Mitochondrial neuroprotection

Thesis

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Budapest, 2008

Introduction

The leading cause of focal brain ischemic injury is the thromboembolic and atherothrombotic occlusion of the intra- and extra-cranial arteries. The consequent pathology depends on its duration and the extent of the ischemia. When brain perfusion is enduringly below physiological threshold level, pathophysiological processes, leading to loss of brain function and neuronal death, set in. The decrease of oxygen and glucose level leads to an energy crisis. The main feature of the crisis is the loss of ATP, phosphorcreatin and glycogen. Furthermore, the anaerobic glycolysis produces a large amount of lactate, resulting in lactate acidosis. The further consequence of the energy crisis in that in the ischemic tissue the cell membranes are depolarized, with a consequent disruption of ion transport, ending up in a toxic level of Ca2+ in the cytoplasm and an increasing extracellular K+ level extraceluularly. The restoration of perfusion leads to the increase of the cells' oxygen supply, which in turn causes an increased free radical production. These processes altogether result in cell injury and consequent cell death by way of necrosis or apoptosis. These latter outcomes depend on the extent of the damage and the cells' ability of ATP production, as apoptosis requires energy whereas necrosis occurs in lack of energy production.

The present dissertation explores various aspects of the aforementioned processes and possible treatments with neuroprotective drugs.

Aims

The main objective of this work is to investigate the neuroprotective effects of vinpocetin and deprenyl. These compounds are used in the neurological practice. The exact mechanisms of the two molecules are not understood and explained in detail.

In line with this, the detailed aims of the thesis are the following:

- to elaborate and validate a new combined method for visualization of free radicals (ROS) and mitochondrial membrane potential (MMP),
- to investigate the concentration dependent effect of vinpocetin by measuring ROS and MMP,
- to demonstrate with PET that vinpocetin binds to peripheral benzodiazepine receptors,
- to assess the changes of brain perfusion and glucose metabolism in vinpocetin treated stroke patient before and after treatment,
- to investigate the concentration dependent effect of deprenyl and deprenyl-N-oxide with the help of ROS and MMP in PC-12 cell culture,
- to measure the antiapoptotic effect of deprenyl and deprenyl-N-oxide in an animal stroke model,
- to demonstrate that the main metabolite of deprenyl, deprenyl-N-oxide, blocks the metabolic pathway of deprenyl.

Methods

Cell culture

PC 12 (ATCC, Manassas, VA, USA) cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, Invitogen GMBH, Austria) with 10% calf serum (Gibco, Invitogen GMBH, Austria), 5% horse serum (Gibco, Invitogen GMBH, Austria), 2 mM L-glutamate (Gibco, Invitogen GMBH, Austria), and antibiotics. Nerve growth factor (NGF, Sigma-Aldrich Co, St. Luis, MO, USA) was also a component in the tissue culture medium. The cells were cultured on collagen covered glass (d=12 mm); collagen was prepared from rat tail as we published previously.

Hypoxia/re-oxygenation procedure

In vitro hypoxia was produced by Argon gas using the method, published by Kusumoto. Argon gas filled chambers were used to create hypoxic condition. Following 1 h of oxygen deprivation the cultures were returned to the normal condition for 24 h (reoxygenization). Control cultures were maintained in the incubator. A blood-gas analyzer (ABL Radiometer, Copenhagen) was used to measure oxygen pressure in the medium fluid.

Animals

Transient ischemia was induced in gerbils (bodyweight between 40 and 60 gr). For permanent ischemia male Wistar rats, weighing

between 320 and 460 gr, were used. The treatment and animal-care complied with EU standards. The experimental procedure was reviewed and approved by the local Ethics Committee.

Permanent middle cerebral artery occlusion

Anaesthesia was induced with 4% and maintained with 2–2.5% of halothane in 70% NO and 30% O, using a face mask. A standardised technique was used to perform the permanent middle cerebral artery occlusions. Ischaemic lesions were induced by electrocoagulating the left middle cerebral artery on the surface of the brain. Briefly, by craniectomy a well about 3 mm in diameter was made just above the left middle cerebral artery occlusion. The dura mater was removed. Subsequently, the middle cerebral artery occlusion was identified and a bipolar coagulator was applied to it in order to obstruct the distal branches above the lenticulostriatal branches.

