PHD THESIS

PHOTOSTRESS INDUCED RETINAL DEGENERATION. ANTIOXIDANT, HORMONAL AND ENVIRONMENTAL FACTORS IN ANIMAL EXPERIMENTS

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Debrecen, 2005.

PhD thesis

Photostress induced retinal degeneration. Antioxidant, hormonal and environmental factors in animal experiments

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Debrecen, 2005.

1. INTRODUCTION

Retinitis pigmentosa is a heterogeneous group of progressive hereditary retinal degenerations, its prevalence is about 1:3000 in industrial countries. The early symptoms are night blindness and peripheral, concentric visual field defects due to death of rod photoreceptor cells. The ERG is abnormal and the retina displays irregular pigment accumulation, strarting in the periphery. As the condition progresses, the visual field loss becomes complete because of cone photoreceptor cell death and the final outcome is blindness.

In 1966, Noell et al. discovered that albino rats exposed to constant illumination underwent dramatic retinal degeneration. In this animal model, as well as in inherited retinal degenerations, the “final common pathway“ of photoreceptor cell death is apoptosis. For this reason, the animal light damage model is widely used to study the mechanisms of cell death in retinitis pigmentosa.

N^G-nitro-L-arginine-methyl ester (L-NAME), a competitive inhibitor of nitric oxide synthase (NOS), has been reported to protect against retinal degenerations caused by damaging light. Nitric oxide (NO) is produced from L-arginine by the family of NOS enzymes, forming free radical NO and citrulline. NOS isoenzymes have been detected in retina and neuronal NOS may be responsible for producing NO in photoreceptors and bipolar cells.

Several experimental studies carried out in humans, as well as in vivo experiments in animals, show a protective effect of female steroid hormones against cardiovascular, cerebrovascular and other neurologic diseases. Premenopausal women show lower incidence of stroke and estrogen therapy in postmenopausal women resulted a decreased risk of heart disease. Hormonal influences on photostress-induced photoreceptor damage have also been studied. Female albino rats were either hypophysectomized (HYPEX) or ovarectomized (OVEX) before puberty. The retinal damage in OVEX and HYPEX rats were significantly less than in intact rats. In a later study though, OVEX rats receiving 100 μg estradiol benzoate, had significantly less destruction of photoreceptor cells, than OVEX oil-treated animals. Progestreone administration (2.5 mg/day) alone had no effect on light-induced photoreceptor degeneration. A novel estradiol analog, MITO-4565 administered by intravitreal injections proved to be cytoprotective in S334ter rats.

There are a number of factors that determine the degree of retinal damage caused by light exposure. One of these is the animal's pre-exposure light history. Rats raised in bright cyclic light or bright light-adapted adult rats are protected against light damage, compared to their dim/dark reared littermates. Examination of these two groups revealed a number of biochemical and morphological differences. These differences are adaptive responses and could be reversed in a few weeks if animals are moved to the opposite lighting environment. Mice have some distinct advantages in research of retinal degenerations. Their genome is easily manipulated and there is a vast amount of information available through the Mouse Genome Project. These are the reasons for we used mice to examine neuroprotective factors that may be up- or down-regulated by light environment.

2. PURPOSE OF STUDY

Our aim was to understand the mechamisms taking place in photoreceptors in retinal degenerations. We tried to inhibit or at least to slow this process by means of giving two different drugs and changing the lighting environment of animals.

2.1 Testing the protective effect of antioxidant L-NAME in wild type rats and in rats suffering from rhodopsin mutations:
previous studies have shown that L-NAME, an inhibitor of NOS, protects retinas of albino rats from damaging levels of light. The aims of our study were two-fold, to confirm the results mentioned above and to test if L-NAME's protective effect works on animals suffering from retinal degeneration.

2.2 We examined the neuroprotective effect of progesterone in male albino rats:
human statistical data and results of animal experiments show the protective effect of female steroid hormones against cardiovascular, cerebrovascular and other neurologic diseases. We tested if this effect works in the retina of male albino rats exposed to bright continuous illumination under experimental conditions, previously shown to cause apoptosis of rod photoreceptor cells.

2.3 We analized the effect of environmental lighting conditions on the degree of retinal degeneration:

previous studies reported that albino rats raised in bright cyclic light were protected from apoptosis, evoked by light exposition. In our present study we determined if this effect exists in albino mice, too.

