

SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Pharmacological interventions for retinopathies

Dr. Balázs Varga

SUPERVISOR: Prof. Dr. Árpád Tószaki



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Pharmacological interventions for retinopathies

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11 a.m., October 28, 2014

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1 p.m., October 28, 2014

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1. Introduction and objectives

Retinal injury is a pathological factor in a significant proportion of ophthalmological diseases. In our experiments we studied effective pharmacological interventions for retinal damaging effects such as glutamate excitotoxicity and ischemia-reperfusion injury.

Glutamate and its receptors play a key role in many neurological diseases as well as pathological conditions of the eye. Monosodium glutamate (MSG), which can be administered by subcutaneous injection or directly into the eye, leads to the destruction of the inner retinal layers. Blocking the effects of glutamate, directly or indirectly, has been shown to attenuate this retinal damage. Similarly, ischemia/reperfusion (I/R) injury and the resulting deterioration of tissue microcirculatory capacity are major contributing factors in many diseases, including retinal vascular occlusion, glaucoma, and diabetic retinopathy. Despite an evolving understanding of I/R-associated disease pathogenesis, no evidence-based strategies for the prevention and therapy of I/R-related ocular diseases are available at the time of this writing.

Pituitary adenylate cyclase-activating polypeptide (PACAP), an endogenous neuropeptide with highly effective neurotrophic and neuroprotective effects, has been shown to be retinoprotective in several models of retinal degeneration. Inland research- provided several lines of evidence that PACAP is protective in MSG-induced retinal lesion, also they demonstrated the protective effect of PACAP in neurochemically identified cell types in the retina. Although these above-mentioned data prove that PACAP has retinoprotective effects, it is not known whether these morphological changes correlate with functional amelioration. From the clinical point of view, it is very important to assess the possible functional improvement by exogenous PACAP administration. Therefore, using MSG-induced retina degeneration, a well-known model of retinal excitotoxicity, the

aim of the present study was to investigate the functional retinoprotective effects of PACAP by electroretinographic measurements.

Our research group formerly carried out experiments with melanocyte-stimulating hormone alpha (α -MSH), a peptide hormone naturally occurring in the human organism. α -MSH is known to protect against ischemic damage of the brain, kidney, and gastrointestinal tract. Additionally, authors have demonstrated α -MSH-mediated preservation of ischemic-reperfused myocardium. Ocular I/R pathologies typically cause significantly adverse effects on retinal tissue as well. However, at the time of this writing, the ability of α -MSH to protect the retina has not been comprehensively investigated. A goal of our experiment was to demonstrate the protective effect of α -MSH on I/R-induced retinal injury in a rat model. The present study demonstrates the protective effects of α -MSH on the recovery of I/R-induced retinal damage in a rat model. A major goal of this research was to identify an effective α -MSH dose range to achieve significant preventive and therapeutic effects in the test animals. These evaluations were conducted using electroretinography (ERG), as described below. Following the identification of an optimal dose range for the outcomes measured, assessments of related physiological parameters were investigated, including α -MSH effects on the degree of I/R-induced retinal edema, structural degenerative changes on the retina, and HO-1 protein expression. Results shown here demonstrate a correlation between α -MSH, therapeutic outcomes, and HO-1 expression. A corollary objective of the present study was to suggest whether non-HO-1-dependent mechanisms downstream of α -MSH might also contribute to protective effects of the hormone. Finally, a hypothesis that α -MSH is an evolutionarily conserved stress response hormone was evaluated by studying the effect of post-ischemic treatment with α -MSH.

2. Materials and methods

2.1. Animals

The experiments with PACAP were conducted using newborn Wistar rats. Wistar rat litters were kept in a 12 h light–dark cycle with food and water ad libitum. Animal housing, care and application of experimental procedures were in accordance with institutional guidelines under approved protocols of University of Pecs.

The experiments with α -MSH were conducted using male Sprague Dawley rats (CrI/SD). Animals weighing 300–350 g were purchased from Charles River Laboratories International, Inc. (Wilmington, Massachusetts). All animals received humane care in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the NIH guidelines. All of the protocols used in the present study were approved by the Institutional Animal Care Committee of University of Debrecen in Debrecen, Hungary. The rats were fed regular rodent chow ad libitum with free access to water until the initiation of experimental procedures.

2.2. Ischemia and reperfusion (I/R)

The animals were anaesthetized with intramuscular injections of ketamin/xylazine (50/5 mg/kg) at the outset of each experimental procedure. Subsequently, the retinal artery supplying blood to left eye of each animal was surgically occluded using a protocol previously applied by the authors of related studies (Szabo et al. 2004). Briefly, eyelids were retracted using sutures, followed by the use of a traction-type occluder (a silk suture thread through a polyethylene guide cannula) for retinal artery blockage. The suture was placed behind the eyeball, loosely around the optic nerve, central retinal artery, ciliary arteries, and the retrobulbar connective tissue. By pulling the suture and pressing the tube against the surface of the optic nerve, ischemia was induced and maintained for the required length of time. Ischemia was

verified macroscopically with a 120-D lens. Reperfusion of retinal tissue was accomplished by post-ischemic release of the occluder, allowing resumption of blood flow through the retinal artery.

