Original Paper

The Neuroprotective Potential of Rho-Kinase Inhibition in Promoting Cell Survival and Reducing Reactive Gliosis in Response to Hypoxia in Isolated Bovine Retina

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Key Words

Electroretinogram • Neuroprotection • H-1152P • Astrocyte • Müller cell • Microglia • Cathepsin-B

Abstract

Aims: To investigate the outcomes of Rho-kinase inhibition in the electrophysiological *ex vivo* model of the isolated perfused vertebrate retina under hypoxia. **Methods:** Bovine retinas were perfused with an oxygen saturated nutrient solution with or without the Rho-kinase inhibitor H-1152P. The retinas were stimulated repeatedly until stable amplitudes were reached and the electroretinogram was recorded at five minute intervals. Hypoxia was induced for 15, 30, and 45 minutes, after which the oxygen saturation was restored. The extent of the cell damage and glial reactivity was determined by Ethidium homodimer-1 staining, immunohistochemistry, and Western blot. **Results:** Hypoxia caused a time-dependent reduction of the b-wave amplitudes, which could not be prevented by the H-1152P. Although the Rho-kinase inhibitor maintained higher b-wave amplitudes, these effects did not reach statistical significance. Hypoxia also resulted in an increase in cell damage and the activation of the glial cells in the untreated retinas whereas the administration of H-1152P significantly reduced the extent of these events. **Conclusion:** H-1152P exerted a neuroprotective effect against necrosis on the isolated bovine retina under hypoxia together with a reduction in glial cell reactivity. However, the inhibitor could not prevent the hypoxia induced retinal dysfunction possibly due to the interference with synaptic modulation.

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Introduction

The retina is one of the most metabolically active tissues in the body, consuming large quantities of energy to convert the light signals into a visual information conveyed to the brain [1, 2]. The high-energy demand of particularly the photoreceptors, which respond to the light and the retinal ganglion cells, the axons of which constitute the optic nerve that transmits the visual output to the brain, is fulfilled mainly by the oxygen-dependent generation of adenosine triphosphate (ATP) [3, 4]. This in turn necessitates a constant blood flow through the retinal vasculature and choroid, with the former providing oxygen and nutrients to the ganglion cells and the inner retinal neurons and the latter supporting the photoreceptors in the outer retina [2]. However, the impairment of vascular integrity or haemodynamics can result in the reduction of the oxygen tension below the levels normally present in a cell, tissue, or organism and lead to the state defined as hypoxia. The insufficient oxygen supply would interfere with the metabolic events required for maintaining the homeostasis in the retina and can therefore compromise the cell survival and function particularly after longer durations [2, 5-8]. Hypoxia is indeed associated with a variety of pathological conditions in the eye, such as glaucoma [9, 10], diabetic retinopathy [11], choroidal neovascularisation, and age-related macular degeneration [12, 13], which may severely and irreversibly impair the vision. The development of strategies aimed at preventing the death of neurons and the restoration of the normal function under such unfavourable conditions therefore gains an increasing concern. Although a growing number of neuroprotective agents have yielded positive results in preclinical studies, only a few drugs were examined in human clinical trials [14]. Yet, no clinical trial has shown neuroprotective efficacy in the ocular diseases until now [15], which necessitates the identification of novel cellular targets central to the pathological processes induced by hypoxia in the retina.

Rho-kinase (ROCK1 and ROCK2) is an intracellular serine/threonine kinase that functions as a downstream effector of the RhoA-GTPase, and regulates mainly the assembly of the actin cytoskeleton, which underlies a wide range of cellular responses to the extracellular cues, such as contraction, migration, proliferation, and gene expression [16, 17]. Rho-kinase is best characterized for its role in promoting the contractility of the smooth muscle cells by favouring the phosphorylation of myosin and the formation of the actin-myosin bundles that provide the cells with contractile strength [18]. Accordingly, the suppression of Rho-kinase activity using pharmacological inhibitors like Fasudil and Y-27632 effectively promoted the vasodilation in numerous experimental models [19-21], and the clinical use of the former inhibitor yielded promising results for the treatment of several cardiovascular disorders and subarachnoid haemorrhage in humans [22, 23]. Recent studies also demonstrated an improvement in ocular blood flow at the optic nerve head in response to different Rho-kinase inhibitors [24, 25]. In addition, the selective inhibition of Rho-kinase allowed for the regeneration of the retinal ganglion cell axons in numerous models of optic nerve injury [26-28] and prevented the retraction of the photoreceptor axons in an *in vitro* model of acute retinal detachment [29]. Moreover, an increase in retinal cell survival in response to the Rho-kinase inhibition has recently been demonstrated under different injury paradigms in the retina including NMDA-induced neurotoxicity, serum deprivation, optic nerve crush, and transient retinal ischemia [28, 30-34]. These findings altogether highlight the involvement of Rho-kinase at a convergence point of diverse pathological events that can be associated with hypoxia. The selective inhibition of Rho-kinase therefore appears as a very efficient strategy to concurrently achieve the multiple aspects of neuroprotection in the retina, such as the prevention of neurite retraction, promotion of neuronal survival, suppression of excessive glial reactivity which might otherwise result in detrimental effects on the rescued neurons [31], and the maintenance of the retinal blood flow. However, the outcomes of Rho-kinase inhibition on retinal function under hypoxia have not been elucidated yet.

The synaptic activity within the retina can be evaluated continuously by recording the electroretinogram (ERG) [6]. The b-wave is the ERG-component, which represents the post-photoreceptor activity in the retina and was found to be having the highest degree

of susceptibility to ischemia/hypoxia. Consistently, the recovery of the b-wave depends on the severity of the experimental model and the duration of hypoxia/ischemia. The reduction in the amplitude of the b-wave is therefore regarded as a sensitive indicator of reduced oxygen delivery in humans as well as the *in vivo* and *in vitro* models [6, 35, 36]. In our study, we evaluated the outcomes of Rho-kinase inhibition in an *ex-vivo* model of retinal hypoxia, using H-1152P to modulate the Rho-kinase activity. H-1152P is a dimethylated derivative of fasudil and has the highest degree of specificity among the currently available pharmacological Rho-kinase inhibitors [37]. The *ex-vivo* effects of Rho-kinase inhibition on the retinal function were determined by monitoring the b-wave amplitudes in isolated and perfused bovine retinas during the hypoxia and reperfusion intervals. Moreover, the retinas were subsequently analysed for the extent of cell survival and glial reactivity to evaluate the outcomes of Rho-kinase inhibition on the cellular responses to hypoxia.