Transient, bilateral common carotid artery occlusion

Anesthesia was introduced and maintained with 4% and 2 to 2.5% of halothane, respectively in the mixture of 70% NO and 30% O2, using a face mask. The rectal temperature was maintained using a heating pad. The common carotid arteries of both sides were exposed, while the vagus nerve fibers were carefully protected. The common carotid arteries were occluded with Codman aneurysm microclips. Ten minutes later, the clips were removed and reperfusion was allowed. Anesthesia was terminated, and the animals returned to their home cage.

Assessment of cell death

Dead cells were visualized with propidium iodide (PI) (Sigma-Aldrich Co, St. Luis, MO, USA) (1,5 μg/ml in saline) for 2 minutes. Viability was calculated on randomly selected fields using a fluorescence microscope, (BIO-RAD MRC 1024 confocal system was used Bio-Rad Corp., Hertfordshire, England on a Nikon OPTIPHOT inverted microscope Donsanto Corp., Nattick, Massachusetts) with 490 nm excitation and 585 nm barrier filters (100x magnification). About 80-100 cells were valuated in each field. Cell-death was expressed as a mean of percentage of PI labeled cells in 3 separate cultures in 12 samples.

Simultaneous staining to assess ROS level and mitochondrial membrane potential

A combined staining method was used to asses ROS and mitochondrial membrane potential, as we published recently (Szilágyi 2006). Briefly, DMEM was removed and 300 μl of JC-1 (Molecular Probes, Invitogen GMBH, Austria) in a concentration of 10 μg/ml was dissolved in physiological saline and added to the cultures for 10 minutes. After the cultures were rinsed with saline, they were stained with 300 μl of 20 mmol/l CeCl3 (Sigma-Aldrich Co, St. Luis, MO, USA) solution (dissolved in lactated Ringer) for 2 minutes. The CeCl3 solution was removed, and the cell culture was rinsed again with saline. Finally the cells were fixed in a 0,25% buffered glutaraldehyde solution for 2 minutes. The fixed cells on glass were covered with the Vectashield mounting medium for

fluorescence study (Vector Laboratories, Inc. Burlingame, CA) and put on glass slides.

Fluorescence microscopy on cell culture

For simultaneous visualization – green fluorescence from JC-1 monomer and red fluorescence from JC-1 J-aggregate – a long pass filter system was used (excitation lines 488 and 568 nm, T1 trichroic and T2a 560 DRLP mirror, 522 DF 35 and 585 LP emission filter set). For detection of cerium labeled cells, single channel detection was utilized in the reflectance mode (excitation line 488 nm of Krypton-Argon laser, B1 block and T1 trichroic mirror, Blue reflection filter set). For immunohistochemistry, 488- and 568-nm lines of a Krypton–Argon laser were applied sequentially. Detection was performed with a standard filter set.

For quantitative cell culture analysis high-resolution (820x) images were taken. The quantitative analysis of reflectance of cerium complex and red/green fluorescence signal was detected and the built-in evaluation software was used for calculation (LaserSharp Processing Bio-Rad Corp., Hertfordshire, England). The average intensities of reflectance (reflecting cerium precipitates), the ratio of green (JC-1 monomers) and red fluorescence (JC-1 J-aggregates) were measured within randomly selected 100-100 cells. In both the experimental and control group from 6 parallel cultures.

Immunohistochemistry

96 hours after the transient ischemia the gerbils were decapitated under deep anesthesia. The brains were removed and fixed in 10% formaldehyde. Ten μm-thick sections were cut from the paraffin embedded hippocampus region and mounted on precleaned, 3-aminopropyltriethoxy-silane-coated glass slides for con-focal laser scanning microscopic (CLSM) studies. The apoptotic cells in the hippocampus were detected by TUNEL reaction broken deoxyribonucleic acid DNA -ends were labeled using an in situ fluorescent terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate conjugated to the green fluorophore fluorescein isothiocyanate DNA nick-end labeling TUNEL kit Boehringer-Mannheim (F. Hoffmann- La Roche Ltd, Basel, Switzerland) , and caspase-3, Immunotech Mouse anti-caspase-3 primary antibody (Beckman Coulter Inc., Fullerton, CA, USA) and secondary antibody goat anti mouse Alexa 568 (Molecular Probes, Invitogen GMBH, Austria) was used.