3. METHODS

3.1 Light damage study

3.1.1 L-NAME protection: a group of 5 week old albino rats was exposed to 2700 lux light stress for 24 hours. The control group stayed under its original dim lighting condition. Within each group, half of the animals were injected with L-NAME (100mg/body weight) 30 min before light exposure and the other half with the same amount of its inactive isomer, intraperitoneally (ip.). ERGs were recorded before and after cessation of exposure, at the end of the experiment eyes were enucleated.

3.1.2 Progesterone protection: In order to minimize the effect of endogen progesterone synthesis, male (albino) rats at the age of 5 weeks were chosen for the experiment. Animals were divided into two groups. Within each group, half of the animals were injected ip. with progesterone (60 mg/body weight) and the other half with the vehicle alone, for 4 days. The third injection was given 30 min before light stress and the fourth injection was given immediately after finishing that. Control animals were injected in the same manner, but remained under the dim cyclic light. Baseline ERGs were recorded 4 days before starting drug administration and follow-ups were carried out 5 days after the end of light stress. Eyes were then enucleated.

3.1.3 Role of light history: BALB/c mice were born in dim cyclic light. At the age of 1 week, half of the litters were moved to bright cyclic light (400 lux). At 5 weeks of age, mice in both rearing environment were divided into two groups. The light-stressed group was exposed for 72 hours of 3000 lux light. The control group was maintained under the original light condition, either in dim or bright cyclic light. After light stress, eyes were removed.

3.2 Transgenic (P23H and S334ter) rat study

Transgenic rats were born and raised in 400 lux cyclic light in our animal facility. At 3 weeks of age, WT, P23H and S334ter animals were devided into two groups, one of which was given L-NAME, the other D-NAME. These drugs were administered by daily (100 mg/body weight) ip. injections and in their drinking water (daily

consumption averaged ~ 40 mg/body weight) for 4 weeks. After the last doses ERGs were determined and eyes were enucleated 3 days later.

3.3 Electroretinography

Rats were dark-adapted and anasthetized under dim red light, pupils were dilated. Five stimuli were presented in ascending order of intensity (-40 dB, -24dB, -8dB, 0 dB, 10 dB) with a 60 sec interval between flashes in a Ganzfeld sphere.

3.4 Histology

Eyes were placed into PerFix and embedded in paraffin. Five μm thick sections were cut along the vertical meridian, through the optic nerve head (ONH), and stained with hematoxylin and eosin. In rats the thickness of the outer nuclear layer (ONL) and the length of the rod inner segment (RIS) + rod outer segment (ROS) were measured at 0.5 mm distances from the ONH to the inferior and superior ora serrata. Same parameters were calculated in mice, but in 0.33 mm distances.

3.5 Tissue harvest

The retinas from each pair of eyes were bluntly dissected from pigment epithelium/ choroid/ sclera, then frozen in liquid nitrogen and stored at –80 ^{0}C until processed.

3.6 Terminal dUTP nick end labeling (TUNEL) assay

Apoptosis of retinal cells was determined by TUNEL assay on 5 μm thick paraffin embedded sections.

3.7 DNA laddering

Retinas were homogenized in lysis buffer (50mM Tris-HCl (pH 8.0), 10mM EDTA, 0.5% SDS and 0.5mg/ml proteinase K). The resultant homogenates were extracted with phenol/chloroform to remove the redundant protein and the contaminated RNA was digested by incubating with 20μg/ml RNase A for 2 h at 37 ^{0}C. Finally, the genomic RNA was run on 2% agarose gel containing 0.5μg/ml ethidium bromide.

3.8 Fatty acid measurement

ROS were prepared from each pair of retinas by discontinuous sucrose gradient centrifugation. The fatty acid composition of ROS total lipids were determined by gas-liquid chromatography.

3.9 Statistical analysis

Results were plotted as mean ± S.D. Unpaired t-test was used for assessing significant differences across groups for the ERG and histology data. Paired t-tests were used where appropriate for comparison of baseline and follow-up ERGs of the same animal. Values below p=0.05 were reported as significant. The Scheffé test was used for the analysis of fatty acid results.