Materials and Methods

Chemicals and reagents

The analysis grade chemicals used for the preparation of the perfusion medium were purchased from Merck (Darmstadt, Germany). H-1152P was purchased from Calbiochem (Merck) and reconstituted in sterile tri-distilled water. The antibodies and the fluorescent reagents used were as follows: Rabbit anti-Cathepsin-B (Acris Antibodies, Herford, Germany), mouse anti-CD11b (Serotec, Düsseldorf, Germany), rabbit anti-cleaved caspase-3 (Cell Signalling Technology, Danvers, MA), rabbit anti-GFAP (DAKO, Hamburg, Germany), mouse anti-Vimentin (Sigma-Aldrich, Munich, Germany), Cy3-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, Dianova, Hamburg, Germany), Alexa 546-conjugated anti-mouse IgG (Molecular Probes, Invitrogen, Darmstadt, Germany), horseradish peroxidase-conjugated secondary anti-rabbit or anti-mouse antibodies (Jackson ImmunoResearch). The protease and phosphatase inhibitor cocktails were purchased from Roche Applied Sciences (Mannheim, Germany). All the remaining chemicals were obtained from Sigma-Aldrich.

ERG of isolated perfused bovine retina

Bovine eyes were obtained from a local slaughterhouse and harvested immediately post-mortem. They were transported in darkness in a serum-free standard medium containing 120 mM NaCl, 2 mM KCl, 0.1 mM MgCl₂, 0.15 mM CaCl₂, 1.5 mM NaH₂PO₄, 13.5 mM Na₂HPO₄, and 5 mM glucose. Our study was in accordance with guidelines from the public health department. Preparations were performed as described previously [38].

Each ERG was recorded in the surrounding nutrient medium via two silver/silver-chloride electrodes on either side of the retina. The recording chamber, containing a piece of retina, was placed in an electrical and optical insulated box. Perfusion velocity was controlled by a roller pump and set to 1 ml/min.

Temperature was kept constant at 30°C. The perfusing medium was pre-equilibrated and saturated with oxygen and could be monitored by a Clark oxygen electrode to demonstrate the hypoxic condition of the nutrient solution. Retinas were dark-adapted, and ERGs were elicited at 5-min intervals using a single white flash for stimulation. The flash intensity was set to 6.3 mlx at the retinal surface using calibrated neutral density filters. The duration of light stimulation (500 ms) was controlled by a timer. ERGs were filtered and amplified (100-Hz high-pass filter, 50-Hz notch filter, 100,000× amplification) using a Grass CP 511 amplifier. Data were processed and converted using an analog-to-digital data acquisition board (NI USB-6221; National Instruments, Austin, TX, USA) and a personal computer. The ERGs were recorded and analysed by DASYLab Professional Version 10.0.0 (National Instruments, Austin, TX, USA).

Retinas were superfused with serum-free nutrient solution and stimulated repeatedly until stable amplitudes were recorded. The oxygen supply into the nutrient solution was stopped for 15 min, 30 min or 45 min and nitrogen was added to the nutrient solution. Meanwhile, the responses were recorded. Thereafter, perfusion with the standard solution was resumed at least for 75 min to monitor b-wave recovery during washout. The b-wave amplitude was measured from the trough of the a-wave to the peak of the b-wave.

To determine the neuroprotective effect of H-1152P we performed the same experimental setup, in which we applied the inhibitor at the concentrations of 1 or 5 μM to the nutrient solution, which was used

for the perfusion of the retinas during the entire measurement period. We studied the effects of H-1152P on ERG parameters varying the time of hypoxia as described before. Recovery of the ERG was followed up for at least 75 min during the washout period. A new retina was used for each experiment with n=5 retinas per group except for the 5 μ M H-1152P and the 45-minute hypoxia groups, for which n=4 retinas were used.

Ethidium homodimer-1 staining

Following the ERG-measurements, the retinas were mounted onto cellulose nitrate filters (Sartorius, Göttingen, Germany, 0.45 μ m pore size, pre-soaked in 0.01M phosphate buffered saline (PBS) and cut into approximately 1x1 cm pieces) with the ganglion cell layer exposed. The retinas were incubated for 30 minutes in 4 μ M Ethidium homodimer-1 (EthD-1) (Molecular Probes) diluted in 0.1% glucose-PBS, rinsed briefly with 0.1% glucose-PBS twice, fixed with 2% paraformaldehyde (PFA)-PBS for 15 minutes followed by 4% PFA-PBS for 15 minutes. After three rinses in PBS for 5 minutes, the retinas were permeabilized in 0.1% Triton X-100/PBS for 10 minutes and counterstained with DAPI (1 μ g/ml in PBS) for 10 minutes to detect the nuclear morphology. The retinas were rinsed twice in PBS, mounted in Mowiol, and analysed by fluorescence microscopy (Leica, Wetzlar, Germany). Quantification of the stained cells was performed in 8-10 areas of 0.16 mm² per retina using the Image J software (National Institutes of Health, Bethesda, Maryland, USA, <http://imagej.nih.gov/ij/>, 1997-2012) Image J. Data are presented as the mean $\pm$ standard error of mean (SEM).

Immunohistochemistry on paraffin sections

The retinas were fixed in formalin, embedded in paraffin, sectioned at 6 μ m on a microtome (Leica, Model RM2125RT) and mounted on Fisherbrand SuperFrost Plus glass slides (Fisher Scientific, Houston, TX). After deparaffinization in xylol for three times for 5 minutes and rehydration in a graded series of alcohol descending from 100% to 50%, the slides were rinsed with PBS and blocked with PBS containing 3% BSA and 0.3% Triton X-100 (BSA-PBST) for 30 minutes at room temperature (RT). The sections were incubated with the primary antibodies diluted in the blocking buffer overnight at 4 °C. Negative controls were incubated in the blocking buffer alone. The sections were rinsed in PBS and incubated with the fluorescent-secondary antibodies. The nuclei were counterstained with DAPI (1 μ g/ml in PBS) for 5 minutes and the slides mounted with Mowiol were analysed by fluorescence microscopy (Leica).