Fluorescence microscopy on gerbil hippocampus and rat brain

For simultaneous visualization – green fluorescence from TUNEL staining and red fluorescence from caspase-3 staining – a long pass filter system was used (excitation lines 488 and 568 nm, T1 trichroic and T2a 560 DRLP mirror, 522 DF 35 and 585 LP emission filter set). Brain sections were observed on the high power fluorescence microscopy (60x). We analyzed 24 images from each animal. The TUNEL and caspase-3 positive cells were counted automatically with Image J 1.37 software (NIH, USA).

Human PET study

Fifteen ischemic chronic stroke patients participated in the present study. The investigations were performed at the Department of Neurology, the Central Laboratory for Nuclear Medicine, and PET Centre of the Debrecen University Medical School, whereas the final part of image processing and analysis was done at the Department of Neuroscience and Department of Clinical Neuroscience, Psychiatry Section, Karolinska Institute. The patients (11 men, 4 women, mean age: 59.7±13.2 years, weight: 68.8±7.2 kg, height: 165.5±3.4 cm, BMI: 25.2±5.3) had an ischemic stroke (12.4±17.3 months), prior to the present investigations. The average volume of the primary stroke lesion was 74.3±61.9 cm3. The patients and their closest relatives were fully informed about the objectives, details, and risks of the study and they gave a written consent, in agreement with the Helsinki Declaration. The study was approved by the Ethical Committee of the Debrecen University Medical School. The experimental design was double-blind. The experimental code was broken after the phase of image analysis. The present patient group was selected from a large pool of chronic stroke patients treated in the Department of Neurology of the Debrecen University Medical School. In each patient the infarcted region was in the territory of the middle cerebral artery (MCA). For the purpose of the present study, the physical and neurological statuses of the selected patients were assessed by the Orgogozo Scale, the Scandinavian Neurological Scale (SNS), the Motor-scale, and the Barthel Index. MRI scan (T1, T2, PD), carotid Doppler sonography (CDS, Ultramark 4 Plus), and

transcranial Doppler (TCD, at 50 mm depth above the main trunk of the MCA) and routine laboratory tests were performed. The study included a 15-day hospitalization period. Following this period no further follow-up was performed on the patients. All subjects received an intravenous infusion of 500 ml physiological solution (Salsol) for 45 min each day for 14 days. One original subgroup of the subjects (8 patients; placebo group) did not have any vinpocetin in the infusion, whereas the other original sub-group (7 patients; vinpocetin group) had 1 mg/kg body-weight vinpocetin.

Monkey PET study

The investigations were performed athe the PET Center of the Psychiatry Section, Department of Clinical Neuroscience, Karolinska institute. Two female cynomolgous monkeys (weight: 4.0 and 7.1 kg; age: 4 and 8 years, respectively) were used in consecutive PET measurements. The study was covered by permission of the Animal Research Ethical Committee of the Northern Stockholm Region (1998/14).

The PET system used was ECAT EXACT HR47 (Siemens), with a spatial resolution (in-plane) of about 3.8 mm full width at half maximum (FWHM). The system was used in the three-dimensional mode, and the reconstructed volume was displayed as 47 horizontal sections with a center-to-center distance of 3.125 mm. The axial field-of-view was 15 cm. Radioactivity in the brain was measured continuously according to a preprogrammed sequence. Each PET measurement was made according to a 63-min-long data acquisition

protocol consisting of 15 time frames (3±1 min, 4±3 min, 8±6 min). Attenuation correction was obtained from a 10- min transmission scan using a 68Ge radiation source. The monkeys were anaesthetized with repeated intramuscular injections of a combination (50–50%; 0.8–1.0 ml/ h) of ketamine (KetalarR, Parke-Davis; 50 g/ml) and xylazine (RompunR vet., Bayer; 20 mg/ml). The monkeys were positioned in the PET system with the help of a head fixation device that maintained the same head position during and between the measurements. The image planes were parallel to that defined by the canto-meatal line. In each monkey, a cannula was fixed in a sural vein for intravenous administration of the tracer.

