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Mitogen-activated Protein Kinase p38 α and Retinal Ischemic Preconditioning

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Abstract

In previous studies, inhibition of mitogen activated protein kinase (MAP) p38 significantly improved recovery and attenuated apoptosis after retinal ischemia in rats. Yet, ischemic preconditioning (IPC) attenuated the ischemia-induced increase in p38 expression. We hypothesized that p38 was required for induction of ischemic tolerance by IPC. We examined the mechanisms of involvement of p38 in IPC neuroprotection. IPC or ischemia was induced in rat retina *in vivo*. Recovery after ischemia performed 24 h after IPC was assessed functionally (electroretinography) and histologically at 7 days after ischemia in the presence or absence of inhibition of p38. We examined the role of p38 α in the mimicking of IPC produced by opening mitochondrial KATP channels using diazoxide, or stimulation of p38 activation by anisomycin. The importance of adenosine receptors in p38 activation after IPC was assessed using specific blockers of adenosine A1 and A2a receptors. Interfering RNA (siRNA) or SB203580 were used to block p38 α . Phosphorylated p38 levels were measured. Phosphorylated p38 protein increased with IPC. Interfering RNA (siRNA) to p38 α prior to IPC, or inhibiting p38 activation with SB203580, with ischemia following 24 h later, significantly attenuated the neuroprotective effect of IPC. Anisomycin administered to increase p38 mimicked IPC, an effect blocked by SB203580. IPC mimicking with diazoxide, an opener of mitochondrial KATP channels, was diminished with p38 α siRNA. Adenosine receptor blockade did not decrease the elevated levels of phosphorylated p38 after IPC. Specific inhibition of p38 α suggests that this MAPK is involved in the protective effects of IPC, and that p38 is downstream of mitochondrial KATP channels, but not adenosine receptors, in this neuroprotection.

Keywords

ischemic preconditioning; p38; retinal ischemia

INTRODUCTION

Retinal ischemia is associated with vascular diseases causing visual loss. The retina's blood and oxygen supply may be decreased in diabetic retinopathy, central retinal artery occlusion,

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carotid artery stenosis, glaucoma, and sickle cell retinopathy. Previously, we demonstrated that a profound endogenous protection against a later injuring ischemia can be produced in the rat retina by earlier non-damaging ischemia (i.e., ischemic tolerance produced by ischemic preconditioning; IPC) (Roth et al. 1998; Zhang et al. 2002; Roth et al. 2006). This endogenous protection in the retina provides the opportunity to uncover new mechanisms, pathways, targets and agents useful to treat retinal ischemia.

Mitogen-activated protein kinases (MAPK) couple cell-surface receptors and chemical and physical cellular stressors to critical regulatory targets and gene transcription changes, and play a key role in cell survival and adaptation (Chang and Karin 2001). MAPK are classified primarily into three signaling arms: extracellular-signal-regulated kinase (ERK), and the two stress-activated c-Jun N-terminal kinase (JNK) and p38, (Chang and Karin 2001) arms. Signaling within these arms is mediated by successive kinase phosphorylation (i.e., activation) of target proteins (Irving and Bamford 2002; Kumar et al. 2003). MAPK activation is also influenced by protein kinase A and C, receptor tyrosine kinases, oxygen free radicals, inflammation, and cytokines (Ping et al. 1999; Cross et al. 2000; Tanaka 2001, Lee et al, 2003).

Previously, we found distinct temporal, cell-specific signaling patterns of ERK, JNK, and p38 activation after retinal ischemia or IPC. Moreover, we found a key role for adenosine receptors and mKATP channels as IPC initiators. But, numerous signaling pathways are believed to underlie the ischemic tolerance produced by IPC (Gidday 2006). Blocking p38 or ERK activation, but not JNK, completely attenuated ischemic injury and dramatically reduced apoptosis-related gene expression (Roth et al. 2003). However, we also showed that p38 was activated by retinal IPC (Zhang et al. 2002). Consistent with these results, others found that IPC activated pro-apoptotic neuronal caspases in the brain (McLaughlin et al. 2003; Tanaka et al. 2004). Following axotomy, p38 α subtype levels were increased in retina in rats, and blockade of p38 activity decreased retinal ganglion cell loss (Kikuchi et al. 2000). Thus, p38 appears to be a critical link in retinal cell survival/death (Roth et al. 2003), where its activation contributes to injury following ischemia, but its earlier activation produces changes that contribute to subsequent protection from ischemia. In this study, we hypothesized that p38 α activation is essential for the induction of ischemic tolerance in the retina by ischemic preconditioning, and that activating p38 can also protect the retina against ischemic injury. For these experiments we targeted the specific isoform, p38 α , that has been shown to be the most important isoform in the retina (Kikuchi et al. 2000). In addition, in order to further extend the analysis of retina IPC, signaling pathways related to IPC and p38 α were also evaluated. Under these conditions, the effects of adenosine receptor antagonism, mitochondrial KATP (mKATP) channel opening, and changes in total and activated/phosphorylated p38 protein were measured (Kumar et al. 2003; Li et al. 2003; Whitlock et al. 2005; Dreixler et al. 2009b). The important roles of p38 α in IPC and ischemia suggest its complex role related to cell survival and cell death under these conditions.