Immunohistochemistry on flat-mounted retinas

For the immunohistochemical detection of microglial reactivity in the nerve fiber layer of flat-mounted retinas, the protocol of Wang et al. [39] was performed with slight modifications. Briefly, the flat-mounted retinas were fixed in paraformaldehyde as above and washed three times for 1 h in PBS containing 0.2% Triton X-100. After blocking in 3% BSA-PBST overnight at 4°C, the retinas were incubated with the antibodies against CD11b diluted 1/5 in the blocking buffer for 48 h at 4°C whereas the negative controls were incubated with the blocking buffer alone. Retinas were washed in PBS three times for 1 h each and incubated with the secondary antibodies indicated above overnight at 4°C. The nuclei were counterstained with DAPI for 10 minutes and the images were acquired under 400x magnification using a fluorescence microscope (Leica).

Quantification of microglia reactivity

The morphological quantification of microglia on the images of the flat-mounted retinas was performed by calculating two different form factors as described [40]. Briefly, the form factor 1 (FF1) is derived from the following equation:

$$FF1 = \frac{4\pi \times \text{cell area}}{(\text{cell perimeter})^2}$$

The FF1 ratio yields values between 0 and 1, acquiring the latter value for a perfectly round (ameboid) cell and decreasing as the number and/or length of the ramifications, hence the cell perimeter increases. The FF1 is therefore a sensitive indicator of initial ramification, acquiring lower values with the presence of even very short cytoplasmic processes. However, it does not always reflect the true degree of microglia reactivity, since an oval shaped cell without ramifications also gets a considerably lower FF1 value compared

to the round, reactive microglia [40]. For this purpose, we also quantified the microglia reactivity in terms of the FF2, which was calculated from the following ratio:

$$\text{FF2} = \frac{\text{cell area}}{\text{convex area}}$$

The convex area is the area of a polygonal object defined by the cell's most prominent and extended projections. For an ameboid (reactive) cell, both the normal and the convex areas would have similar values and the FF2 approaches 1, whereas the long ramifications would significantly increase the convex area, resulting in the reduction of the FF2 ratio [40]. The FF2 values therefore serve as a more specific indicator for the true, long ramifications. Quantification of the microglia area, perimeter, and the convex area was performed individually for each cell using the Image J software on a minimum number of $n=16$ cells/retina that were entirely localized within the boundaries of an image. The FF1 and FF2 ratios were calculated as above individually for each cell and denoted as percentage. For each retina, the mean FF1 and FF2 values of the quantified cells were calculated. For each treatment group, the mean $\pm$ SEM of the FF1 and FF2 values were calculated from the individual mean values obtained from $n=3$ independently processed retinas per group.

Immunoblotting

The retinas were rinsed briefly in PBS and homogenized in ice-cold tissue lysis buffer (50 mM Tris-HCl, pH 7.4, 2 mM MgCl_2 , 1% NP-40, 10% glycerol, 100 mM NaCl, and 1% protease and phosphatase inhibitor cocktails added just before use) using 40 μl buffer per retina. The total lysates were cleared by centrifugation at 12000g for 20 minutes. The protein concentration of the supernatant was determined using the BCA assay (Pierce, Rockford, IL) according to the manufacturer's instructions. Five μg of protein was run in 5-12% denaturing and non-reducing gels at a constant voltage of 120 V for 1-1.5 hours and transferred onto nitrocellulose membranes using a semi-dry blotting apparatus (Biotec-Fischer, Reiskirchen, Germany) at 25 V for 30 minutes. Ponceau S staining was carried out to verify equal protein loading. Immunoblotting and signal detection by enhanced chemiluminescence were performed as described previously [31], by incubating the membranes with the primary antibodies against cathepsin-B (1:1000), GFAP (1:2000), or vimentin (1:1500) overnight at 4°C followed by horseradish peroxidase-conjugated secondary anti-rabbit or anti-mouse antibodies (1:2500) for 1 h at RT.

Experimental layout and statistical analysis

The experimental layout results in data from 8 experimental groups. There are three different hypoxia times (15, 30 and 45 minutes) and three different concentrations for the Rho-kinase inhibitor H-1152P: 0 μM (control group), 1 μM , and 5 μM . However, in the 15 minutes hypoxia group, the high dose of H-1152P (5 μM) was not applied. Within each group measurements were taken every 5 minutes from 5 (start of experiment) up to 150 minutes. The first six measurements before hypoxia were used as baseline measurements, followed by the group specific time of hypoxia and by a follow-up period of 105 minutes in the 15 minutes hypoxia group or 75 minutes in the 45 minutes hypoxia group.

For statistical analysis we used the last baseline measurement (30 minutes), last measurement under hypoxia (45, 60 or 75 minutes) and the maximal comparable follow-up time (120 minutes for 15 minutes hypoxia, 135 minutes for 30 minutes hypoxia and 150 minutes for 45 minutes hypoxia). We fitted two ANOVA models to the data. Our first model (Model 1) involves the dose 1 μM and control only. Hereby we assume unstructured covariance and the exploratory between grouping factors substance (control, 1 μM) and hypoxia duration (15, 30 and 45 minutes) and the within grouping factor hypoxia-status (before, at the end of hypoxia, at the end of recovery). The second model (Model 2) is used to analyse the high dose effect, so we assume an AR(1) covariance structure and use the model building between grouping factors substance (control, 1 μM , 5 μM) and hypoxia duration (30 and 45 minutes) and the within grouping factor hypoxia-status (before, at the end of hypoxia, at the end of recovery). During fitting we performed a model diagnostic as well. To describe the data, we calculated the percent changes of the b-wave amplitudes after hypoxia or after recovery respectively from baseline. Differences between the b-wave after recovery as well as between the post-hypoxia and baseline or between the experimental series at the different concentrations and the control were analysed by linear contrasts. The statistical significance of the damaged cells in the ganglion cell layer was analysed by two factor main effects ANOVA models followed by linear contrasts on main

Fig. 1. Effects of hypoxia and H-1152P on the course of the electroretinogram. The figures indicate the course of the b-wave amplitude before and after hypoxia. The dashed lines mark the experiments with continuous application of H-1152P (1 μ M and 5 μ M, green and blue, respectively) compared to the control (continuous line, red). The grey background marks the duration of hypoxia (A: 15 minutes; B: 30 minutes; C: 45 minutes). Average of representative series. The standard deviation is given for each mean value.

effects. The statistical significance of microglia reactivity was analysed by the two-tailed t-test, assuming equal variance between the samples. Statistical analysis was done using the SAS System 9.2 for Windows 7 (X64VSPRO, SAS Institute, Cary, NC, USA). P-values ≤ 0.05 were assessed as significant.