Result

1. Effect of vinpocetin, deprenyl and DNO on PC 12 cell death

The cytoprotective effect of vinpocetin at 10-5,10-6, 10-7 M, DNO at 10-5, 10-8, 10-12 M and deprenyl at 10-3, 10-8 and 10-12 M concentration was measured in PC12 cell culture, subjected by hypoxia, followed by reoxygenation. The all treatment decreased significantly the cell death in an inverse dose dependent manner. The lower concentration resulted in a stronger cell-protective effect as compared to the higher concentration. .

2. Effect of vinpocetin, deprenyl and DNO on mitochondrial membrane potential

The ratios of red/green signals were significantly increased in the vinpocetin, deprenyl and DNO treated groups compared to the untreated control. More red, less green signals reflect the maintained mitochondrial membrane potential due to treatment.

3. Vinpocetin, deprenyl and DNO effect on ROS release

The reflectance intensity of cerium-perhydroxyde refers to the ROS level in the injured cells. In both, normoxic and in hypoxia/re-oxygenation groups the reflectance of cerium derivates were significantly decreased in cells treated with vinpocetin, deprenyl and DNO.

4. Effect of deprenyl-N-oxide on mitochondrial membrane integrity after hypoxia

In both "pre-treated" and "post-treated" PC 12 cells were protected by DNO from the hypoxia induced injury or cell death. In both cases in a dose of 10-12 M DNO was used. In the DNO-pre-treated cells JC1 labeling returned to the normal already after 30 minutes of hypoxia while in the post-treatment group about 60 minutes was needed to reach the normal range of JC1 staining (red/green ratio).

5. Effect of deprenyl-N-oxide on apoptosis in hippocampus region

TUNEL and caspase double labeled (apoptotic) cells were counted in the CA1 and CA2 regions in the hippocampus and expressed in percentage of number total cells in control, shame operated gerbils and in animals after 10 min occlusion of both common carotid arteries. Percentage of apoptotic neurons were also calculated after

DNO treatment. No or only occasional apoptotic cell were found in normal, or shame operated animals, while transient brain ischemia resulted in increase of apoptosis in both CA1 and CA2 segments of hippocampus. DNO treatment decreased the neuronal injury in both regions this effect was significant in the CA2 segments.

6. Effect of deprenyl on apoptosis in rat brain

The lesion size decreased after 48 h of continuous treatment with deprenyl by 50% on average. The average lesion size in treated animals was 36.5 mm^3, whereas it was 65.8 mm^3 in control rats. Two days after the permanent middle cerebral artery occlusion, a well-defined ischemic lesion could be characterized on Cresyl violet-stained sections. In the infracted area, no neural elements could be identified; however, endothelial cells of capillaries and microvessels and glia cells were morphologically well preserved. In some of the sections, polymorphonuclear leucocytes were detected in various numbers, mostly in the periphery of the infracted brain tissue. The number of TUNEL-labeled cells averaged over 60 samples was 17±12 in treated rats, whereas it was 28±25 in control rats. The number of TUNEL– caspase-3 double-labeled cells averaged over 30 samples was 3±3 in treated rats, whereas it was 8±6 in control rats.

7. Effect of vinpocetin on brain perfusion and glucose metabolism

The highest blood flow increases after treatment in the placebo group were found in the mesencephalon, pons, and cerebellum, whereas in the vinpocetin group they were found in the aforementioned structures (mesencephalon, pons, cerebellum), and

the unaffected thalamus, caudate nucleus, and the stroke region. The 3-way ANOVA showed an apparent tendency for the increases in rCBF values after treatment, with special regard to the thalamus. Nevertheless, the treatment-related changes in the contralateral hemisphere in the vinpocetin group showed a marked tendency for more accentuated increases than in the placebo group. The comparison of the flow-differences (placebo group/prevs. post-treatment/ as compared to the respective values in the vinpocetin group) also indicated a strong tendency, namely that the flow increases in the vinpocetin group tend to markedly exceed those in the placebo group (for the basal ganglia, thalamus, mesencephalon, occipital and frontal cortex, and whole unaffected hemisphere). The basal whole-brain CMRglc values were 6.96±0.37 in the whole patient group, 7.30±0.32 mg/100 g/min in the placebo group and 6.50±0.73 mg/100g/min in the vinpocetin group. Following the infusion, in contrast to the CBF changes, the CMRglc decreased globally as well as regionally in both patient groups. In the affected hemisphere the CMRglc decreases were more expressed in the placebo group than in the vinpocetin one. This effect was seen principally in the stroke region.

