Analysis of the early phase of ischemic-reperfusion brain damage using histological, neurochemical and functional methods

PhD thesis

Angéla Benedek

Semmelweis University

Doctoral School of Pharmaceutical and Pharmacological Sciences

Supervisor: Prof. Péter Mátyus, D.Sc.

Consultant: Gábor Szénási, Ph.D.

Opponents:

Head of Examination Committee: Prof. Klára Gyires, D. Sc.

Members of Examination Committee: Prof. Pál Perjési, C. Sc.

Prof. Valéria Kecskeméti, Ph.D.

Budapest

2008

Summary

Under the supervision of Professor Péter Mátyus, D.Sc.
Semmelweis University, Department of Organic Chemistry
Doctoral School of Pharmaceutical and Pharmacological Sciences

The failure of efforts to develop a drug for the treatment of stroke can be explained, in part, by the difficulty of delineating sufficient and firm animal data required for establishing the initiation of a clinical stroke trial. Our aim was to study how precisely the ischaemic-reperfusion damage (IR) is reflected by the methods routinely applied in the preclinical practice. At Division of Preclinical Research of EGIS Pharmaceuticals Plc, we evaluated the IR damage of brain tissue using triphenyl-tetrazolium chloride (TTC) staining and histological analysis in rats after a 1-hour middle cerebral artery occlusion followed by different reperfusion periods. Neuronal function of the territory judged infarcted by TTC staining was investigated by the help of corticostriatal brain slice with radioactively labelled dopamine, *in vitro*. The animals underwent *in vivo* assessment applying 4 different neurological scales, which were completed with foot fault, beam balance, locomotor activity tests.

The results of TTC staining performed by transcardiac perfusion or in brain slices were different in the cortex and striatum, and considering the size of the core and penumbra. After transcardiac TTC staining the infarct volume temporarily decreased during the early reperfusion period. At 24 hours of reperfusion astrocytes died within the area judged infarcted by TTC staining, and despite extensive tissue damage, several morphologically viable neurons and intact mitochondria were observed. Dopamine content decreased and dopamine turnover increased in the infarcted striatum, but dopaminerg nerve terminals retained functional activity. All neurological scales scored sensorimotor deficit similarly in rats after stroke. Beam balance, foot fault and locomotor activity tests can not substitute for neurological scales.

Taken together, the pathological process in consequence of an ischaemia-reperfusion challenge can be accurately assessed using a number of complementary methods.

Introduction

In these days stroke creates a serious problem considering mortality and the size of medical expenses. Approximately 80 % of stroke cases are ischemic, frequently caused by middle cerebral artery occlusion. The main symptoms of the disease are contralateral paraesthesia, paralysis of the face or the extremities, aphasia, and visual loss; stroke often leads to disability or death. Tools for the treatment of the disease are currently limited. The efficacy drugs influencing the pathological process caused by ischemia-reperfusion (IR) (neuroprotective treatment) – is not proven in clinical trials despite promising results of preclinical drug research.

The severity of blood flow disturbances is determined by three independent factors: the speed of vessel occlusion, its duration, and the absolute regional blood flow of the territory prior to injury. IR damage due to occlusion of the cerebral vessels causes complex cellular metabolic alterations, which overlap in time and space. Formation of reactive oxygen radicals upon reperfusion further aggravates the disturbance of vital functions of cells caused by ischemia. In the centre of ischaemia (core) cells become irreversibly damaged when blood flow and the oxygen consumption falls below the "infarction threshold". Penumbra, which is the target of possible neuroprotective interventions, surrounds core.

We chose middle cerebral artery occlusion (MCAO) method, which served as an animal model of human focal cerebral ischemia. The aim of our study was to investigate tissue damage in the striatum and cerebral cortex in consequence of a 1-hour middle cerebral artery occlusion followed by 0-24 hours of reperfusion using histological, neurochemical and functional tests.