Results

Functional outcomes of hypoxia for different durations

The perfusion of the isolated bovine retina was performed under stable environmental conditions. Osmotic pressure, temperature, and pO_2 remained unchanged during perfusion.

In our experimental setting stable ERG-amplitudes were reached within two hours perfusion and the superfused retinal preparations responded constantly to light stimulation for more than ten hours [38].

Our first model shows that the b-wave varies significantly between baseline, under hypoxia and at the end of recovery ($F=21.23$, ndf 2, ddf 24, $p<0.0001$, Fig. 1A-C). Furthermore, the mean b-wave changes significantly depending on the duration of hypoxia ($F=10.98$, ndf

2, ddf 24, $p=0.0004$, Fig. 1A-C) and these two factors interact significantly with each other ($F=4.25$, ndf 4, ddf 24, $p=0.0097$, Fig. 1A-C). We observed no significant difference in the course of these events in response to the Rho-kinase inhibitor compared to the untreated retinas ($F=1.44$, ndf 1, ddf 24, $p=0.2422$, Fig. 1A-C).

To be more specific, we observed a significant mean change in b-wave from baseline to the last measurement under hypoxia ($t=6.33$, df 24, $p<0.0001$, Fig. 1A-C) and from the last measurement under hypoxia to the last observation under recovery ($t=-2.65$, df 24, $p=0.0139$, Fig. 1A-C), but no difference between baseline and recovery ($t=0.70$, df 24, $p=0.4904$, Fig. 1A-C). Hereby the mean change between baseline and the last measurement under hypoxia is mainly caused by the difference in the 45 minutes group (1 μM : $t=5.25$, df 24, $p<0.0001$; control group: $t=4.10$, df 24, $p=0.0004$, Fig. 1C) and the 30 minutes group (1 μM : $t=5.14$, df 24, $p<0.0001$; control group: $t=3.98$, df 24, $p=0.0005$, Fig. 1B).

After recording the stable b-wave amplitudes for 30 minutes, hypoxia was induced by applying nitrogen to the nutrient solution (Fig. 1A). During 15 minutes of hypoxia, the b-wave amplitudes were reduced from 19.35 μV (SD 4.07) to 15.2 μV (SD 3.43) in the untreated retinas ($t=-0.50$, df 24, $p=0.6224$) and increased from 17.4 μV (SD 4.45) to 25.0 μV (SD 10.85) during the additional application of 1 μM H-1152P to the nutrient solution ($t=-0.87$, df 24, $p=0.3935$). However, the period of hypoxia was not long enough to induce a considerable effect on the b-wave amplitude ($t=0.70$, df 24, $p=0.4904$).

Longer periods of hypoxia (30 and 45 minutes) resulted in progressively greater decreases of the b-wave amplitude (Fig. 1B and 1C). To be more concrete, after 30 minutes of hypoxia, we detected a significant decrease of the b-wave amplitudes from 10.2 μV (SD 1.04) to 6.2 μV (SD 0.69) ($t=5.13$, df 24, $p=0.0005$), which was fully reversible at the end of the washout to 9.55 μV (SD 3.14) ($t=0.93$, df 24, $p=0.3624$). The application of 1 μM H-1152P did not prevent the reduction of the b-wave amplitude during 30 minutes of hypoxia and we detected a significant decrease of b-wave amplitudes from 14.7 μV (SD 6.91) to 6.95 μV (SD 0.74) ($t=5.14$, df 24, $p<0.0001$, Fig. 1B). However, a rapid recovery was observed within 15 minutes of reperfusion and the ERG-amplitudes at the washout ($t=0.56$, df 24, $p=0.5818$) reached the level before hypoxia. Yet, the treatment with 1 μM H-1152P did not improve the course of the b-wave amplitudes compared to the control retinas ($F=1.44$, ndf 1, ddf 24, $p=0.2422$).

After 45 minutes of hypoxia (Fig. 1C), the b-wave was significantly reduced from 13.1 μV (SD 4.09) to 7.1 μV (SD 2.40) ($t=3.98$, df 24, $p=0.0005$), but a full recovery of the b-wave amplitudes to 13.80 μV (SD 8.39) ($t=1.18$, df 24, $p=0.2511$, compared to baseline) was observed at the end of the washout, which was comparable to the untreated retinas. Administration of H-1152P at 1 μM could not prevent a significant decrease of the b-wave from 16.9 μV (SD 7.48) to 10.8 μV (SD 6.50) ($t=5.14$, df 24, $p<0.0001$) during hypoxia.

Our second model aims to compare the efficacy of different doses of the Rho-kinase inhibitor under hypoxia for 30 and 45 minutes (Fig. 1B and C). We observed, that the b-wave varies significantly between baseline, under hypoxia and at the end of recovery ($F=47.94$, ndf 2, ddf 44, $p<0.0001$). Therefore, we did not observe any significant difference in the mean b-wave depending on the duration of hypoxia ($F=0.29$, ndf 1, ddf 20, $p=0.5954$) or between the different concentrations of the inhibitor ($F=0.85$, ndf 2, ddf 20, $p=0.4424$).