4. RESULTS

4.1 Administration of L-NAME

4.1.1 Acute light damage studies

2700 lux continuous illumination for 24 hours caused a significant reduction in ERG responses in both exposed groups. Although the loss of function appeared to be less in the L-NAME group, the difference between the ERG responses of the light damaged D-NAME treated and light damaged L-NAME treated groups was not statistically significant. Photostress resulted in significant loss of photoreceptor cells in both treatment groups, with the least loss occuring in the animals treated with L-NAME. The damage was most severe in the superior hemisphere. We calculated the average ONL thickness for each animal. In the superior hemisphere the D-NAME group had only 35% of the control ONL thickness, while in the L-NAME treated group this value was as high as 64%. These findings show that L-NAME preserves morphology in the acute light exposure paradigm.

4.1.2. Transgenic rat study

The ERG responses of P23H and S334ter animals were significantly lower than those from the WT controls. The S334ter amplitudes were more severely depressed than those of the P23H rats. Administration of L-NAME had no significant effect on the preservation of retinal function in either type of transgenic rats, compared to the same strain of animals receiving D-NAME. There was a greater loss of photoreceptor cells in the transgenic animals too, compared to the WT, with the greatest loss occuring in

the S334ter group. There was no significant difference in the effects of L-NAME and D-NAME within any of the three groups. Thus, L-NAME, which protects against light damage, does not protect against inherited retinal degeneration in the two groups of rats with rhodopsin mutations.

4.2 Testing the effect of progesterone in light stress

When comparing follow-up ERG data of light-stressed progesterone treated and light-stressed nontreated rats, the difference was not statistically significant (a wave: $p=0.12$, b wave: $p=0.08$). In the light-exposed groups the retinas were severely damaged, the degeneration was the most pronounced in the superior hemisphere. The average ONL thickness and RIS+ROS length for each group was calculated. The difference between the progesterone-treated, exposed and the nontreated, exposed groups' morphologic data was not statistically significant (ONL: $p=0.61$, RIS+ROS: $p=0.51$). These results are in concordance with those of the ERG tests, which collectively show that progesterone is not protective against retinal degeneration caused by excessive light exposure.

4.3 Analysis of the effect of environmental lighting conditions

Exposure to 3000 lux light for 72 hours resulted in a significant damage to the retinas of both light history groups, with the dim-reared group being the most affected. There was modest reduction in ONL thickness and the appearance of shortened rod outer segments in the bright-reared group. Compared to non-exposed bright-reared controls, the average ONL thickness was reduced by 17% ($p<0.0001$) and the average RIS+ROS length was reduced by 28% ($p<0.0001$). Although in the dim-reared animals there was a dramatic loss of photoreceptor cells. Compared to dim-reared controls, there was a 59% reduction ($p<0.0001$) in average ONL thickness and 71% reduction in the average RIS+ROS length ($p<0.0001$). Comparing the two light-stressed groups, in the dim-raised animals, the average ONL thickness was 50% less ($p<0.0001$) and the average RIS+ROS length was 56% less ($p<0.0001$) than that in bright-reared, light-stressed animals.

To provide further data on the number of apoptotic cells in the experimental groups, we performed TUNEL assay. There were no TUNEL-positive cells observed in the retinas of dim- or bright reared control mice. In contrast, TUNEL-positive cells were found in retinas of both groups that were exposed to constant light, with the greatest

number occuring in the dim-reared animals. DNA fragmentation patterns determined by neutral gel electrophoresis confirmed the TUNEL assay findings. DNA from animals raised in dim cyclic light showed greater laddering than DNA from bright cyclic reared mice.

We measured the DHA content of ROS of the four groups of mice. The highest levels of DHA were found in the two control groups and there was no significant difference between the dim-reared (36 mol%) and the bright-reared (35 mol%) animals. Following 72 hours of constant light exposure, the DHA content was significantly reduced in both group compared to controls, with the greatest loss occuring in the dim-reared animals (16 vs. 23 mol% after light stress).