MATERIALS and METHODS

Retina Preconditioning and Ischemia

Procedures (Roth et al. 2006; Dreixler et al. 2008) conformed to the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Research and were approved by our Animal Care Committee. Sprague-Dawley rats (200-250 gm) from Harlan (Indianapolis, IN) were maintained on a 12 h on/12 h off light cycle. For preconditioning, intraocular pressure (IOP) was increased for 8 min to 160 mm Hg using an elevated 1000-ml bag of sterile normal saline connected to a 30-g needle placed under direct vision in the center of the anterior chamber as described earlier (Dreixler et al. 2008; Dreixler et al. 2009a; Dreixler et al. 2009b). For

retinal ischemia, performed 24 h after IPC, IOP was increased to 110 mm Hg for 45 min in rats anesthetized with chloral hydrate, 400 mg/kg i.p.

Electroretinography Before and after Retina Ischemia

Procedures have been described in detail previously (Roth et al. 2003; Roth et al. 2006; Dreixler et al. 2008). Animals were dark-adapted at least 2 h before electroretinogram (ERG) recordings. For baseline and post-ischemic (i.e., after 7 d) follow-up ERG, and during pre-conditioning, rats were injected i.p. with ketamine (35 mg/kg), and xylazine (5 mg/kg). Corneal analgesia was achieved with 0.5% proparacaine (Alcon, Ft. Worth, TX), and pupils were dilated with 0.5% tropicamide (Alcon), and cyclomydril (0.2% cyclopentolate HCl and 1% phenylephrine HCl (Alcon). Body temperature was maintained at 36.5-37.0 C with a servo-controlled heating blanket (Harvard Apparatus, Natick, MA).

The scotopic ERG was recorded from responses to 10- μ s white light flashes from a Nicolet Ganzfeld stimulator (Madison, WI) with the rat's head centered 6 in from it on a Nicolet Spirit 486 System as previously described (Dreixler et al. 2008; Dreixler et al. 2009a; Dreixler et al. 2009b). Intensity of the unattenuated light flash was 1.00 log cd.s/m²; the high pass filter setting was 1 kHz, and low pass, 1 Hz. Baseline b-wave amplitudes in both eyes were 800 to 1100 μ V. (Roth et al. 2003; Roth et al. 2006; Dreixler et al. 2008). Wave amplitudes 7 d after ischemia were measured and reported as a percentage of the baseline, non-ischemic wave amplitude.

p38 Kinase Activation and downstream signaling Measurements

Western blotting procedures described in previous studies (i.e., with the exception of the tissue lysis method for the kinase assay) were utilized (Junk et al. 2002; Zhang et al. 2002; Roth et al. 2003). A non-radioactive p38 MAP Kinase Assay Kit (#9820; Cell-Signaling, Danvers, MA) measured p38 activity. Briefly, retinæ removed from euthanized rats were treated with lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1mM β -Glycerolphosphate, 1mM Na₃VO₄, 1 μ g/mL Leupeptin) and sonicated 5 s on ice. Phosphorylated p38 was immunoprecipitated from the supernatant with immobilized, anti-phosphorylated (Thr180/Tyr182) p38 MAP Kinase primary antibody (Cell Signaling). For specific measurement of p38 α , we used a similar procedure but immunoprecipitated with monoclonal mouse p38 α antibody (BD Biosciences, San Jose, CA) and red protein G affinity gel (Ezview; Sigma-Aldrich). The pellet was washed twice with lysis and kinase buffers (25mM Tris, pH 7.5, 5mM β -Glycerolphosphate, 2mM DTT, 0.1 mM Na₃VO₄, and 10 mM MgCl₂), then resuspended in 50 μ L of kinase buffer supplemented with 200 μ M ATP and 1 μ L kinase substrate. After 30 min incubation at 30°C, the reaction was terminated with 25 μ L 3X SDS sample buffer, and 30 μ L of each sample was loaded onto SDS-PAGE gels for Western blot. Membranes were treated with anti-phosphorylated (Thr71) ATF-2 antibody (Cell Signaling, 1:1000), HRP-conjugated secondary antibody (Cell Signaling, 1:2000) and HRP-conjugated anti-biotin antibody (Cell Signaling, 1:1000).

Inhibition of p38 signaling or p38 silencing

We used a well known inhibitor of p38 α and p38 β , SB203580, which inhibits activated, phosphorylated p38 from phosphorylating downstream targets (Lee et al. 2000). Doses that inhibited p38 in retina had been determined by us earlier (Roth et al. 2003). A 4- μ L injection of SB203580 (24 mM) or PBS (vehicle control) was made into the mid-vitreous of both eyes using a microsyringe (Hamilton, Reno, NV) as previously described (Roth et al. 2006). We also silenced p38 α using p38 α interfering RNA (siRNA), which was validated to reduce p38 protein as described below.

