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THE HYPOXIC TRANSCRIPTOME OF THE RETINA: IDENTIFICATION OF FACTORS WITH POTENTIAL NEUROPROTECTIVE ACTIVITY

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1. INTRODUCTION

Most blinding diseases of the retina share a common feature – the loss of photoreceptor cells by apoptosis. Although degenerative diseases like age-related macular degeneration (AMD) and Retinitis Pigmentosa (RP) are among the main causes for severe visual impairment and blindness, no effective therapeutical treatments are available to prevent loss of vision in human patients. Protection of retinal cells against cell death is a promising strategy to develop therapies aiming at the rescue of retinal function. For the successful design of neuroprotective strategies, it is essential to understand the molecular events leading to the degeneration of retinal cells. To study signaling pathways and molecular mechanisms during the degenerative processes, several mouse models of inherited retinal degeneration are used (Fauser et al. 2002). These models are complemented by models of induced photoreceptor apoptosis like the model of light-induced degeneration (Wenzel et al. 2005). The advantage of the induced models is the synchronized apoptotic response of many photoreceptor cells to the death stimulus. This might raise the activation of regulatory factors above detection threshold allowing their detailed investigation.

Recently, we showed that hypoxic preconditioning protects photoreceptor cells against light-induced cell death by preserving retinal function and morphology (Grimm et al. 2002). Similarly, hypoxic pretreatment can also protect other tissues like heart or brain against various toxic insults (Gidday et al. 1994; Emerson et al. 1999; Cai et al. 2003; Dong et al. 2003) suggesting that hypoxic preconditioning might induce a general protective response. Such a response might involve hypoxia-inducible-factor 1a (HIF-1a) which is stabilized in the retina after hypoxic exposure (Grimm et al. 2002). As a

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transcription factor, HIF-1 α (together with HIF-2 α) is a key element of the hypoxic response regulating expression of many target genes. Such genes are involved in different physiological functions like angiogenesis, general metabolism and apoptosis. One target gene of HIF-1 α and/or HIF-2 α has been identified in erythropoietin (Epo). Epo was shown to prevent death of precursor cells of erythrocytes in bone marrow and was thus recognized as a potent anti-apoptotic factor (Koury et al. 2002). Since expression of Epo is strongly induced after hypoxic preconditioning in the retina and Epo receptor expression was localized mainly to photoreceptor and ganglion cells (Bocker-Meffert et al. 2002; Grimm et al. 2002; Grimm et al. 2004), Epo was considered to be a factor protecting the retina against degeneration. Supporting this conclusion, application of recombinant Epo protected photoreceptor cells against light-induced degeneration. However, protection was not as complete as after hypoxic preconditioning suggesting that exposure to reduced oxygen levels may differentially regulate expression of additional genes involved in the protection of photoreceptor cells (Grimm et al. 2002; Grimm et al. 2004).

The goal of the present study was to analyze the retinal response to hypoxia and to identify differentially regulated genes that might be involved in retinal neuroprotection.

2. MATERIAL AND METHODS

2.1. Hypoxic preconditioning and Affymetrix microarrays

Animals were treated in accordance with the regulations of the Veterinary Authority of Zurich and with the statement of 'The Association for Research in Vision and Ophthalmology' for the use of animals in research. BALB/c mice (Harlan, The Netherlands) were exposed to 6% oxygen for 6 h. Retinas were isolated at 0 h, 2 h, 4 h or 16 h after the period of hypoxic preconditioning. Normoxic controls were treated in parallel and collected at the same time points. For each condition three retinas of three different mice were pooled. Total RNA was prepared, processed according to standard procedures and hybridized to Affymetrix GeneChip® Mouse Genome 430 2.0 microarrays. The experiment was conducted three times independently resulting in three microarray replicates per condition and a total number of 9 retinas per condition.

2.2. Quality control and Affymetrix chip analyzes

To analyze the quality of the results after gene chip hybridization the online tool RACE (Remote Analysis Computation for gene Expression data) of the University of Lausanne was used.