2.3. Electroretinography (ERG)

The animals were prepared for electroretinographic measurements by anesthesia with 50/5 mg/kg of ketamin/xylazine, administered as intramuscular injections. Both pupils of each animal were dilated with 0.5 % cyclopentolate hydrochloride (Humapent, Teva, Hungary) and 10 % phenylephrine hydrochloride (Neosynephrin-HCL, Ursapharm, Saarbruecken, Germany). Detailed description of the system used by our workgroup for recording electroretinograms is as follows. Five silver needle electrodes were used for each measurement. In clinical practice ERG is praised for being a noninvasive technique, however, in our case, where eyes of the animals are used as experimental samples for different molecular biological assessments, this was not a requirement. And we found that in contrast to regular contact lens type electrodes, electrical contact, and signal throughput of needle electrodes carefully and superficially inserted into corneal surface, is much better. Retinal signals were analyzed using two measuring electrodes (one on each eye), inserted into corneal surfaces so as to avoid scleral damage or corneal perforation. Two reference electrodes were positioned on the earlobes of each animal (one on each earlobe) and the main ground electrode at the glabella. Effective electrical contacts and protection of eyes from dehydration was provided by a solution of methylcellulose (0.5 %). After a short dark adaptation period (20 min), the eyes were illuminated with a self-built stroboscope (20 cd/m², 0.5 Hz). Electrical signals corresponding to retinal changes passed through an ERG-recording system (ADInstruments, Australia) composed of an amplifier and an analog–digital converter (Bridge Amp and PowerLab, ADInstruments, Australia), displayed on a PC monitor, and

analyzed using a software, PowerLab Chart (Version 5.2.2., ADInstruments, Australia).

Obtained electroretinograms contain well identifiable, definite spikes composed of a negative peak followed by a highly positive peak which then fade out through another negative peak, and a steady, moderate extraneous signal (background noise). The highly positive peak is the maximum of b wave, preceded by a negative peak (the negative maximum of a wave). These electrical activities (spikes) occur consistently after light stimulus with the same rhythm, same frequency the stroboscope has (0.5 Hz). The most intense electrical activity on a common ERG recording to cause such highly positive peak is typically the b wave. However, a waves or other minor components of the ERG can not always be clearly identified: e.g., amplitude of the probable a-waves was in the same range as extraneous signal (background noise) present in each of the recordings, which in some cases hindered clear interpretation of a wave ERG data. Nevertheless, it should also be mentioned that in this study there was no need to be able to measure such subtleties as c waves or oscillatory potentials, only changes in electrical activity of retina, which is related to retina function. Actual amplitudes of b waves were measured between the (preceding) negative and the highly positive peaks of a spike. Selection of the spikes was done by a computer macro, written by our workgroup, which then – using the given criterias – selected the b-waves and recorded their data. This made it possible to evaluate even hundreds of spikes in ERG-recording of each animal.

The outcome of ERG experiments formerly conducted by the authors with the same recording system demonstrated that the aforementioned experimental strategy provides reproducible, cost-effective data on retinal function, closely correlated to the survival of retinal cells.

2.4. Processing for Examination by Light- and Electronmicroscopy

In the α -MSH-experiment samples designated for examination by light or fluorescent microscopy were produced according to the following general protocol: the fixation of retinal tissue was accomplished via trans-aortic cardiac cannulization, accompanied by a surgical incision in the right ventricle to allow a free outflow of blood. The vasculature of each animal was flushed (perfused) with physiological saline (0.9 % NaCl) to clear their system of blood. In each case, perfusion time was limited to 20-s interval to minimize nervous tissue edema. Physiological saline rinses were conducted concurrently with the fixation of retinas with 100 ml Bouin solution, followed by an extraction of bulbi and coronalis sectioning, bisecting each eyeball along the ora serrata. The vitreum of each eye was removed, and the bulbs were fixed, alcohol dehydrated, and paraffinized. Some paraffinized bulbi were processed into 7- μ m sagittal sections, which were then dyed with hematoxylin–eosin (HE) and examined by light microscopy. According to previous reports, I/R-induced edema formation is well recognized and documented in the inner plexiform layer of the retina (Shakib and Ashton 1966; Juarez et al. 1986). The average thickness for each eye was measured in the inner plexiform layer in sagittal sections at near the optic disc and expressed in micrometers using a manual scale on each glass slide. Paraffinized bulbi from a second group of eyes were sectioned into 60- μ m sagittal slices and prepared for electron microscopic (EM) examination by fixation of tissue lipids with osmium tetroxide (OsO₄). (Fixation of tissue proteins was done earlier by the formaldehyde constituent of the Bouin solution.) Samples fixed with OsO₄ were alcohol dehydrated and embedded into Araldite epoxy, and then heat treated at 56 °C for 2 days to induce polymerization (hardening). The samples were subsequently processed by ultramicrotome into (ultrathin) sections adequate for EM-based analysis.