A significant effect of hypoxia on the ERG-amplitudes was observed when the concentration of H-1152P was increased to 5 μM ($t=6.80$, df 44, $p<0.0001$) and the b-wave amplitude remained significantly reduced at the end of washout ($t=3.07$, df 44, $p=0.0036$). After the exposure to the higher concentration the effect of the inhibitor was significantly different compared to the control ($t=2.06$, df 44, $p=0.0452$). Though the reduction of the amplitude was lower in the presence of 1 μM H-1152P, this effect was not significant compared to the control ($t=1.55$, df 44, $p=0.1284$, Fig. 1B and C), but the b-wave was higher in response to the inhibitor. Moreover, the effect of 5 μM after hypoxia was reversible at the washout ($t=0.92$, df 44, $p=0.3648$) in contrast to the control. A reduction of the b-wave amplitude during hypoxia could also not be prevented despite the application of 5 μM H-1152P ($t=6.80$, df 44, $p<0.0001$, Fig. 1B and C) and we found a pronounced reduction of

Fig. 2. The neuroprotective effect of the Rho-kinase inhibitor in the ganglion cell layer of hypoxic retinas. (A) Ethidium homodimer-1 (EthD-1) staining performed on the flat mounts of the retinas immediately after the functional measurements. The damaged cells exhibited an increase in the uptake of the fluorescent dye EthD-1. Nuclei were counterstained with DAPI. Hypoxia resulted in a time-dependent increase in the extent of cell damage in the untreated retinas, whereas the treatment with the Rho-kinase inhibitor H-1152P led to a significant reduction in the percentage of dead cells in the ganglion cell layer. Bar indicates 25 μ m. (B) Quantification of the percentage of the cell death in the ganglion cell layer after 15 to 45 minutes of hypoxia. Data represent the mean $\pm$ SEM of n=2 retinas after 15 minutes of hypoxia and the retinas treated with 5 μ M H-1152P during 45 minutes of hypoxia; n=3 retinas for the untreated group (0 μ M) during 30 and 45 minutes of hypoxia; n=4 retinas that received 1 and 5 μ M of H-1152P during 45 and 30 minutes of hypoxia, respectively; and n=5 retinas treated with 1 μ M inhibitor during 30 minutes of hypoxia. *p=0.0006, **p=0.0001.

the b-wave amplitudes compared to the ERG-amplitudes before hypoxia. However, the effect of hypoxia was reversible at the end of the washout.

Reduction in hypoxic cell damage in response to the Rho-kinase inhibitor

Based on the previous studies demonstrating the susceptibility of particularly the retinal ganglion cells to hypoxic conditions [2], we initially focused on the extent of cell survival in the ganglion cell layer as determined by the Ethidium homodimer-1 staining performed on the retinas immediately after the functional measurements. Ethidium homodimer-1 is a cell-impermeable dye that can only enter the cells with damaged membranes, where it undergoes a 40-fold enhancement of red fluorescence upon binding to nucleic acids [41].

We observed no significant difference in the extent of cell damage among the retinas treated with different concentrations of the inhibitor during a given period of hypoxia ($F=0.40$, $\text{ndf } 2$, $\text{ddf } 21$, $p=0.6741$), but a significantly different mean percentage of dead cells depending on the duration of hypoxia ($F=22.11$, $\text{ndf } 2$, $\text{ddf } 21$, $p<0.0001$), with a hypoxic period of 45 minutes resulting in a significant increase in the percentage of dead cells compared to 15 minutes ($t=3.39$, $\text{df } 21$, $p=0.0028$) and 30 minutes ($t=2.64$, $\text{df } 21$, $p=0.0154$).

Furthermore, the mean percentage of dead cells differed significantly between the untreated retinas ($F=35.43$, $\text{ndf } 2$, $\text{ddf } 2$, $p<0.0001$) and the retinas treated with the Rho-

Fig. 3. Changes in the pattern of Cathepsin-B expression after 45 minutes of hypoxia and in response to the Rho-kinase inhibitor. (A) Immunostainings on the paraffin sections of a normal retina at time point 0 (T0) and retinas exposed to hypoxia for 45 minutes with (+) or without (-) 1 μ M H-1152P, demonstrating the gradual increase in the levels of the cytoplasmic Cathepsin-B after hypoxia particularly in the ganglion cell layer (GCL) as well as the photoreceptor outer segments underneath the outer nuclear layer (ONL), and the reduction in this process by the Rho-kinase inhibitor. INL: inner nuclear layer. Bar= 25 μ m. (B) Representative immunoblot of the whole retinal lysates demonstrating the upregulation of cathepsin-B in response to hypoxia (-) compared to the normal retina at T0. H-1152P (1 μ M, +) led to a considerable decrease in the levels of cathepsin-B after 45 minutes of hypoxia. Ponceau S staining was performed to verify equal protein loading.

kinase inhibitor during hypoxia ($F=12.94$, $ndf\ 2$, $ddf\ 20$, $p=0.0002$). Hereby, the prolonged exposure to hypoxia resulted in a gradual increase in the extent of cell damage in the ganglion cell layer, from $1.04 \pm 0.7\%$ dead cells after 15 minutes (mean $\pm$ SEM of $n=2$ retinas, $t=5.96$, $df\ 20$, $p<0.0001$) to $15.17 \pm 1.63\%$ dead cells after 30 minutes (mean $\pm$ SEM of $n=3$ retinas, $t=8.41$, $df\ 20$, $p<0.0001$) and to $20.7 \pm 0.85\%$ dead cells after 45 minutes of hypoxia (mean $\pm$ SEM of $n=3$ retinas, $t=3.51$, $df\ 20$, $p=0.0022$). However, the administration of the Rho-kinase inhibitor could significantly reduce the percentage of dead cells compared to control ($t=4.07$, $df\ 20$, $p=0.0006$ with 1 μ M H-1152P; $t=4.68$, $df\ 20$, $p=0.0001$ with 5 μ M H-1152P) without a considerable difference between the concentrations of 1 μ M and 5 μ M ($t=1.41$, $df\ 20$, $p=0.1729$, Fig. 2A and B).