To analyze gene expression profiles and to identify differentially regulated genes, we used Genespring 7.0 (Agilent Technologies) software based on the Mas5.0 algorithm. In brief, hypoxic samples (H, hypoxic) were compared to their corresponding normoxic controls (N, normoxic) resulting in 4 different analyzes: N0 vs. H0, N2 vs. H2, N4 vs. H4 and N16 vs. H16. In the first step, genes were tested for their presence or absence in the samples. Genes were considered to be expressed and allowed to pass the filter only when all 3 replicates of a particular condition (normoxic, hypoxic) got a present call. Genes that passed the first filter were then analyzed and filtered according to their fold change (at least factor 2) and to their p-value (≤ 0.05). Additionally, a statistical correction

according to Benjamini/Hochberg (Benjamini and Hochberg 1995) was applied. To sort differentially regulated genes according to their biological function the online tool Onto-Express from Intelligent Systems and Bioinformatics Laboratory (Draghici et al. 2003a; Draghici et al. 2003b; Draghici et al. 2003c) was used (<http://vortex.cs.wayne.edu/projects.htm>).

2.3. Real-time PCR

Gene regulation was confirmed by real-time PCR on cDNA prepared from total retinal RNA, using a LightCycler 480 instrument (Roche Diagnostics AG, Rotkreuz, Switzerland) and LightCycler 480 SYBR Green I Master Mix (Roche Diagnostics AG). Primers were designed using the Universal ProbeLibrary Assay Design Centre (Roche Diagnostics AG) <https://www.roche-applied-science.com>. Relative expression was calculated according to the expression of b-actin using the LightCycler software package.

2.4. Light exposure and quantification of cell death

p21^{-/-} mice on a mixed Bl/6;129S2 background were purchased from Jackson Laboratory (Bar Harbor, USA). Mice homozygous for the light sensitive Rpe65^{450Leu} variant (Wenzel et al. 2001; Samardzija et al. 2006) were used to establish the breeding colony. After hypoxic preconditioning (see 2.1.) mice were reoxygenated in darkness (dark adaptation) for 4 hours prior to illumination. 45 minutes prior to illumination, pupils were dilated in dim red light using 1% Cyclogyl (Alcon, Cham, Switzerland) and 5% Phenylephrine (Ciba Vision, Niederwangen, Switzerland). Mice were exposed to 13'000 lux of white fluorescent light for 2 hours.

36 hours after light exposure apoptotic cell death was quantified in isolated retinas using a sandwich ELISA system (Cell Death Detection ElisaPlus, Roche Diagnostics, Basel, Switzerland, used according to the manufacturer's recommendation) to determine the relative amount of free nucleosomes in the cytosolic fraction of isolated retinas.

2.5. Morphology

Eyes were removed and fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3 at 4°C overnight. For each eye, the superior central and the inferior central retina adjacent to the optic nerve were trimmed and embedded in Epon 812. Sections (0.5 µm) were prepared from the inferior central retina, stained with toluidine blue and analyzed using an Axioplan microscope (Zeiss, Oberkochen, Germany).

3. RESULTS

3.1. Statistical Analyzes

Hypoxic preconditioning was shown to completely protect photoreceptor cells against light-induced cell death when the light stimulus was given after a reoxygenation period of 4 h (Grimm et al. 2002). In contrast, mice exposed to light at 16 h after hypoxic preconditioning were not protected suggesting that the protective effect of hypoxia is transient and short-lived. Based on these findings we decided to analyze the retinal transcriptome at 4 different time points after hypoxic preconditioning. The first three time points were within the frame of full neuroprotection (0 h, +2 h, +4 h) whereas the last

Table 1. Total number of genes differentially regulated at 0 h, 2 h, 4 h and 16 h after hypoxia.