In the PACAP-experiment rats were sacrificed with an overdose of anesthetic; eyes were removed and immediately dissected in ice-cold phosphatebuffered saline and fixed in 4% paraformaldehyde dissolved in 0.1 M of phosphate buffer (Sigma, Hungary). Tissues were embedded in Durcupan ACM resin (Fluka, Switzerland), cut at 2 μm and stained with toluidine blue (Sigma, Hungary). The sections were then mounted in Depex medium (Fluka, Switzerland) and examined in a Nikon Eclipse 80i microscope (Tokyo, Japan). Measurements were taken from the digital photographs with the NIH Image 1.55 program (Tokyo, Japan). Six tissue blocks from at least three animals were prepared, and central retinal areas within 1 and 2 mm from the optic disc were used for measurements (n=2–5 measurements from one tissue block). Sections where the ganglion cell layer (GCL) appeared thicker than a single cell row were excluded from evaluation. The following parameters were measured: (1) cross-section of the retina from the outer limiting membrane to the inner limiting membrane, (2) the width of individual retinal layers and (3) the number of cells/100 μm of section length in the GCL.

2.5. Western blot

Enucleated bulbi were suspended in a homogenisation buffer composed of Tris, 25 mM; NaCl, 25 mM; Naorthovanadate, 1 mM; NaF, 10 mM; Na-pyrophosphate, 10 mM; Okadaic acid, 10 nM; EDTA, 0.5 mM; PMSF, 1 mM; protease inhibitor cocktail; and distilled water (Sigma-Aldrich, St. Louis, Missouri). Samples of homogenized bulbi (50 μg protein per sample) and protein standards were electrophoretically fractionated using SDSpolyacrylamide gels with 12 % acrylamide-to-bis ratios. Protein standards included PageRuler Prestained Protein Ladder (Fermentas GmbH, Germany).

Fractionized proteins were subsequently transferred onto Protran Nitrocellulose Membranes (Whatman International Ltd., UK) in a protocol using Mini Trans-Blot Electrophoretic Transfer Cell (Bio- Rad Laboratories Ltd.). Blocking of each blot was accomplished using Tris-buffered saline with Tween 20 for 1.5 h with 5 % (w/v) nonfat dry milk. Subsequently each blot was incubated overnight at 4 °C with primary anti-HO-1 antibodies (Sigma-Aldrich), diluted 1:1,000 in TBS-T. Subsequently, each blot was incubated for 1 h with horseradish peroxidase-conjugated secondary antibody, diluted 1:2,000 in TBS-T, and 5 % (w/v) nonfat dry milk. After incubation and a short wash period, blots were treated with enhanced chemiluminescence reagents (Millipore, Billerica, MA). Detection was carried out via autoradiography for varying lengths of time with Medical XRay Film (Agfa-Gevaert N.V., Belgium). Films were scanned and evaluated with Scion Image software (Scion Corporation, Torrance, CA). GAPDH was used as loading control (Sigma-Aldrich).

2.6. Measurement of Heme Oxygenase Activity

Activity of heme oxygenase (HO) in bulbal tissue was measured according to the following general protocol: enucleated bulbi were homogenized in a solution containing HEPES, 10 mM; sucrose, 32 mM; DTT, 1 mM; EDTA, 0.1 mM; soybean trypsin inhibitor, 10 µg/ml; Leupeptin, 10 µg/ml; Aprotinin, 2 µg/ml; pH 7.4 (homogenisate buffer). All reagents were purchased from Sigma-Aldrich. The supernatant was collected by centrifugation of the homogenate for 30 min at 20,000×g at 4 °C. Assessment of HO activity was performed on each sample of supernatant, according to procedures used by Tenhunen et al. Briefly, HO activity was estimated based on a spectrophotometric measurement of bilirubin formation in a reaction mixture containing: an aliquot of the supernatant, plus glucose-6-phosphate, 2 mM; glucose-6-phosphate dehydrogenase, 0.14 U/ml; heme, 15 µM; NADPH, 150

μM , rat liver cytosol as a source of biliverdin reductase, 120 $\mu\text{g/ml}$; MgCl_2 , 2 mM, and KH_2PO_4 , 100 mM. After incubation in darkness for 60 min, the reaction was stopped by putting the samples on ice. Bilirubin formation was calculated on the basis of difference between optical densities obtained at 460 and 530 nm. The amount of bilirubin (in nanomoles) produced per hour per milligram protein was defined as one unit of HO activity. Differences seen between results of two activity measurements from two different samples but from the same tissue type may be attributed to HO-1 activity since other isoforms of the enzyme are of the noninducible, constitutive form.

2.7. Experimental protocols and groups

2.7.1. PACAP-experiment

Purpose of the PACAP-experiment was to assess the functional retinoprotective effect of PACAP with the help of electroretinography.

Rat pups received 2-mg/g MSG (n=16) diluted in 100- μl saline subcutaneously three times, on postnatal days P1, P5 and P9. On the same days as the MSG treatment, half of the animals received 100-pmol PACAP38 diluted in 5- μl saline into their right eyes intravitreously using a Hamilton syringe. Animals received the same volume of vehicle to their left eyes. Dose and schedule of MSG and PACAP treatments were based on our previous experiments (Babai et al. 2005). No treatment was applied in control animals (n=5).

ERG measurements were performed 2 months after treatments. After the ERG measurements animals were sacrificed and their eyes were enucleated for further histological investigations, as described before.

