mechanisms. Further investigations are required to elucidate these processes. However, it may be concluded that OxAc doses which provide many beneficial effects and neuroprotection in numerous studies can influence the important somatosensory system under normal conditions.

Many promising studies have suggested the advantages of the effects of Glu scavengers, and especially 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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