The rupture of the lysosomal membranes and the leakage of the lysosomal enzyme Cathepsin-B into the cytoplasm is a common feature of necrotic cells, which was also observed in different models of hypoxia/ischemia induced neurodegeneration [42-44]. To gain insight into the extent of cell damage in all the retinal layers, we therefore analysed the level of Cathepsin-B in the retinal transverse sections. In the normal retinal pieces fixed immediately after the isolation at time point 0 (T0), we detected a moderate degree of Cathepsin-B immunoreactivity mainly as a cytoplasmic punctate staining in all the cellular layers, consistent with the localization of this protein predominantly to the lysosomes. However, the induction of hypoxia resulted in a notable increase in the levels of cytoplasmic Cathepsin-B after 45 minutes, with a more diffuse expression pattern particularly in the ganglion cells, suggesting the leakage of Cathepsin-B into the cytoplasm. Though the administration of H-1152P could not completely prevent the upregulation of Cathepsin-B under hypoxia, the levels of this protein remained considerably lower particularly in the ganglion cell and photoreceptor layers after 45 minutes of hypoxia in contrast to the untreated retinas. Moreover, the expression pattern of Cathepsin-B in the treated retinas was also mainly punctate similar to the normal retinas at T0, suggesting that the administration of H-1152P could suppress the leakage of Cathepsin-B into the cytoplasm, which was also confirmed by the immunoblots of the total retinal lysates (Fig. 3A and B).

Fig. 4. Decrease in the reactivity of the astrocytes and Müller cells in response to the Rho-kinase inhibitor. (A) Immunostainings for the glial marker GFAP (red) on the paraffin sections of the retinas demonstrating the hypoxia dependent activation of the Müller cells spanning the entire retinal width in the untreated retinas (control) compared to the normal retinas at T0. The elevated GFAP-immunoreactivity in the nerve fiber layer of these retinas (overlying the GCL) may indicate the bundles of the end-feet of the Müller cells as well as an increase in the reactivity of the astrocytes. In the presence of the inhibitor, the pattern of GFAP immunoreactivity was similar to that observed in the normal retinas at T0, which was confined mainly to the nerve fiber layer with very weak levels of expression in the Müller cell processes extending into the inner nuclear layer (INL). The nuclei were counterstained in blue with DAPI. ONL: outer nuclear layer. (B) Immunostainings for vimentin demonstrating the upregulation of this protein in the Müller cell processes as well as the nerve fiber layer of the untreated retinas after 30 minutes of hypoxia (control), and the H-1152P dependent reduction in this event. (C) Representative immunoblots confirming the upregulation of the main GFAP isoform (49 kDa, indicated by the arrow) and vimentin in response to hypoxia (0) compared to the normal retinas at timepoint 0 (T0). Administration of H-1152P at 1 and 5 μ M (1, 5) led to a considerable decrease in the levels of these intermediate filaments after 45 minutes of hypoxia. Ponceau S staining was performed to confirm equal protein loading.

Decrease in hypoxia-dependent glial cell reactivity in response to the Rho-kinase inhibitor

The glial cells execute numerous supportive functions, which are essential for neuronal function and survival. Though the up-regulation of glial reactivity is a rapid response to various stress conditions as a protective attempt, an excessive glial reactivity may exert detrimental effects on the neurons. A common feature of the reactive astrocytes and Müller cells, the radial glia spanning the entire depth of the retina, is the up-regulation of the intermediate filament glial fibrillary acidic protein (GFAP) [45-47]. To determine the extent of astrocyte and Müller cell reactivity in our hypoxia model, we therefore performed immunolabeling for GFAP on the sections of the retina fixed immediately after the reperfusion period.

In the normal retinas at T0, we detected the GFAP-immunoreactivity mainly in the nerve fiber layer where the astrocytes and the end-feet of the Müller cells interact with the ganglion cells. The induction of hypoxia for 15 minutes did not result in a considerable difference in this expression pattern in either the untreated or the treated retinas. However, the 30-minute exposure to hypoxia led to the significant upregulation of GFAP in both the Müller cells extending across the full depth of the retina and in the nerve fiber layer as thick bundles which might represent the aggregated end-feet of the Müller cells as well as the activated astrocytes. This expression pattern was also prevalent after 45-minutes of hypoxia. In contrast, the administration of the Rho-kinase inhibitor (1 μ M and 5 μ M) considerably

Fig. 5. Decrease in the microglial reactivity in response to the Rho-kinase inhibitor. (A) Morphology of the microglia was determined by the immunostainings for the marker protein CD11b on the flat-mounted retinas immediately after the functional recordings. Hypoxia resulted in the activation of microglia as detected by the gradual retraction of the cellular protrusions and the acquisition of a more amoeboid morphology over a period of 45 minutes (control). The Rho-kinase inhibitor H-1152P reduced the extent of microglia reactivity as demonstrated by the maintenance of the long cellular ramifications at all the hypoxic periods tested. Bar indicates 25 μ m. (B) Quantification of microglial reactivity based on the form factor 1 (FF1), which reflects the minor differences in the number and/or length of the ramifications. Data represent the mean SEM of $n=3$ retinas for each group. * $p=0.0485$, ** $p=0.0477$, *** $p=0.0284$. (C) Quantification of microglial reactivity based on the form factor 2 (FF2), which is a more specific indicator of true, long ramifications. Data represent the mean SEM of $n=3$ retinas for each group. * $p=0.0177$, ** $p=0.0088$.

interfered with the upregulation of GFAP particularly in the Müller cells after a hypoxic period of 30 and 45 minutes (Fig. 4A). Similarly, the intermediate filament vimentin, which is another marker of astrocyte and Müller cell reactivity [47], was significantly upregulated in the untreated retinas after 30 minutes of hypoxia, whereas the treatment with 1 μ M H-1152P reduced the level of vimentin in the nerve fiber layer and Müller cells (Fig. 4B). Upregulation of the main GFAP isoform (49 kDa) and vimentin after 45 minutes of hypoxia and the H-1152P dependent decrease in this process was also confirmed by immunoblotting (Fig. 4C).