2.7.2. MSH-experiment 1. phase

The first major strategy used in the present study was conducted to find

the best dosage and to evaluate the capacity of α -MSH to mitigate I/R-induced ocular pathologies. This strategy included the following major features: the animals were divided randomly into two major treatment groups, groups I-a and I-b (n=30 in each group). Group I-a served as a dose–response study group and included five subgroups: a control group and four treatment groups (n=6 in each subgroup). Twelve hours before induction of ischemia, vehicle-treated controls were given subcutaneous (s.c.) physiological saline, while the other four groups were administered s.c. 50, 250, 500, and 1,000 μ g/kg α -MSH (Sigma-Aldrich), respectively. α -MSH used for experiments described here was prepared as a solution of the hormone in physiological saline. Group I-b, which was considered to estimate the effects of α -MSH, included two subgroups (n=15 in each subgroup). Twelve hours before induction of ischemia vehicle-treated controls were given physiological saline (s.c.), while the other subgroup was administered 500 μ g/kg α -MSH (s.c.), an effective dose previously identified in group I-a by electroretinographic measurements. Animals in group I-a were subjected to 30 min of ischemia in one eye as described above, followed by a 24-h period of reperfusion and ERG measurements as described above. Group I-b animals received 90 min of ischemia in one eye, followed by the same 24-h reperfusion treatment that group I-a animals were administered. The basis for selection of the two aforementioned ischemic time intervals is based on a previous observation by the authors of the present study that retinal edema, a focus of study in this research, fails to develop following only 30 min of ischemia but develops definitively in rat eyes treated for 90 min. However, a 90-min ischemic period induces near blindness, making ERG measurements impractical. Hence, the design strategy for part 1 of the present study incorporated two major groups of animals segregated according to the length of the ischemic period prior to reperfusion. Bulbi collected from group I-b were divided into three groups (n=5 in each group), and segregated according to processing for evaluation of

α -MSH influence on I/R-related pathologies. The first group of samples was subjected to histological examination using HE-stained sections and light microscopy; the second group was osmium-fixed and analyzed by electron microscopy; and a third group of bulbar samples was used as source material for a Western blot assay as described above.

2.7.3. MSH-experiment 2.phase

Part II experiments were conducted to evaluate the efficacy of post-ischemic α -MSH-treatment against I/R injury and also to assess the significance of HO-1 in the effect of α -MSH. This experimental design strategy included the following major features: Animals (group II; n=20) were divided randomly into four major treatment cohorts (n=5 in each group), designated as shown below with drugs administered according to the indicated timeline. Control animals were treated intraperitoneally (i.p.) with vehicle of SnPP (described below) 5, 3, and 1 day before induction of ischemia, and then with physiological saline (vehicle of α -MSH) subcutaneously at the outset of postischemic reperfusion. A second group of animals were treated with vehicle of SnPP (i.p.) 5, 3, and 1 day before induction of ischemia and then with 500 μ g/kg α -MSH (s.c.) at the outset of postischemic reperfusion. α -MSH used for experiments described here was prepared as a solution of the hormone in physiological saline. A third group of animals were treated with 50 μ mol/kg SnPP (i.p.) 5, 3, and 1 day before the induction of ischemia, and then with 500 μ g/kg α -MSH (s.c.) at the outset of postischemic reperfusion. SnPP (Frontier Scientific Inc., Logan, Utah) is a potent inhibitor of HO-1. SnPP was dissolved in 0.1 N sodium hydroxide, adjusted with HCl to pH 7.4 and diluted with physiological saline. Each batch of SnPP was prepared within 1 h of use and protected from light. A fourth group of animals were treated with 50 μ mol/kg SnPP (i.p.) 5, 3, and 1 day before induction of ischemia, and then with physiological saline (s.c.) at the

outset of postischemic reperfusion. Retinal ischemia was applied in each group for 30 min followed by 24 h of reperfusion. Then ERG and retinal tissue HO activity measurement were performed to evaluate the effect of the abovementioned treatment combinations on I/R injury.

2.8. Statistical Analysis

A one-way analysis of variance with a Tukey posttest was used for Gaussian data results from the D'Agostino and Pearson omnibus normality test. Data with nonparametric distribution was analyzed using the Kruskal–Wallis test along with the Dunn's posttest

3. Results

3.1. Results of PACAP-experiment

By standard histological technique, all the characteristic layers of the mammalian retina were well visible in normal control preparations: pigment epithelium, photoreceptor layer (PL), outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL) and finally ganglion cell layer (GCL). When compared to control animals, the overall thickness of the retina was significantly reduced in pups exposed to 3xMSG, with the entire inner retina suffering a significant loss. With the almost entire disappearance of the IPL, the fusion of the INL and GCL could be observed. Several signs of degeneration, empty cell body-shaped spaces, could be observed in the nuclear layers (ONL and INL). From the inner limiting membrane (ILM) to the outer limiting membrane (OLM), distance was approximately 47% of the normal retinas. All of the retinal layers were also markedly reduced (ONL=75%, OPL=66%, INL=26%, IPL=15%). The number of cells/100 μm in the GCL was decreased approximately to 38%. Our results revealed that the MSG-induced severe retinal degeneration could be

improved by local 3xPACAP treatment, in accordance with our previous observations. Intravitreal 3xPACAP administration caused a significant amelioration of the retinal layers (ONL=89%, OPL=95%, INL=74%, IPL=60%). The IPL remained visible; the INL and GCL were clearly separated at all places. The number of cells in the GCL was significantly more than in the MSG-treated retinas (65%) compared to the control ones.