| Condition | Altered Genes with Benjamini/Hochberg | False positive | Altered Genes without Benjamini/Hochberg | False positive |
|-------------|---------------------------------------|----------------|--|----------------|
| H0 vs. N0 | 83 | 4 | 772 | 96 |
| H2 vs. N2 | 7 | - | 189 | 24 |
| H4 vs. N4 | 0 | - | 74 | 9 |
| H16 vs. N16 | 0 | - | 24 | 3 |

Shown are the numbers of genes passing the stringent Benjamini/Hochberg correction in comparison to a normal t-test. The columns 'false positive' show the expected number of genes which have passed the respective test but which are nevertheless not differentially regulated. H0, H2, H4, H16: retinas at 0h, 2h, 4h and 16h, respectively, after hypoxic preconditioning. N0, N2, N4, N16: control normoxic retinas.

time point was taken after neuroprotection has ceased (+16 h). Quality controls were used to confirm the successful performance of the experiment. Analyzes, which compare the global similarity of all gene chips indicated that hypoxic replicates were very similar within their groups and highly different to their normoxic controls (data not shown).

Genespring 7.0 software was used to detect genes that were differentially regulated by hypoxic exposure. In this first statistical analysis without mathematical correction 772 genes appeared to be differentially regulated immediately after hypoxia. This number decreased during reoxygenation to 189 genes after 2 h, 74 genes after 4 h and 24 genes 16 h after hypoxia (Table 1). After application of the highly stringent statistical method of Benjamini/Hochberg (Benjamini and Hochberg 1995), we identified a total of 83 genes as significantly regulated immediately after hypoxic preconditioning. Already at 2 h after hypoxia, only 7 genes remained to have significantly different expression levels and at 4 h and 16 h after hypoxia the retinal transcriptome of preconditioned mice was indistinguishable from normoxic controls (Table 1). Both statistical methods indicated that differential regulation of gene expression is strongest immediately after hypoxia. During reoxygenation, the retinal transcriptome quickly returned to the normoxic gene expression pattern suggesting a short-lived molecular effect of hypoxic preconditioning.

3.2. Biological classification

It is eminent to sort differentially regulated genes according to their biological relevance. Using the statistical online tool "Onto express" we classified the identified genes with respect to their various biological functions like apoptosis, transport, cell cycle and others (Fig. 1). The highest number of regulated genes was sorted into the group of transport-related genes followed by apoptosis-related processes. This may be of high relevance since hypoxic preconditioning induces a neuroprotective response in the retina, which most likely involves a number of genes related to the control of apoptotic events. Having in mind, that genes can be present in more than one biological group, the number of sorted genes does not reflect the real number of genes identified as differentially regulated. Individual genes of the group of 'anti-apoptotic' or the group of 'induction of apoptosis' were also found in the group 'apoptosis'. Table 2 summarizes differentially regulated genes that are considered anti-apoptotic. Note that some of these genes are repressed by the hypoxic pretreatment. Table 3 shows the most prominently regulated pro-apoptotic genes. In total, 13 genes with a known anti-apoptotic function

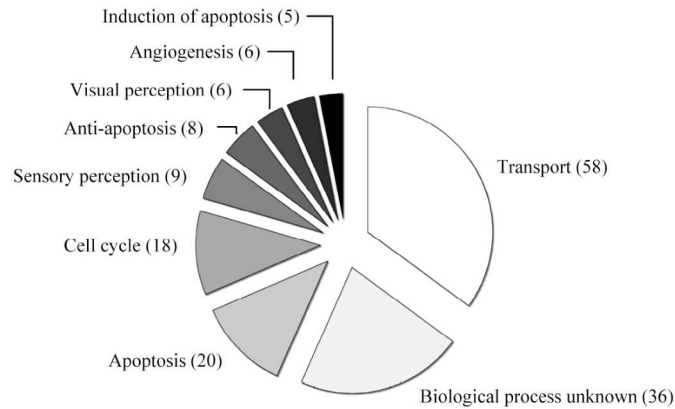


Figure 1. Classification of differentially expressed genes according to their biological function. Genes regulated immediately after hypoxia (0 h) without statistical correction were classified using Onto-Express. Only most prominent and statistically significant ($p \leq 0.05$) groups are shown. Numbers of genes in each group are given in brackets.