5. DISCUSSION

5.1 Administration of L-NAME

Although the exact molecular steps that lead to photoreceptor cell death still need to be clarified, several hypotheses suggest that excessive light absorbtion induces free radical formation, which in turn causes photoreceptor cell injury. Neuronal NO, one of these free radicals, works normally through influence on the phototransduction cascade by activating guanylate cyclase. In our experimental paradigm, L-NAME, but not its inactive isomer D-NAME, protected rod photoreceptor cells from light damage, but functional protection was not statistically significant. Neither studies published previously used ERG to evaluate a protective effect of L-NAME on retinal function. The explanation of our results could be that since the structural protection was significant only in the superior hemisphere, this area may not be large enough to have an influence on the global response, represented by the ERG response. On the other hand, similarly to our data, several animal models of retinal degeneration given with different growth factors were found to have significant structural rescue, without a significant recovery in ERG response. The underlying mechanism could be a subject of further investigation.

To determine if the neuroprotective effect of L-NAME could be extended to inherited retinal degenerations caused by rhodopsin mutations, we used two transgenic strains (P23H and S334ter). We did not find a significant difference between the L-NAME and D-NAME treated groups in the same transgenic genotype, either functionally or structually. Like L-NAME, PBN was also protective against light stress, but

ineffective in inherited retinal degenerations. This differential drug effect may provide some useful information regarding the mechanism of cell death due to photoreceptor specific mutations. For example, since PBN and L-NAME did not prevent degeneration in P23H and S334ter rats, it is unlikely that free radical mechanisms are involved in their retinal degeneration. The different responses to the drugs in the light damage and mutant paradigms point out a fundamental difference in these two processes. Although apoptosis is the "final common pathway", there is a considerable divergence in the upstream events that lead to cell death. Free radical generation and lipid peroxidation have been implicated in light damage, but do not seem to be involved in inherited retinal degeneration, at least in the two rhodopsin mutant strains we studied.

5.2 Testing the effect of progesterone in light stress

Our data showed that illumination caused statistically significant decrease in ERG amplitudes in both light-damaged groups, although there was no significant difference between progesterone- and vehicle treated animals data. These results are supported by our structural data. The findings detailed above do not support the hypothesis that progesterone administration provides protection against light-induced retinal degeneration in male albino rats. It seems probable that light-induced retinal stress, contrary to other stresses (i.e. brain injury, stroke, epileptic seizures) cannot be ameliorated by progesterone administration. Although in this study progesterone alone was not protective, it is possible that the interaction between the female steroid hormones, progesterone and estrogen is responsible for the neuro- and cardioprotective effects.

5.3 Analysis of the effect of environmental lighting conditions

Structural and biochemical evidence presented in this study show that preconditioning albino mice with bright cyclic light prevents light-induced apoptosis. Albino mice are capable of undergoing adaptive responses to environmental lighting conditions, as other authors have previously demonstrated in albino rats. As in the mice raised from 7 to 35 days of age in bright cyclic light there was no evidence of apoptosis and the mice of the two experimental groups had the same genotype, it seems probable that any differences between dim and bright cyclic reared animals should reflect adaptive responses to their different light history. One of these adaptive mechanisms is

photostasis. This phenomenon means that the retina reacts to high or low photon flux by altering the content of rhodopsin in the ROS, to assure capture of a constant number of photons each day. Although we did not measure rhodopsin levels in our mice, photostasis probably also played a role. There is another way of adaptation, lowering the DHA content in ROS, to reduce the substrate for lipid peroxidation. These changes serve anatomical and biochemical adaptation of retina to bright light stress, to reduce the susceptibility to light-induced apoptosis. The process responds rapidly to changes in environmental light intensity and shows remarkable plasticity. In our study, following light challenge, there was a greater loss of DHA in the dim-reared animals, suggesting that their anti-oxidant defenses were less effective in preventing lipid peroxidation.

A number of endogenous responses to chronic light rearing conditions or to acute light stress have been reported for retinas of mice and rats, and can be roughly divided into three categories. (i) Structural and molecular changes that reduce the efficiency of photon capture and visual transduction, (ii) Up-regulation of endogenous pathways that protect against apoptosis, and (iii) Down-regulation of endogenous pathways that are sensitive to stress-induced apoptosis. Identifying the molecular etiology of these responses and learning how to control their expression could provide a rational basis for treatment of a variety of inherited retinal degenerations. In the study described herein, we establish that the albino mouse provide some distinct advantages over albino rats for these types of investigations.

6. NEW FINDINGS

6.1 NOS inhibitor L-NAME provides pronounced, but statistically non significant functional protection against photoreceptor damage, induced by photostress in wild type albino rats.

6.2 L-NAME is not protective in retinal degenerations caused by rhodopsin mutations in P23H and S334ter transgene albino rats.