According to the results of ERG measurements the scotopic b-wave is a measurement of the field potential that primarily arises from rod bipolar cells in response to the flashes of light. Figure 2 shows scotopic b-wave in control, MSG-treated and MSG+PACAP groups. We measured the amplitude of the waves. In control eyes without any treatment, the value of the b-wave was $220.9 \pm 19.5 \mu\text{V}$. Using MSG treatment, b-wave value decreased to $37.76 \pm 8.3 \mu\text{V}$. Intravitreal PACAP treatment showed a protective effect against MSG deterioration, and the b-waves significantly increased $60.9 \pm 12.0 \mu\text{V}$ compared to the MSG treatment alone. In order to further directly characterize rod photoreceptor function, we measured the scotopic a-wave of the electroretinogram which arises almost exclusively from the rod photoreceptors. Figure 2b shows the response to an intense flash, indicating maximal scotopic response. The saturated a-wave amplitude from control eye's value ($65.0 \pm 6.0 \mu\text{V}$) deteriorated to $9.4 \pm 3.0 \mu\text{V}$. The protective effects of PACAP were demonstrated: the a-wave values were increased to $16.8 \pm 6.0 \mu\text{V}$. Representative ERG waveforms are shown from control (Fig. 3a), MSG-treated (Fig. 3b) and MSG+ PACAP-treated (Fig. 3c) retinas. ERG waveforms appeared reduced after MSG administration, shown by further change in the amplitudes and latencies of both a- and b-waves. PACAP treatment ameliorated the MSG-induced functional changes, resulting in slight but significant differences between MSG-treated and MSG+PACAP-treated retinas. There was no statistical difference in photopic a-wave or b-wave

responses when MSG-treated and PACAP-intravitreally injected eyes were compared.

3.2. Results of MSH-experiment 1. phase

I/R injury as measured by ERG b-wave measurements degraded retinal function to 31.64 % of that exhibited by nonischemic (baseline) eyes of control animals. Administration of α -MSH dose responsively improved retinal function in I/R-injured eyes of animals to a value of 66.18 % of control baseline—a significant outcome achieved in I/R-injured eyes of animals treated with a 1,000 $\mu\text{g}/\text{kg}$ α -MSH ($p < 0.001$ vs. control I/R). I/R-mediated retinal damage was severe to such an extent that α -MSH treatment could not fully restore function of the tissue to normal levels. Moreover, doseresponsive improvement in tissue functional parameters leveled off at a dosage of 1,000 $\mu\text{g}/\text{kg}$, beyond which it is anticipated that negligibly more favorable changes would be observed. The optimal effective dose range identified in the present experimental setting was observed to be 250–500 $\mu\text{g}/\text{kg}$ of α -MSH. Measureable effects of α -MSH-treatment on the b waves of ERG were also observed at a dosage as low as 50 $\mu\text{g}/\text{kg}$ (35.64 % of control baseline); however, the magnitude of improvements was not statistically significant at this dosage, only at higher doses, such as 250 (48.00 %, $p < 0.001$ vs. control I/R) and 500 $\mu\text{g}/\text{kg}$ (64.36 %, $p < 0.001$ vs. control I/R). Treatment with 1,000 $\mu\text{g}/\text{kg}$ mediated a significant protective effect on retinal function, but since no statistically significant improvement in outcome was observed between 500 and 1,000 $\mu\text{g}/\text{kg}$ (64.36 vs. 66.18 %), it has been concluded that a 500- $\mu\text{g}/\text{kg}$ dosage is sufficient and probably optimal for inhibition of I/R-mediated retinal damage.

According to histological results retinal layers become thickened as a result of I/R-induced interstitial edema formation. 500 $\mu\text{g}/\text{kg}$ α -MSH inhibits

the development of I/R-induced damage, principally retinal edema and resultant thickening. Significant differences between control and treated non-I/R values were not observed.

I/R-mediated damage to mitochondria of inner retinal cells was clearly observed on electronmicroscopic images. Due to formation of interior vacuoles damaged mitochondria could be seen in inner retinal cells of I/R injured retinal tissue from control animals. By contrast, inner retinal cell mitochondria from I/R-injured retinas taken from animals treated with 500 $\mu\text{g}/\text{kg}$ α -MSH appeared intact with no vacuolization. EM ultrastructure of retinal pigment cells and photoreceptors revealed no apparent morphological changes in cells in the outer retinal layers following I/R injury. Significant differences between control and treated non-I/R ultrastructure were not observed.

Western blot analysis of HO-1 expression in ocular tissue revealed significantly greater amounts of HO-1 protein in I/R-injured bulbi taken from animals receiving 500 $\mu\text{g}/\text{kg}$ α -MSH treatment than from vehicle-treated rats ($p < 0.05$). Significant differences between control and treated non-I/R values were not observed (data not shown).