Table 2. Differentially regulated genes with anti-apoptotic functions.

| Gene | FC 0 h | FC 2 h | FC 4 h | FC 16 h |
|----------------------------|--------|--------|--------|---------|
| p21 | 34.4 | - | - | - |
| Bcl2-like 10 | 6.3 | - | - | - |
| Snai2 | - | 3.1 | - | - |
| Vegfa | 2.6 | - | - | - |
| Bhlhb4 | 2.3 | - | - | - |
| Bfar | 2.2 | - | - | - |
| Camk1d | - | 2.2 | 2.0 | - |
| Birc6 | - | 2.1 | - | - |
| C/EBP | 2.0 | 2.3 | - | - |
| p21-activated kinase 7 | 0.46 | - | - | - |
| Apoptosis inhibitor 5 | 0.34 | - | - | - |
| Glutaminyl-tRNA-Synthetase | 0.32 | - | - | - |
| Birc4 | 0.22 | - | - | - |

Shown are the gene-chip deduced fold-changes (FC) at 0h, 2h, 4h and 16h after hypoxic preconditioning. p21 is the most prominently up-regulated gene within this group. -, not detected

Table 3. Differentially regulated genes with pro-apoptotic functions.

| Gene | FC 0 h | FC 2 h | FC 4 h | FC 16 h |
|----------------------|--------|--------|--------|---------|
| Pmaip1 | 12.9 | - | - | - |
| Sh3glb1 (endophilin) | 5 | 4.7 | - | - |
| Bnip3 | 2.3 | - | - | - |
| BCL2-like 11 | 2.2 | - | - | - |
| Foxo3a | 2.1 | - | - | - |

Shown are the gene-chip deduced fold-changes (FC) at 0h, 2h, 4h and 16h after hypoxic preconditioning. -, not detected

were differentially regulated by hypoxia. Nine genes were induced whereas 4 genes were repressed (Table 2). Test of VEGF, p21 and Bcl2110 gene expression by real-time PCR confirmed the chip data and their up-regulation in response to the hypoxic pretreatment (Fig. 2). In addition to the anti-apoptotic genes, expression of 5 genes expected to be pro-apoptotic was induced during hypoxia (Table 3).

Having both, pro- and anti-apoptotic genes regulated by hypoxia suggests that the preconditioning protocol induces a complex response priming the tissue to resist a toxic insult but also preparing the cells to induce apoptotic cell death if needed. We hypothesize that reducing the number of cells may be required in case of prolonged hypoxic exposure to ensure that at least some cells can be supplied with sufficient oxygen to function.

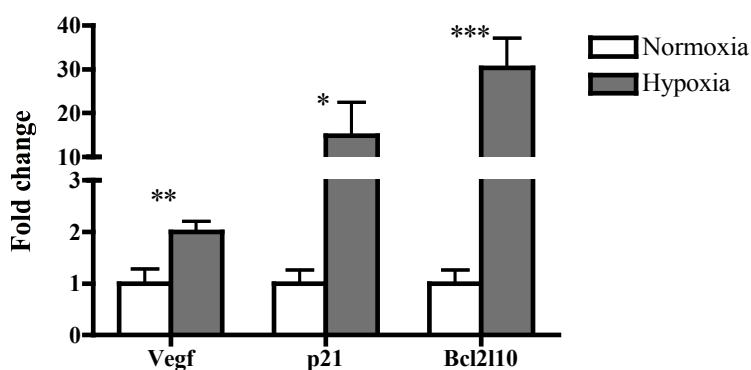


Figure 2. Expression of apoptosis-related genes immediately after hypoxic preconditioning relative to normoxic controls as determined by real-time PCR. Expression of Vegf, p21 and Bcl2110 (as indicated) was determined in three normoxic and three hypoxic retinas. Amplifications were done in duplicates. Normoxic samples were set to 1 and hypoxic samples represent the relative fold change due to hypoxia.