Preclinical research of the neuroprotective agents differs from human studies in many respects; these differences render difficult the prediction of preclinical data to clinical conditions. There are fundamental differences between clinical and pre-clinical studies, as the preclinical practice is to use standardized and artificial cerebral ischemia in young, healthy and mostly male rodents. These methods cause cell damage in certain, well defined areas. In contrast to that subjects enrolled into clinical trials are mostly

elderly males and females affected by co-morbidity, risk factors, and some patients suffer from a second stroke. In animal studies the test items most frequently are administered as an intraperitoneal injection. Dosage tested and plasma levels achieved are commonly higher than that tolerated by patients. Randomized, blinded or crossover administration protocols are rare in animal studies. Conduction of clinical trials is regulated by international guidelines; therefore standardized neurological scales are applied as outcome measures. Contrary to clinical protocols, protocols of animal studies are highly diverse. The simple and fast triphenyl-tetrazolium chloride (TTC) staining is used in some studies while complex histological techniques and analyses are applied in others. Several scales are known for scoring deteriorations in sensorimotor functions. Some of those put emphasis on special parameters, e.g. sensory functions, gait- and orientation disturbances, mild changes or the number of deaths. However, investigations of the intellectual and the cognitive functions are limited in animal studies. Moreover, in preclinical experiments the efficacy of a neuroprotectant primarily means reduction of infarct size (penumbra) and rarely the improvement of sensorimotor functions. In clinical studies the efficacy of a certain drug is judged on the basis of its beneficial effects on the neurological symptoms of stroke, and on the quality of life.

Investigation of a neuroprotectant influencing the excitotoxic-oxidative cascade can be exclusively carried out in the early phase of IR damage, and it is an essential part of the development of drug treatment aiming at improving the quality of life after stroke. Therefore, animal experiments in the early phase of IR damage are of great importance.

Objectives

During our experiments we compared histological and functional alterations obtained using routinely applied methods in the pharmacological practice after a 1-hour middle cerebral artery occlusion followed by 0-24 hours of reperfusion in rats. Our main questions were as follows:

Is TTC staining a suitable tool for the reliable detection of cerebral infarction?

1. Does application of TTC staining by a transcardial perfusion or in brain slices alter the results obtained?
2. Whether a brain area judged infracted by TTC staining can be considered infarcted also by using histological methods?
3. Are there functional dopaminergic nerve terminals within the area judged necrotic by TTC staining as assesses in complex brain slice preparation, *in vitro*?

Can we substitute neurological scales developed for the measurement of sensorimotor deficits with easily manageable functional tests?

1. To what extent the results of the neurological scales developed for the measurement of sensorimotor deficits are uniform?
2. Can easily manageable methods such as beam balance and foot fault tests replace or supplement the application of complex neurological scales?
3. Is any effect of IR damage on the locomotor activity in rats?

Methods

In our experiment we used male, SPRD rats. The animals underwent a 1-hour middle cerebral artery occlusion (MCAO) followed by different durations of reperfusion, and then we investigated its histological and functional consequences in the sensorimotor cortex and striatum supplied by the occluded vessel.
The right middle cerebral artery (MCA) was occluded using the intraluminal suture technique described by Longa et al. with minor modifications. During the surgical procedure the animals were anaesthetized with ether. The vessels were reperfused by gentle withdrawal of the filament (reperfusion) after 60 minutes.

For the determination of the extent of IR damage, we applied TTC staining, which was done in two ways: in case of *in vivo* method, a 4 % TTC solution was injected intracardially, and in the case of *in vitro* method 1 mm thick coronal sections were cut and stained in 2 % TTC solution. Evaluation of the TTC stained sections was performed using a morphometric program (Digi Cell 4.0). To prove that the TTC solution perfused the previously ischaemic brain area we injected Evans blue solution also intracardially 1 minute after TTC solution was injected.

For the general histological analysis sections cut from paraffin blocks were stained using toluidine blue and Luxol Fast Blue staining as described by Klüver-Barrera. Fluoro-Jade staining was applied for specific detection of neurodegeneration. Astroglial cells were visualized on the basis of intermedier filament responsible for maintaining the shape of the glial cells using GFAP (glial fibrillary acidic protein) immunohistochemistry. Morphological examinations were completed with electromicroscopic analysis. Dopaminergic neurons in the striatum were visualized by the help of immunohistochemistry. This method is based on tyrosine hydroxylase enzyme detection, which enzyme plays a key role in dopamine synthesis.

The concentration of dopamine and its metabolites in corticostriatal slices were measured by high performance liquid chromatography coupled with an amperometric detector system. Function of the afferent corticostriatal pathway was studied in complex brain slice preparations in two-compartment chambers, which includes a part of the

prefrontal cortex, corpus callosum and striatum in a continuous slice. During the experiment, we measured radioactivity (Bq/g) of the tritiated dopamine released with a liquid scintillation beta-counter.