6.3 Administration of progesterone does not provide protection against photoreceptor apoptosis evoked by exposition to bright light in male albino rats.

6.4 Albino mice reared in bright cyclic light are protected against photostress-induced retinal degeneration.

7. PRACTICAL UTILIZATION OF THE FINDINGS

Retinitis pigmentosa has a poor prognosis, by the age of 50 years about half of the patients have a visual acuity 0.1 or worse, and a 2-3° central field. There is no effective therapy, so far. Ascorbic acid, DMTU, PBN, Ginko biloba, vitamin E and methylprednisolone provided protection against photostress induced retinal degeneration in animal experiments. In human medicine ascorbic acid, Gingko biloba and vitamin E are used because of their antioxidant effect, but those drugs didn't bring a break-through, either. An ideal drug would block or at least slow down the process of apoptosis, let the patients live longer with a reasonable visual acuity.

At the level of L-NAME used in our study, the degree of protection was not as great as found for PBN, ascorbic acid or DMTU, which may limit its usefulness in future studies, but we got closer in understanding the molecular mechanism of apoptosis, since NO definitely playes a role in it.

Administration of progesterone was the most promising as it has been used in the medical practice for a long time, though for other indications. Unfortunately progesterone proved to be protective only in cerebro- and cardiovascular diseases, but not in retinal degeneration.

By showing that albino mice reared in bright cyclic light have neuroptotection against photostress, new ways of investigation in retinal degeneration were opened up. The mouse genome can easily be manipulated and is better known, thus it provides several advantages over rats.

8. LIST OF PUBLICATIONS AND PRESENTATIONS

Publications included in the thesis

1. Káldi, I., Dittmar, M., Pierce, P., Anderson, R. E.: L-NAME protects against acute light damage in albino rats, but not against retinal degeneration in P23H and S334ter transgenic rats. *Experimental Eye Research* 2003, 76 (4): 453-461. IF: 2.180

2. Káldi, I., Martin, R.E., Huang, H., Brush, R.S., Morrison, K.A., Anderson, R.E.: Bright cyclic rearing protects albino mouse retina against acute light-induced apoptosis. *Molecular Vision* 2003, 9: 337-344. IF: 2.722

3. Káldi, I., Berta, A.: Progesterone administration fails to protect albino male rats against photostress-induced retinal degeneration. *European Journal of Ophtalmology* 2004, 14(4): 306-314.
IF: 0.483

Other publications

1. Káldi I., Török M.: Glare induced poor visual acuity in patients with incipient and progredient cataracts. *Szemészet* 1995. 132: 197-199. IF: -

2. Nagy, E.V., Tóth, J., Káldi, I., Damjanovich, J., Mezôsi, E., Lenkey, A.,Tóth, L., Szabó, J., Karányi,Z., Leövey, A.: Graves' ophthalmopathy: eye muscle involvement in patients with diplopia. *European Journal of Endocrinology* 2000. 142 (6): 591-597.
IF:2.133

Cumulativ impact factor: 7.518
International citation: 16

Presentations

1. Káldi, I., Török, M. Glare induced visual decrease in patients with incipient and progredient cataract *(poster) Xth Congress European Society of Ophthalmology Milano, Italy June* 25-29, 1995.

2. Halász, B., Káldi, I. Ocul-Info ophthalmological patient registry *(oral presentation) Annual Congress of the Hungarian Ophthalmological Society* Pécs, 1997.

3. Káldi, I., Török, M. Evaluation of visual field loss by Humphrey Field Analyser-730 in glaucoma *(oral presentation) Annual Congress of the Hungarian Ophthalmological Society* Kaposvár, 1998.

4. Ranchon, I., Káldi, I., Anderson, R.E. P23H and S334ter rhodopsin transgenic rats are differently susceptible to cyclic or continuous light. Effect of PBN on the degeneration induced by the mutation or light *(poster) Association for Research in Vision and Ophthalmology (ARVO)*, Miami, Fl., USA, 2002.

5. Káldi, I. Anderson, R.E. L-NAME protects against acute light damage in albino rats, but not against retinal degeneration in P23H and S334ter transgenic rats. *(oral presentation) Annual Congress of the Hungarian Society of Free Radical Research* Nagykôrös, Hungary, 2003.