3.3. The neuroprotective impact of p21

One of the most prominently induced genes with reported anti-apoptotic properties was p21, a cyclin-dependent kinase inhibitor. To analyze its role in neuroprotection by hypoxic preconditioning, p21 knockout mice were exposed to hypoxia, reoxygenated for 4 hours and exposed to 2 hours of 13,000 lux of white light. As controls, p21^{-/-} mice were kept in normoxic conditions before light exposure. Light-induced photoreceptor apoptosis was analyzed 36 hours after exposure by light microscopy (Fig. 3A) and by the semi-quantitative determination of free nucleosomes (Fig. 3B). In contrast to normoxic control mice, hypoxic preconditioned p21 knockout animals did not produce a significant amount of free nucleosomes after light-exposure suggesting that lack of p21 did not interfere with the neuroprotection by hypoxic preconditioning. These findings were confirmed by the morphological analysis of retinal tissue after light exposure (Fig. 3A). In contrast to normoxic controls where light exposure induced the formation of pyknotic nuclei and a severe disruption of rod inner and outer segments, retinal morphology of preconditioned knockout mice was completely protected.

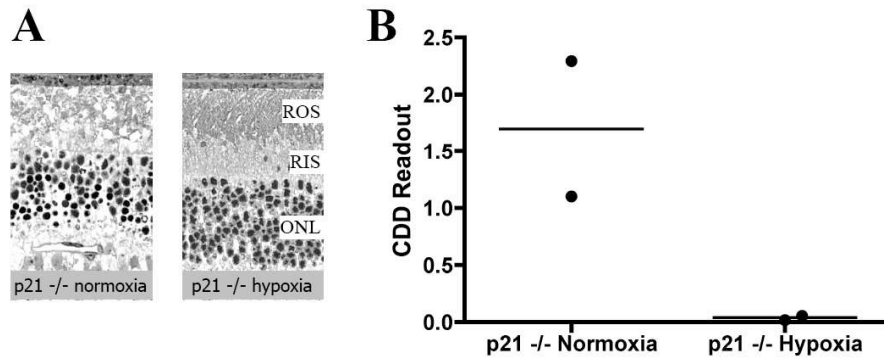


Figure 3. Analysis of the role of p21 in retinal neuroprotection by hypoxic preconditioning. p21^{-/-} mice were either kept in normoxia or were preconditioned with 6% O₂ for 6 hours before light exposure. A) Retinal morphology was analyzed 36 hours after exposure. Normoxic mice (left panel) showed strong signs of degeneration with many pyknotic photoreceptor nuclei and severe destruction of inner and outer segments. Retinas of hypoxic preconditioned mice (right panel) were completely protected against light-induced degeneration. B) Cell death detection (CDD) assay to analyze the amount of free nucleosomes in the cytoplasmic fraction 36 h after light exposure. Normoxic mice showed a high content of free nucleosomes whereas hypoxic preconditioned mice showed no detectable release of nucleosomes. Shown are data points of two individual mice (dots) with the respective average (line). ROS: rod outer segments; RIS: rod inner segments; ONL: outer nuclear layer.

4. DISCUSSION

Photoreceptor cells are among the cells with the highest need for oxygen. Highest oxygen concentrations are measured at the level of the outer segments which are closest to the choroidal blood vessels. With increasing distance from the RPE and the choroidea, oxygen concentrations drop rapidly and photoreceptor cells can experience borderline hypoxic conditions at night, the time of highest energy consumption (Yu and Cringle 2005). To react and/or adapt to such conditions, the retina needs a system that can sense oxygen levels and induce appropriate endogenous mechanisms in response to varying conditions. In a normal physiological situation, such a response may include an adaptation of the metabolism reducing or increasing the consumption of energy. In harsher conditions, the retina may prepare the cells to survive by the induction of an endogenous neuroprotective response. If unfavorable conditions persist, the sacrifice of some individual cells may be required in order to safe the function of the retinal tissue as a whole. Understanding the retinal response to hypoxia may lead to the identification of potent endogenous neuroprotective mechanisms. Artificial and controlled stimulation of such mechanisms may be beneficial for human patients suffering from loss of vision due to retinal degeneration. Furthermore, hypoxia is one of the driving forces for neovascularization, a major complication of many retinal diseases including diabetic retinopathy and AMD (Zhang and Ma 2007). Identification of the molecular mechanisms controlling the hypoxia-related production of angiogenic factors like VEGF may lead to the development of new and efficient treatments for these diseases.