4.3. Results of MSH-experiment 2. phase

The outcome of part II ERG studies suggest three major trends with respect to effect of each treatment on retinal integrity: relative to waveforms recorded for nonischemic retinas (Control Baseline), I/R injury, and SnPP-mediated HO-1 inhibition decreased the amplitude and altered the waveform characteristics in ways indicative of degraded retinal tissue function. By contrast α -MSH treatment shifted retinal responses toward improved retinal function. Interestingly, administration of SnPP together with

α -MSH did decrease but did not thoroughly abolish this protective effect of α -MSH. Mean percentage of b waves relative to control baseline (i.e., effect of postischemic treatment) was 67.69 % of control baseline values in animals treated with 500 $\mu\text{g}/\text{kg}$ α -MSH ($p < 0.001$ vs. control I/R). This value is even a little bit higher than what could be observed in outcomes of the part I experiments (i.e. effect of pre-ischemic treatment), which was 64.36 % of control baseline values in animals treated with 500 $\mu\text{g}/\text{kg}$ α -MSH ($p < 0.001$ vs. control I/R). Mean percentage of b waves relative to control baseline measured in I/R-injured retinas from vehicle-treated rats (Control Ischemia) of second experimental studies were 33.44 %. Percentage of SnPP-treated I/R retinas were observed to be 19.76 % ($p < 0.001$ vs. control I/R). Dual-treatment (SnPP+MSH) significantly lowered the b wave percentages vs. MSH-only treatment (55.77 vs. 67.69 %; $p < 0.01$). However, percentage of SnPP+ MSH-treated I/R retinas proved to be significantly higher than that of SnPP-only treatment (55.77 vs. 19.76 %; $p < 0.001$). Significant differences between control and treated non-I/R values were not observed.

According to the outcome of HO activity measurement in ocular tissue of rats used for part II experiments tissue activity of the enzyme was amplified by I/R (119.58 % of control baseline), and even more by treatment of animals with 500 $\mu\text{g}/\text{kg}$ α -MSH (159.42 %). Latter effect was diminished by combined administration of α -MSH with the HO-1 inhibitor SnPP (108.77 %). However, there was a slight difference between SnPP+MSH- and SnPP-only-treated groups (108.77 and 96.60 %, respectively). Significant differences between control and treated non-I/R values were not observed.

4. Discussion

We showed, for the first time, that intravitreal PACAP treatment induced a significant functional amelioration in MSG-induced retinal degeneration. Numerous studies have examined potential retinoprotective strategies from both the morphological and functional points of view. The degree of degeneration and the morphological retinoprotection often correlate with functional improvement. Trophic factors like fibroblast growth factor have been shown to induce both morphological and corresponding ERG improvement in retinal degeneration. However, functional measurements do not necessarily correspond to the morphological outcome in all cases in either human patients or animals studies. Even opposing effects are known from the literature, for example, ciliary neurotrophic factor suppresses ERG waves in spite of promoting photoreceptor survival. Our results show that PACAP treatment led to a very pronounced morphological protection in the retina in MSG-treated rats. The functional outcome, although not parallel with the morphology, was also significantly improved after PACAP treatment. In the present study, we found that the changes in the scotopic waves were partially counteracted by PACAP treatment, but not the photopic changes. In the scotopic responses, the amelioration by PACAP was observed both in the a-wave and the b-wave, representing the photoreceptor and the inner retinal function, respectively. Glutamate mediates excitatory synaptic transmission at the photoreceptor/bipolar cells and at the bipolar/ganglion cell synapses. MSG treatment, in accordance with earlier observations, led to a very severe degeneration in the inner retina and less severe degeneration in the photoreceptor layer. The excitotoxicity induced by high concentration of glutamate is thought to be caused by both direct and indirect mechanisms, depending on the presence and density of glutamate receptors. Numerous studies have shown the distribution of glutamate receptors in the retina, with high density in the inner retinal layers. This may explain the high vulnerability of the inner retina also shown in our study. In our earlier studies, where retinas

were processed for histological analysis at 3 weeks of age after neonatal MSG treatment, this severe degeneration in the inner layers was revealed with an apparently intact photoreceptor layer. In the present study, when survival time was longer, degeneration was also observed in the photoreceptor layer, and it was further supported by the decrease in the a-wave in the electroretinogram. By the long-term degeneration of the input elements of the photoreceptors, the photoreceptors themselves will suffer degeneration, which could be clearly seen in our present study. PACAP treatment could attenuate the MSG-induced changes, both at morphological and functional level. PACAP has been shown to have various effects in the retina and in the retinal pathways. PACAP protected all inner retinal layers, in correlation with previous results showing the distribution of PAC1 receptor in the retina. Although the functional amelioration was more subtle than the morphological, the present observations are important from the clinical point of view showing, for the first time, that PACAP treatment is able to improve the functional properties of the retina in excitotoxic damage.

The experiments conducted in 1 phase MSH-experiment examined the capacity of α -MSH to protect against I/R-induced retinal injury. In these studies, α -MSH was observed to significantly preserve functions of the eyes subjected to I/R injury. Evidence for this protective effect is provided by ERG experiments that demonstrated an α -MSH dose-dependent increase in I/R-damaged retinal b wave magnitude. These results represent the first demonstration of α -MSH-mediated protection against I/R injury to the retina. The aforementioned outcomes are significant in the context of a 2013 report describing the capacity of resveratrol, a plant polyphenol, to therapeutically increase b wave amplitude and ameliorate the effects of retinal ischemia induced by high intraocular pressure through a mechanism that prominently includes upregulation of HO-1. Present study demonstrate that HO-1 activity,

although it is not an exclusive component of α -MSH-mediated protection of retinal tissue, increases correlating with α -MSH administration.

HO-1 expression in injured tissue is a highly conserved adaptive response that is observed in many tissues in a very diverse range of organisms. Previous work by the authors to develop therapeutic potential of this enzyme show that upregulation of HO-1 with an inducer mediates powerful therapeutic effects in vivo, further supporting the potential clinical application of agents that upregulate HO-1.