8. Effect of vinpocetin on brain uptake and PBR receptor binding

The two pretreatment conditions resulted in fundamentally different patterns. Compared to the baseline condition following the injection of [11C] PK11195, pretreatment with vinpocetin resulted in a lower global brain radioactivity uptake (0.77% and 0.60% of the total injected radioactivity, respectively). Compared to the baseline

condition following the injection of [11C] vinpocetin, in the case of pretreatment with PK11195, the global brain radioactivity uptake was markedly higher (3.84% and 5.96%, respectively). Despite the fact that approximately five times more vinpocetin enters the brain than PK11195, pretreatment with the known PBBS ligand PK11195 reduces markedly the BP values for vinpocetin in the brain, supporting the fact that the two ligands bind to the same receptor site (and that PK11195 does enter the brain albeit much less than vinpocetin).

Conclusion

From the findings, the following conclusions can be drawn:

- A new, combined method for visualizing ROS and MMP has been deviced; its usefulness to provide a quantitative analysis has been demonstrated;
- Vinpocetin shows a dose dependent neuroprotective affect as demonstrated by the measurement of cell death using ROS and MMP. The most potent concentration was the 10-7M,
- The peripheral benzodiazepine receptor binding of vinpocetine was demonstrated in a PET study,
- It has been shown that there are marked trends but no significant changes in brain perfusion and glucose metabolism in vinpocetin treated stroke patient between pre-tratment and post-treatment situations,

- Deprenyl and deprenyl-N-oxide show a dose dependent neuroprotective effect on cell death, as assessed by ROS and MMP. The most potent concentration was the 10-7M,
- The antiapoptotic effect of deprenyl and deprenyl-N-oxide was determined in an animal stroke model,
- It has been demonstrated that the main metabolite of deprenyl, deprenyl-N-oxide, is blocking the metabolic pathway.

Summary

In my PhD work I have investigated the cell protective effect of vinpocetin, deprenyl and deprenyl-N-oxide. The present investigations focused on these drugs' cellular effects, with special regard to their mitochondrial effects. In first part of the PhD period I established a new method by combining two well known methods: JC-1 staining (a mitochondrial transmembrane potential dye) and CeCl3 staining (which could assess the free radical production) (Géza Szilágyi, László Simon, Péter Koska, Géza Telek, Zoltán Nagy, Visualization of mitochondrial membrane potential and reactive oxygen species via double staining Neurosci Lett 2006 May;399(3):206-9.). Furthermore, in our laboratory we established fast and well reproducible methods for assessing cell death, measuring mitochondrial membrane potential, and free radical staining. The cell biological effects of vinpocetin, deprenyl and deprenyl-N-oxide were investigated in PC-12 cell culture. We proved that all molecules have a concentration dependent protective

effect against hypoxia (Simon L, Szilágyi G, Bori Z, Telek G, Magyar K, Nagy Z. Low dose (-)deprenyl is cytoprotective: It maintains mitochondrial membrane potential and eliminates oxygen radicals. Life Sci. 2005 Dec 5;78(3):225-31.). We suggested and proved a new mechanism of vinpocetin: that vinpocetin is a PBR ligand. With this new mechanism we could explain the cellular effects as well as the changes in aerobic/anaerobic metabolism in the brain after the treatment with vinpocetin (G. Szilágyi, et al. Effects of vinpocetin on the redistribution of cerebral blood flow and glucose metabolism in chronic ischaemic stroke patients: a PET study J Neurol Sci 2005 May;229-230:275-284). We have demonstrated a concentration dependent protective effect of deprenyl-N-oxide, a newly synthesized metabolite of deprenyl. This effect was MAO-B independent. On the basis of our result we suggest that the active metabolite of deprenyl is DNO. Furthermore we hypothesise that DNO has an affect on the mitochondrial transition permeability pore complex.

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