Recently we showed that a transient period of strong hypoxia induces a molecular response in the retina which protects photoreceptors from the otherwise deleterious effect of light exposure (Grimm et al. 2002). A central regulator of this response might be the transcription factor HIF-1a which is stabilized and therefore activated in hypoxic conditions in several tissues including the retina (Grimm et al. 2002; Maxwell 2003; Sharp and Bernaudin 2004). However, since HIF-1a and other transcription factors modulate the expression of a large amount of genes (Ke and Costa 2006), the molecular mechanisms involved in the tissue response to hypoxia are still poorly understood and the molecular basis for hypoxia-induced neuroprotection remains unclear. Our study was therefore designed to increase our knowledge of hypoxia-dependent gene regulation and neuroprotection by analyzing the retinal transcriptome after hypoxic preconditioning.

The highest number of differentially regulated genes was found immediately after hypoxia. At this time, HIF-1a is present at high levels suggesting that HIF-1a may indeed be one of the major factors controlling the hypoxic response in the retina. This is further supported by the rapid decline of HIF-1a levels (Grimm et al. 2002) and of differentially expressed genes during reoxygenation.

The most prominent functional group affected by hypoxia was “transport” which includes genes of general metabolic activity like Slc37a4 (glucose-6-phosphate transporter) or FABP4 (fatty acid binding protein 4). This may reflect an adaptational response of the retina, which may normally occur to a rather mild change in oxygen levels. Despite the strong neuroprotective capacity of hypoxic preconditioning, we also detected a number of differentially regulated genes that are pro-apoptotic and may therefore promote cell death. As discussed above, these factors may be produced to reduce the number of oxygen-consuming cells if low levels of oxygen persist. This might secure sufficient oxygen supply and therefore energy for a smaller cell population which may thus survive and function even in unfavorable conditions. Although this may reduce the general sensitivity of the retina, it may nevertheless rescue some (reduced) function. It might be interesting to analyze whether less severe hypoxic conditions would increase the differential regulation of genes involved in general metabolism and decrease the number of pro-apoptotic genes.

Our focus, however, is the elucidation of the neuroprotective mechanisms in response to a harsh but transient hypoxic period. Therefore, we concentrated during our initial analysis on genes which might be involved in an anti-apoptotic response of the retina. Because p21 was one of the most prominently induced genes with known anti-apoptotic functions (Zaman et al. 1999; Mahyar-Roemer and Roemer 2001), we focused on its potential role in neuroprotection after hypoxic preconditioning. We hypothesized that lack of p21 would reduce the neuroprotective effect if the gene was indeed involved in the protective response. However, mice with a functional knockout of p21 were highly protected against a toxic light insult when preconditioned with hypoxia. This suggests that this Cyclin dependent kinase inhibitor is at least not a major contributor to the neuroprotective response induced by hypoxic preconditioning.

We will continue the analysis of individual genes identified in our screen. Future studies will focus on genes like Bcl2l10 or C/EBP which were up-regulated during hypoxia and which are known to be anti-apoptotic (Song et al. 1999; Naumann et al. 2001; Buck and Chojkier 2003). Additionally, strongly up-regulated genes (not shown) like Paraoxonase 1, which is involved in oxidative stress response and lipid oxidation (Aviram et al. 1998) or Adrenomedullin, which was reported to be neuroprotective in

ischemia models (Miyashita et al. 2006) will be studied for their relevance to retinal neuroprotection after hypoxic preconditioning.

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