The activity of HO-1 is increased in response to a wide variety of stressors, including oxidative and inflammatory insults, as well as metabolic and hemodynamic factors such as high glucose. Nevertheless, in most cases, the pathophysiological activation of HO-1 results only in a transient or marginal increase of HO-1 activity that falls below the threshold necessary to activate downstream signaling components of the HO system at levels capable of achieving significant remission of serious inflammatory pathologies. Thus, strategies for use of HO-1 as definitive prophylaxis or treatment for retinopathies and other diseases are expected to increasingly make use of pharmacological agents capable of increasing expression and activity of the enzyme. Previously, the authors have demonstrated that sour cherry kernel extract is capable of protecting rat retinas against I/R injury in vivo through activation of HO-1. The results of Western blot analysis of the present study revealed significantly increased expression of HO-1 in retinal tissue of I/R-injured eyes from 500 μ g/kg α -MSH-treated rats in comparison to expression of the enzyme in I/R-injured eyes from vehicle-treated animals. This outcome suggests that HO-1 may be upregulated by α -MSH in ways that might be significantly augmented by its concurrent pharmacological induction as a therapeutic strategy.

Histological examination of retinal tissue in the present study revealed that α -MSH-treated animals exhibited significantly reduced retinal edema

following I/R injury. This finding is also potentially significant in the context of the role of HO-1 in protection against retinal edema. These results may contribute to development of novel strategies for alleviation of retinal edema in ocular disorders.

This study also provides EM demonstrations of suppression of I/R-induced damage to mitochondria of inner retinal cells by α -MSH at subcellular level, which is also a novel result.

The experiments conducted in part II of the present investigation examined the capacity of post-ischemic administration of α -MSH to protect against I/R-induced retinal injury. A corollary objective was to suggest whether non- HO-1-dependent pathways might also contribute to protective effects of α -MSH. The results of the part II ERG studies reveal that the amplitude of waveforms, which is a function of healthy retinal function, was decreased by I/R injury but, most strikingly, increased by post-ischemic α -MSH treatment. At the same time, eyes from rats treated with the HO-1 inhibitor SnPP and α -MSH together exhibited reduced ERG waveform amplitude. Also, tissue HO-1 activity correlated with waveform amplitudes. Taken together, clear correlation between α -MSH, mitigation of I/R injury to retinas, and HO- 1 involvement suggest that α -MSH may mediate protection from I/R injury substantially via an HO-1-dependent pathway. Results presented here cannot rule out partial involvement of some other, as-yet unidentified non-HO-1-dependent mechanisms as well. Exploration of this possibility is the focus of future research by the authors. Avenues of investigation are suggested by the work by other investigators, showing that α -MSH has anti-cytokine activity, inhibits proinflammatory protein expression, decreases fever, and modulates neuroimmune activity. The present study provides the first demonstration of the efficacy of α -MSH in protecting against I/R-induced retinal damage when administered either before or after an ischemic event. Moreover, the ability of this hormone to protect the retina

following a period of ischemia lends strength to the hypothesis that α -MSH expression is a major physiological countermeasure to a wide range of stressors, developed early in vertebrate evolution and conserved as an important survival trait. The observation that post-ischemic administration of α -MSH mediates protective effects in the same range as pre-ischemic treatment is encouraging from the perspective of its potential therapeutic use as well. Results of the present study validate the hypothesis that α -MSH mitigates the severity of I/R-induced retinal injury and reinforce evidence for possible involvement of HO-1. Of particular interest is the capacity of α -MSH to reconstitute the function of retinal tissue when administered following a period of ischemia. This finding suggests that α -MSH may have a very wide range of uses in the prevention of I/R-mediated pathologies.

5. Summary

A purpose of the experimental work was to utilize electroretinography, a retinal function measurement. With the help of this method we successfully demonstrated that in the background of the morphological effect of PACAP against MSG-induced retinopathy functional amelioration also exists. This may establish a rationale for possible future use of PACAP as a therapeutic agent in excitotoxic retinal damage. The results of our experiments carried out with α MSH justify the presumption that α MSH ameliorates the severity of ischemia/reperfusion-induced retinal damage functionally as well as on tissue and cellular level, and provide evidence on the remarkable but not exclusive participation of HO-1 in the action mechanism of the hormone. Remarkable is the property of α MSH to be able to restore retinal function after an ischemic period, i.e., post-ischemically applied, which leads to the conclusion that α MSH can potentially be used in a wide range of I/R-mediated pathological conditions applied either as prevention or as treatment.

Register Number: DEENKÉTK/43/2014.