We evaluated the sensorimotor deficit caused by the IR damage using four neurological scales (Reglődi-, Hunter-, Garcia- and Bederson-scale). The neurological scales chosen include the investigation of gait disturbances, the postural signs, and the reduction in muscle tone, the spontaneous motor activity and the general appearance. The evaluation of sensorimotor deficit was completed with foot fault, beam balance and locomotor activity tests, and the correlation of these results was investigated.

Statistical analysis was performed to evaluate the significance of the results using Statistica 6.0 software, and the relevant statistical methods were chosen according to the type of experiments (TTC staining and locomotor activity: ANOVA, Duncan post hoc test; HPLC measurement and corticostriatal slice technique: ANOVA, Tukey-Kramer post hoc test; correlation analysis of neurological scales: Pearson correlation).

Results

In case of transcardial TTC staining, *in vivo*, a large core volume of tissue injury was found immediately after withdrawal of the filament. Core volume steadily decreased up to 16 hours of reperfusion. Over this time period, core volume decreased by approximately 50%. Then, core volume substantially increased from 16 hours to 24 hours of reperfusion.

In case of TTC staining, *in vitro*, core volume of tissue injury was negligible immediately after withdrawal of the filament and at 1 hour of reperfusion. A significant core volume appeared at 4 hours and gradually increased up to 24 hours of reperfusion.

Comparing the result obtained using *in vivo* and *in vitro* TTC staining we found that at 0-1 reperfusion of time tissue damage measured using *in vitro* staining was larger. In a latter phase of reperfusion the picture become complex: after 8 hours of reperfusion core and penumbra volumes are similar in both brain regions, but at 4 hours of reperfusion the core in striatum and at 16 hours of reperfusion the penumbra in cortex significantly differ from each other. After a 24-hour reperfusion the total damaged

volume (core and penumbra in both regions) is similar using the two staining methods, however in case of *in vivo* TTC staining core volume is larger and penumbra volume is smaller (with the exclusion of striatal penumbra volume).

Histological picture progressively worsened over reperfusion time in the area supplied by the right middle cerebral artery. There was extensive damage on the ipsilateral side of the brain at 24 hours of reperfusion. Large number of necrotic, apoptotic and shrunken (dark) neurons were present with widened pericellular spaces, microvacuolisation in the cytoplasm, nuclear pyknosis and oedema in neuropil (status cribrosus). Similarly, dopaminergic neurons visualized by tyrosine-hydroxylase enzyme immunohistochemistry are also severely damaged. However, neurons with well-preserved morphology could be seen in the cortical and striatal core (TTC negative, white) areas in most animals irrespective of the method of histological processing. On the contralateral side there were only minimal histological changes, but mild oedema formation and few degenerated neurons were manifest.

After 24 hours of reperfusion in IR animals, tissue contents of dopamine is decreased significantly in the ipsilateral cortex (TTC white). Dopamine content is significantly reduced in the striatum both ipsilalaterally and contralaterally (TTC white and red). Dopamine turnover significantly increased in all brain regions investigated with the exclusion of the contralateral cortex, which hemisphere was not directly affected by MCAO. With the use of Evans blue staining we proved that the TTC-containing solution reached the previously ischaemic area supplied by the middle cerebral artery.

In corticostriatal slice preparations, by electrical field stimulation of the adjacent cortex approximately the same amount (6-7 fold) of tritiated dopamine was released from both the IR and intact striatum compared to the basal level of tritiated dopamine overflow. As a consequence of MCAO lesser amount of tritiated dopamine was released both during the initial phase and the stimulation phase of the experiment. Additionally, smaller amount of isotope-labelled dopamine remained in the tissue by the end of the experiment. In IR animals, the area under the curve (AUC) of tritiated dopamine released during electrical field stimulation was reduced in the cortex ipsilaterally, in the

striatum contralaterally and ipsilaterally compared to those in intact animals. The most prominent reduction in AUC was observed in the ipsilateral striatum.

After a successful 1-hour MCAO followed by a 24-hour reperfusion, locomotor activity of all animals declined over time, however it was significantly reduced during the third 5-minute period only. We detected a significant difference between the locomotor activities of the intact and the IR animals during the full length of 15-minute experiment.

In case of animals subjected to a 1-hour MCAO and a 24-hour reperfusion, the total neurological score correlated with each other with the exclusion of the Garcia and Bederson scales. Parameters described in the scales are expressed in different proportions of the total score in each scale. In case of the Garcia scale the most prominent correlation was found between the number of parameters and the total score. The results of correlation analysis could not be interpreted in case of parameters such as general appearance, which did not detect differences among the animals. The extent of sensomotor deficit measured using the foot fault and beam balance tests were different. The result of foot fault test did not show any correlation with neurological scores. However, average residential time on the beam correlated significantly with forelimb flexion (Reglődi-scale), hanging on horizontal bar, rotation (Hunter-scale) and symmetry of four limbs (Garcia-scale).