Hypoxia also resulted in the activation of the microglia in the nerve fiber layer as demonstrated by the immunostainings for the microglia marker CD11b on the retinal flat mounts revealing the cellular morphology [48]. The microglia in the untreated retinas exposed to 15 minutes of hypoxia exhibited the quiescent, ramified morphology. However, as the hypoxic period increased to 30 and 45 minutes, the microglia gradually acquired the reactive, amoeboid morphology with the retraction of the protrusions and a decrease in the number of the ramifications. In contrast, the quiescent, ramified microglial morphology was preserved in the retinas treated with 1 μ M H-1152P after all the hypoxic periods (Fig. 5A). These findings were also confirmed by the morphological quantifications of the microglia based on the form factors FF1 and FF2, which mainly reflect the low and high degrees of ramification, respectively [40]. In the untreated retinas, the duration of hypoxia resulted in a significant increase in the values of FF1 from $13.00 \pm 1.40\%$ after 30 minutes to $27.37 \pm 5.89\%$ after 45 minutes (Fig. 5B, mean $\pm$ SEM of $n=3$ retinas for each group, $p=0.0284$).

Similarly, the FF2 values were increased from $44.67 \pm 3.72\%$ after 30 minutes to $59.83 \pm 2.50\%$ after 45 minutes of hypoxia in the untreated retinas (Fig. 5C, mean $\pm$ SEM of $n=3$ retinas from each group, $p=0.0088$), confirming the retraction of the ramifications and the acquisition of a more reactive morphology. In contrast, the microglia in the retinas treated with $1 \mu\text{M}$ H-1152P had significantly lower FF1 values after both 30 minutes ($10.17 \pm 0.26\%$, $p=0.0477$) and 45 minutes of hypoxia (Fig. 5B, $14.33 \pm 2.90\%$, $p=0.0485$, mean $\pm$ SEM of $n=3$ retinas for each group). Though we did not observe a significant decrease in the FF2 values of the microglia in the treated retinas after 30 minutes of hypoxia ($39.97 \pm 1.19\%$, $p=0.1640$), the FF2 values of the treated cells after 45 minutes of hypoxia were also significantly lower (Fig. 5C, $45.47 \pm 4.60\%$, $p=0.0177$, mean $\pm$ SEM of $n=3$ retinas for each group) compared to the microglia in the untreated retinas, demonstrating the suppression of microglia activation by the preservation of both the short and the long, true ramifications in response to the Rho-kinase inhibitor.

Discussion

In our study, hypoxia resulted in a time-dependent reduction of the b-wave amplitudes, which returned to the baseline values during the reperfusion period. This was associated with a significant increase in the extent of cell damage and glial reactivity in the untreated retinas. The Rho-kinase inhibitor exerted a neuroprotective effect in the retinas under hypoxia, with a significant decrease in the amount of the damaged cells and the extent of glial reactivity. Yet, despite the protective effect on the histological level, the inhibitor failed to prevent the hypoxia-dependent functional reduction and did not improve the functional outcome.

Necrosis is the neuronal death mechanism that predominates under conditions of hypoxic/ischemic energy deprivation. However, necrotic events can also activate the components of the apoptotic pathway by energy-independent mechanisms. Accordingly, the combined inhibition of the major cysteine proteases from both the necrotic and the apoptotic pathways is regarded as a more efficient strategy to restrict the neuronal damage after hypoxic/ischemic injury [44]. In our model, the damaged cells exhibited mainly the features associated with necrosis, such as the early decrease in the plasma membrane integrity as detected by the Ethidium homodimer-1 uptake. Though the compromise of the membrane integrity can also be observed at the later stages of the apoptotic cells, our immunostainings for cleaved caspase-3 revealed a very low degree of immunoreactivity in all the treatment groups (data not shown), weakening the possibility that the apoptotic mechanisms were activated at the time points tested. A further support to the hypoxia-dependent necrosis was gathered from the levels of the cytoplasmic Cathepsin-B, a lysosomal cysteine protease which leaks into the cytoplasm due to the rupture of the lysosomal membranes under energy deprivation and may lead to irreversible cell damage through the cleavage of the structural proteins [42-44]. In our model, the leakage of Cathepsin-B in the untreated retinas was prevalent in all the retinal layers after 45 minutes of hypoxia. The absence of the underlying pigment epithelium and the choroid vessels underneath the photoreceptors in the isolated retinas might have resulted in these signs of necrotic damage in the photoreceptors, which are usually not as sensitive as the ganglion cells and the inner retinal neurons in some models of hypoxia/ischemia [2, 49]. Our model therefore simulates a more severe hypoxic condition where not only the retinal vasculature feeding the inner retina, but also the choroid vessels supporting the photoreceptors fail to provide the neurons with oxygen. Nevertheless, despite the adversity of the conditions, the administration of the Rho-kinase inhibitor resulted in a significant reduction in cell damage as indicated by the better preserved plasma membrane integrity. The inhibitor also considerably interfered with the leakage of Cathepsin-B into the cytoplasm, thereby protecting the cells from irreversible damage. Inhibition of Rho-kinase therefore appears to be a simple and efficient strategy to limit the extent of hypoxic cell injury compared to the co-administration of multiple cysteine protease inhibitors.

Rho-kinase is involved in the regulation of the cytoskeletal rearrangements that generate the apoptotic contraction and membrane blebbing [17]. However, the interference with these apoptotic events probably does not underlay the prevailing mechanism of the protective effect of H-1152P in our model, since the damaged cells mainly exhibited the features of necrosis. A likely mediator of cell damage which is targeted by the Rho-kinase inhibitor in our model might be the extracellular glutamate. Glutamate is the major excitatory neurotransmitter in the retina, which is released by the photoreceptors in darkness to activate the bipolar and horizontal cells. The rapid clearance and recycling of glutamate from the synapses is therefore essential for the normal neuronal functioning. The Müller cells are mainly responsible for the glutamate uptake via high affinity transporters, which rely on a very negative membrane potential [50, 51]. However, in retinal ischemia and diabetes, the glutamate uptake into the Müller cells is reduced due to the depolarization of the Müller cells in response to elevated extracellular potassium. The excessive accumulation of glutamate and aspartate in the synapses is implicated in the excitotoxic damage to the retinal ganglion cells. Glutamate excitotoxicity is also a major cause of neuronal loss in the retinal disorders including glaucoma, diabetes, inherited photoreceptor degeneration, and ischemia [52]. Interestingly, the Rho-kinase inhibitor Fasudil could exert a direct neuroprotective effect on primary cerebral neurons through the attenuation of glutamate-induced neurotoxicity [53]. Moreover, glutamate can transiently activate Rho-kinase through the upstream activation of RhoA in hippocampal neurons [54]. These findings favour the possibility that excessive glutamate can also activate the Rho-kinase in the hypoxic retina and the administration of H-1152P can interfere with certain events downstream of glutamate, which remains to be determined.