Item Number:

Subject: Ph.D. List of Publications

Candidate: Balázs Varga

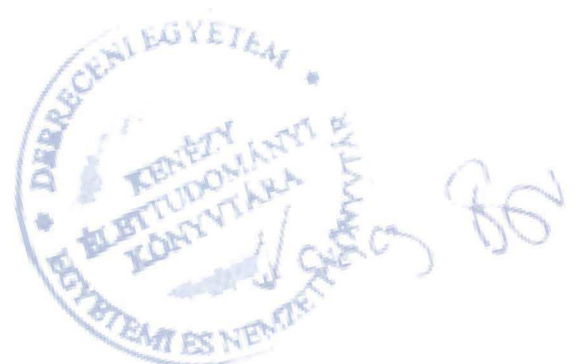
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Mtmt ID: 10037794

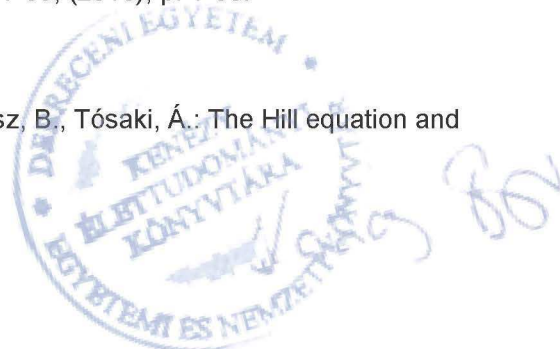
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1. **Varga, B.**, Gesztelyi, R., Bombicz, M., Haines, D., Szabó, A.M., Kemény-Beke, Á., Antal, M., Vecsernyés, M., Juhász, B., Tósaki, Á.: Protective Effect of Alpha-Melanocyte-Stimulating Hormone (α -MSH) on the Recovery of Ischemia/Reperfusion (I/R)-Induced Retinal Damage in A Rat Model.
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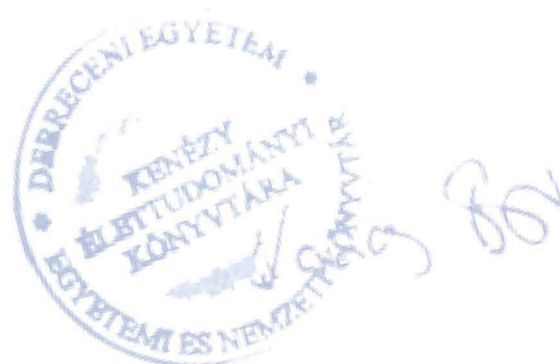


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3. Juhász, B., Kertész, A., Balla, J., Balla, G., Szabó, Z., Bombicz, M., Priksz, D., Gesztelyi, R., **Varga, B.**, Haines, D.D., Tósaki, Á.: Cardioprotective Effects of Sour Cherry Seed Extract (SCSE) on the Hypercholesterolemic Rabbit Heart.
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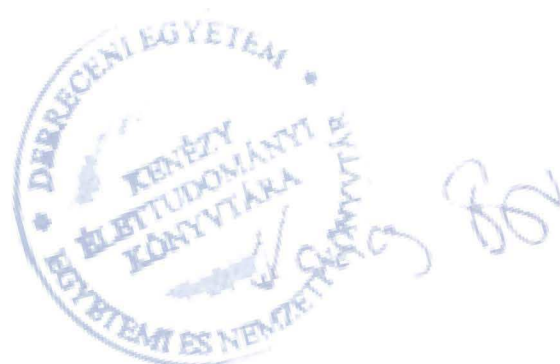
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Total IF of journals (all publications): 33.44

Total IF of journals (publications related to the dissertation): 5.395

The Candidate's publication data submitted to the Publication Database of the University of Debrecen have been validated by Kenezy Life Sciences Library on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

04 March, 2014



7. Acknowledgements

I express my thanks to my supervisor, Dr. Árpád Tósaki full professor (Univ. of Debrecen, Faculty of Pharmacy, Department of Pharmacology), for enabling implementation of my PhD work in the department he led.

I thank Dr. Béla Juhász assistant professor (Univ. of Debrecen, Faculty of Pharmacy, Department of Pharmacology), for following my experimental work all the time and providing guidance always, when needed.

I thank Dr. Rudolf Gesztelyi assistant professor (Univ. of Debrecen, Faculty of Pharmacy, Department of Pharmacology), for always having a minute for me, if I had any – especially if I had statistical – questions.

I thank the cooperation of Dr. Tamás Atlasz and Krisztina Szabadfi (Univ. of Pécs, Department of Physical Education and Sports Sciences), furthermore I thank the staff of departments related to them, who facilitated our collaborative work and appearance of our joint article.

I thank Dr. Miklós Antal full professor and Gréta Kiss (Univ. of Debrecen, Department of Anatomy, Histology and Embryology) for their help in preparation of electronmicroscopic images.

I thank Dr. Anikó Pósa and Csaba Varga (Univ. of Szeged, Department of Physiology, Anatomy and Neuroscience) for their help in heme oxygenase activity measurements.

I thank to my colleagues working in our department, especially dr. Mariann Bombicz and Dániel Priksz.

I thank my family, my wife, Adrienn, my daughters Panni and Alíz, as well as my parents, my brother and my grandparents, for their loving support.

I thank my friends for the time I spent with them with relaxation and recharge.

This study was supported by grants from OTKA (78223 and 104017), Richter Gedeon Foundation, and in part by the project of New Hungary Development Plan, co-financed by the European Union and the European Social Fund of TAMOP-4.2.1/B-09/1/KONV-2010- 0007, TAMOP-4.2.2./B-10/1-2010-0024, TAMOP-4.2.2.A-11/1/KONV-2012-0045 and TÁMOP-4.2.4.A/2-11-1-2012-0001. The research took place under the project with identification number of TÁMOP 4.2.4.A/2-11-1-2012-0001 entitled “National Excellence Program—Convergence program for elaboration and operation of support-system sponsoring Hungarian students and researchers,” funded by the European Union, co-financed by the European Social Fund.