Conclusions

After transient cerebral ischemia induced by a surgical method of middle cerebral artery occlusion, the results obtained by *in vivo* (intracardial) and *in vitro* (in brain slices) TTC staining are markedly different at reperfusion times shorter than 8 hours: in case of in vitro staining the infarct volume temporarily decreased after reperfusion. Within the territory judged infarcted by TTC staining, several morphologically viable neurons were seen. Supposedly the TTC reduction to formazan stops in the early phase of reperfusion when the metabolism of the brain tissue is temporarily or permanently exhausted. Therefore, TTC staining is not a suitable tool for the accurate determination of irreversible brain damage.

Tyrosine hydroxylase immunohistochemistry after a 1-hour middle cerebral artery occlusion followed by a 24-hour reperfusion showed that the striatal dopaminergic terminals were markedly damaged. In IR animals, the dopamine turnover increased in the ipsilateral cortex, striatum and in the contralateral striatum as well. Electrical field stimulation of the cortex elicited approximately the same tritiated dopamine release from both the intact and IR striatum. Taking together, the striatal dopaminergic terminals remained viable at 24 hours of reperfusion even in the area judged infarcted by TTC staining.

We investigated the influence of IR on the sensomotor functions using 4 different, published neurological scales and compared those results with the results of foot fault, beam balance and locomotor activity tests. Total neurological scores chosen correlated well with each other; consequently, all scales measured the severity of IR damage similarly. The results of the foot fault and beam balance tests did not show correlation with any neurological scores; therefore, neither of them is suitable to substitute for the neurological scales.

References

Papers related to thesis

1. **Benedek A**, Móricz K, Jurányi Z, Gigler G, Lévay G, Hársing LG Jr, Mátyus P, Szénási G, Albert M. (2006) Use of TTC staining for the evaluation of tissue injury in the early phases of reperfusion after focal cerebral ischemia in rats. Brain Res, 1116: 159-165. IF: 2,341

2. Matucz E, Móricz K, Gigler G, **Benedek A**, Barkóczy J, Lévay G, Hársing LG Jr, Szénási G. (2006) Therapeutic time window of neuroprotection by non-competitive AMPA antagonists in transient and permanent focal cerebral ischemia in rats. Brain Res, 1123: 60-67. IF: 2,341

3. Gigler G, Móricz K, Ágoston M, Simó A, Albert M, **Benedek A**, Kapus G, Kertész S, Végh M, Barkóczy J, Markó B, Szabó G, Matucz E, Gacsályi I, Lévay G, Hársing LG Jr, Szénási G. (2007) Neuroprotective and anticonvulsant effects of EGIS-8332, a non-competitive AMPA receptor antagonist, in a range of animal models. Br J Pharmacol, 152: 151-160. IF: 3,825

Oral and poster presentations related to the thesis

1. **Benedek A**, Móricz K, Gigler G, Hársing LG Jr, Szénási G, Mátyus P, Albert M. Fokális, ischaemiás-reperfúziós károsodás progressziójának szövettani vizsgálata patkányban. Semmelweis Egyetem PhD-napok, Budapest, 2004. (poster)

2. **Benedek A**. Analysis of the progression of focal cerebral damage induced by ischemia and reperfusion in rats. XXIV. Advanced Course of Medicinal Chemistry and "E. Duranti" Seminar for PhD students, Urbino, Italy, 2004. (poster)

3. **Benedek A,** Móricz K, Albert M, Gigler G, Hársing LG Jr, Szénási G, Mátyus P. Morphological and functional investigation of TTC-stained brain tissue in the early phase of reperfusion after transient focal cerebral ischaemia in rats. Giornate Dottorato, Mini-symposium organized by University of Milan and Semmelweis University, Milano, Italy, 2004. (lecture).

4. **Benedek A,** Móricz K, Jurányi Z, Albert M, Gigler G, Hársing LG Jr, Mátyus P, Szénási G. Evaluation of early brain damage by TTC staining and histology after transient focal cerebral ischaemia in rats. 14th European Stroke Conference, Bologna, Italy, 2005. (poster) Cerebrovasc Dis, (2005) 19: 116.