The protective effect of the Rho-kinase inhibitor might also be related to a reduction in glial reactivity. Indeed, the proliferation and elevated reactivity of Müller cells was associated with the disruption of glutamate recycling in hypoxic retinas in a model of retinal detachment whereas limiting the Müller cell reactivity via oxygen supplementation reduced the abnormalities in glutamate distribution and uptake [55]. H-1152P might have directly interfered with the Müller cell hypertrophy by suppressing the Rho-kinase dependent phosphorylation of GFAP at the head domain and thereby preventing the reorganization of these filaments [56]. Indeed, a novel study demonstrates that several commercially available antibodies against GFAP have a higher affinity towards the phosphorylated protein compared to the unphosphorylated GFAP-isoforms, suggesting that the changes in the levels of GFAP immunoreactivity might in fact reflect the changes in the degree of phosphorylation rather than the total level of this protein [57]. The Rho-kinase may also regulate the cytoskeletal contraction in the microglial cells, resulting in the activation of these cells [31] whereas the inhibitor prevented the transition into the reactive, ameboid morphology. However, the H-1152P dependent decrease in glial reactivity could also have occurred secondarily to the decreased neurotoxicity. Alternatively, the inhibitor might have exerted a direct effect on both the neuronal cells and the retinal glia, resulting in an additive positive outcome on neuronal survival, which remains to be determined.

Yet, the well-preserved cellular integrity and the positive effects on cellular survival in response to H-1152P were not reflected to the functional outcome as estimated by the b-wave amplitudes. H-1152P is an isoquinolinesulfonamide, which structurally resembles adenosine and occupies the ATP-binding domain of Rho-kinase [37]. Interestingly, adenosine can act as a modulator of synaptic transmission in the retina and regulate the blood flow particularly under compromised oxygen delivery [58]. It would be therefore of interest to determine whether H-1152P can interact with the adenosine receptors in the retina, which would have interfered with the synaptic transmission. An alternative reason underlying the inability of the Rho-kinase inhibitor to improve the b-wave amplitudes might be the remodelling of the actin cytoskeleton at the synapses of the depolarizing bipolar cells in an activity dependent manner. The b-wave represents the combined response of the depolarizing bipolar, amacrine, and Müller cells, which are dependent on the hyperpolarization of the photoreceptors upon the perception of light [6, 35]. In response to the light stimulus, the hyperpolarizing bipolar

cells project thin extrusions called spinules into the synaptic processes of the amacrine cells, which possibly strengthens the synaptic connections. In contrast, light causes the retraction of the spinules in the depolarizing bipolar cells concurrent with the formation of filamentous actin in the contracted synaptic pedicles and an increase in protein kinase C (PKC) activity [59]. PKC is indeed a frequently used marker for the bipolar cells, which is localized mainly to the bipolar cell dendrites, axons, and photoreceptor presynaptic terminals, suggesting a key role for PKC in the establishment of the retinal circuitry [60, 61].

The direct inhibition of PKC via H-1152P can be possible *in vitro* using purified molecules, yet the affinity of the inhibitor to other kinases is considerably lower, as reflected by the K_i value of 1.6 nM for the Rho-kinase as opposed to the K_i value of 630 nM for protein kinase A, and 9.27 μ M for PKC in cell-free assays [37]. However, the inhibitor is usually administered at micromolar concentrations in cell-based assays, since it has to compete with the intracellular ATP, which is present in millimolar concentrations in most cells [62]. Owing to its high metabolic activity, the retina is particularly rich in ATP, with the concentrations reaching 1 mM in the photoreceptor outer segments of the bovine retina [63]. In our study, we applied the inhibitor at 1 to 5 μ M based on a previous study on serum deprived retinas *in vitro*, where the application of H-1152P at 1 μ M significantly reduced the Rho-kinase dependent phosphorylation of the cytoskeletal protein adducin without interfering with the PKA-dependent phosphorylation of the same protein [31]. Therefore, the unspecific targeting of PKC in our model remains as a very weak possibility despite the shortage of energy and the reduction in intracellular ATP that the inhibitor will be competing with [64, 65]. Interestingly, PKC can act as an upstream activator of the RhoA/Rho-kinase pathway in diverse cell types [66]. Though the involvement of Rho-kinase in the synaptic remodeling in the retina is to our knowledge not reported, it is likely that the H-1152P could have reversed the actin reorganization in the bipolar cell synapses, resulting in the absence of a functional improvement. Moreover, the majority of significant cell protection by Rho-kinase inhibition was mainly found in the ganglion cell layer in our model. However, the ganglion cell function does not contribute to the course of our recorded electroretinogram. Therefore, a functional improvement due to ganglion cell survival could not be detected in our model although the neuroprotective effect was observed by histological assessment.

In conclusion, our findings provide further support to the neuroprotective potential of the pharmacological Rho-kinase inhibitor H-1152P under hypoxic conditions. Though the presence of the inhibitor did not prevent the functional decrease during the hypoxic phase or promote a faster recovery during the reperfusion, the cells treated with the inhibitor were significantly protected from necrotic damage and are expected to function properly once the normal conditions are restored. Nevertheless, the termination of the treatment soon after the reestablishment of the normal perfusion might be necessary to avoid the possible interference with the synaptic modulation and to thereby restore the neuronal function. The inhibitor could therefore be applied during hypoxic conditions to restrict the extent of retinal damage especially in the ganglion cell layer, which could have a clinical significance in the case of a branch or central arterial retinal occlusion, in which the deterioration of the ganglion cell layer after an arterial occlusion of retina results in a pronounced visual impairment in these patients [67].

Conflict of Interest

All authors have no financial interest related to the manuscript. All authors disclosed any private sponsorship or funding arrangements relating to this research and all authors disclosed any possible conflicts of interest related to this paper.

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