5. **Benedek A**, Móricz K, Jurányi Z, Albert M, Gigler G, Hársing LG Jr, Mátyus P, Szénási G.A korai ischaemiás-reperfúziós agyi károsodás értékelése TTC festéssel

és szövettani módszerekkel. Magyar Élettani Társaság LXIX. Vándorgyűlése, Budapest, 2005. (poster, lecture) - posterkategória nyertes

6. **Benedek A**, Móricz K, Hevesi Tóth B, Gigler G, Hársing LG Jr, Kéry Á, Szénási G. Antioxidant and neuroprotective effects of *Epilobium parviflorum* Schreb, Small Flowered Willow herb extract. Magyar Kísérletes és Klinikai Farmakológiai Társaság, Magyar Experimentális Farmakológia Tavaszi Szimpóziuma, Budapest, 2005. (poster)

7. **Benedek A**, Móricz K, Jurányi Z, Albert M, Gigler G, Hársing LG Jr, Mátyus P, Szénási G. Alkalmas-e a TTC festés a korai ischaemiás-reperfúziós agyi károsodás jellemzésére? Magyar Stroke Társaság és Neuroszonológiai Társaság VII. Konferenciája, Eger, 2005.- poster kategória II. díj. Agyérbetegségek, (2005) 11: 10 (poster, lecture)

8. **Benedek A**, Nagy K, Móricz K, Gigler G, Szénási G, Mátyus P, Hársing LG Jr, Jurányi Z. [^{3}H]Dopamine release in corticostriatal slices after transient cerebral ischemia in rats. International Brain Research Organisation Workshop - Magyar Idegtudományi Társaság XII. Konferenciája, Budapest, 2006. (poster) Ideggyógy Sz, (2006) 59: 11.

9. Gigler G, Móricz K, Albert M, Matucz E, Ágoston M, Simó A, **Benedek A**, Barkóczy J, Hársing LG Jr, Szénási G. Neuroprotective efficacy of EGIS-8332, a non-competitive AMPA antagonist in transient focal cerebral ischemia and multiple sclerosis tests in rats. Budapest Spring Neuropathology Conference, Budapest, 2004. (poster)

10. Móricz K, Gigler G, Albert M, Matucz E, Ágoston M, Simó A, **Benedek A**, Barkóczy J, Hársing LG Jr, Lévay G, Szénási G. Effects of EGIS-8332, an AMPA antagonist, in transient focal cerebral ischemia and multiple sclerosis in rats. 7th International Congress of Neuroimmunology, Venice, Italy, 2004. (poster) J Neuroimmunol, (2004) 154: 86.

11. Móricz K, Gigler G, Albert M, **Benedek A**, Ágoston M, Matucz E, Lévay G, Hársing LG, Szénási G. Az EGIS-8332, egy nem-kompetetív AMPA antagonista neuroprotektív hatása fokális agyi ischaemia és sclerosis multiplex modellben, patkányon. Magyar Idegtudományi Társaság XI. Konferenciája, Pécs, 2005. (poster) Ideggy Szemle, (2005) 58: 68.

12. Móricz K, Gigler G, Matucz E, Ágoston M, **Benedek A**, Lévay G, Hársing LG Jr, Szénási G. EGIS-8332: A terápiás ablak meghatározása patkányokon végzett permanens és tranziens fokális agyi ischaemia teszten. Magyar Stroke Társaság és Neuroszonológiai Társaság VII. Konferenciája, Eger, 2005. (poster) Agyérbetegségek, (2005)11: 21 (poster)

13. Barkóczy J, Ling I, Simig G, Gigler G, Simó A, **Benedek A**, Móricz K, Ágoston M, Végh M, Kapus G, Kertész S, Lévay G, Hársing LG Jr, Szénási G. Neuroprotective effect of EGIS-11229, a 2,3-benzodiazepine type AMPA

antagonist. 230th American Chemical Society Meeting, Washington DC, USA, 2005. (poster)

14. Hársing LG Jr, **Benedek A**, Albert M, Jurányi Z. Release of [^{3}H]dopamine in corticostriatal slices after transient middle cerebral artery occlusion in rats. Annual Meeting of the Society for Neuroscience, Washington, USA, 2006. (poster)

15. Szénási G, **Benedek A**, Nagy K, Móricz K, Albert M, Gigler G, Hársing LG, Jr., Jurányi Z. Survival of dopaminergic nerve terminals in the cortex and striatum after transient cerebral ischaemia followed by 24 hours of reperfusion in rats. 7th World Congress of Neuroscience, IBRO, Melbourne, Australia, 2007 (poster)