We found previously that the use of four siRNA oligomers were successful in cell systems or *in vivo* rat retina to increase the probability of silencing a given gene (Dreixler et al. 2008). We

targeted the specific p38 isoform p38 α , earlier shown to be the most important isoform in the retina (Kikuchi et al. 2000). Target interfering RNA (siRNA) sequences for p38 α (siRNA, Qiagen, Valencia, CA) were AAGCTCTTGCGCATGCCTACT, AAGGTCCTGGAAGAATTCAA, AAGAAGCTGTCGAGACCGTTT and AAGAGCTGACCTACGATGAA. These oligomers were designed using neural-network technology as described previously (Huesken et al. 2005; Dreixler et al. 2008; Dreixler et al. 2009b). Confirmed by BLAST, siRNAs were 100% homologous to the p38 α mRNA sequence. Design was checked for homology to all other sequences of the genome, 3' UTR/seed analysis, single nucleotide polymorphisms, and interferon motif avoidance (Farh et al. 2005; Hornung et al. 2005; Judge et al. 2005) and found to be negative. A 2- μ l, 3 μ M final concentration, mixture of these four different sequences of p38 α siRNA, or of a single non-silencing, negative control sequence (Qiagen) in RPMI media (Invitrogen, Carlsbad, CA) plus RNAiFect transfection reagent (Qiagen), was injected into the mid-vitreous of both eyes using a microsyringe (Hamilton, Reno, NV) as previously described (Dreixler et al. 2008; Dreixler et al. 2009b). A 2- μ l of 3 μ M non-silencing siRNA (random) sequence, AATTCTCCGAACGTGTCACGT, was used as a control.

Immunocytochemistry and Immunohistochemistry

The PC12-T cell line was cultured in RPMI-1640 media supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum, Glutamax, penicillin, and streptomycin (Invitrogen) in a humidified 10% CO₂ incubator at 37°C. Cells differentiated for 48 h with the same culture medium plus nerve growth factor (NGF; 100 ng/ml; Invitrogen) were exposed for 48 h to 400 nM siRNA (non-silencing control or p38 α siRNA) in RPMI media with RNAiFect transfection reagent (Qiagen). Expression of the targeted p38 α was examined using immunocytochemistry as we previously described (Dreixler et al. 2008) using mouse monoclonal anti-p38 α (1:2000; BD Biosciences).

Eyes removed from euthanized rats were evaluated using immunohistochemistry as previously described (Junk et al. 2002; Dreixler et al. 2008; Dreixler et al. 2009a; Dreixler et al. 2009b) using mouse monoclonal anti-p38 α (1:50, BD Biosciences). Antibody processing utilized standard antibody concentrations and antibody exposure times, of both the primary and secondary antibodies, to allow quantification of fluorescent intensities as described in our previous studies and below (Dreixler et al. 2008; Dreixler et al. 2009a; Dreixler et al. 2009b).

p38 protein level measurements

Western blotting procedures described previously (i.e., with the exception of the tissue lysis method of the kinase assay) were utilized (Junk et al. 2002; Zhang et al. 2002; Roth et al. 2003). Membranes were incubated overnight at 4°C with rabbit polyclonal anti-phosphorylated Thr180/Tyr182 p38 (1:300; Promega, Madison, WI), or rabbit polyclonal anti-p38 (1:300; Santa Cruz Biotechnology, Santa Cruz, CA) in 5% nonfat dry milk solution in TTBS. Anti-rabbit horseradish peroxidase (HRP)-conjugated (goat IgG; Jackson ImmunoResearch, West Grove, PA) secondary antibodies were applied at 1:20,000. Chemiluminescence was developed using Super Signal West Pico (Pierce, Rockford, IL). Protein bands were digitally imaged and semi-quantified by densitometry as previously described (Junk et al. 2002; Zhang et al. 2002; Roth et al. 2003). Equal protein loading was checked by immunoblotting with rabbit polyclonal anti-tubulin (1:500; Sigma, St. Louis, MO), or anti-p38 (1:300, Cell Signaling).

Imaging and Image analysis

For imaging the stained frozen cell culture slides and retinal sections (immunohistochemistry), we utilized fluorescence microscopy (Olympus IX81 inverted with 40 X oil lens, Olympus, Center Valley, PA), and a Fast firewire Retiga EXi chilled CCD camera (QImaging, Pleasanton, CA). Excitation/dichroic/emission settings were 530-550 nm - 570 DM-590 LP

for greens (fluorescein). Immunohistochemical fluorescent intensities were measured with NIH ImageJ v.1.33, adapted from our previous methods (Roth et al. 2003; Dreixler et al. 2009a; Dreixler et al. 2009b). Mean fluorescent intensity for the cell slides or retinal ganglion cell and inner plexiform layers were measured. Three 40X images, 200 μ m apart in the same slides or regions of the retina, were measured, found to be repetitive, and thus averaged. All were obtained at the same laser intensity and time exposure for all paired experiments. To measure siRNA differences, the mean intensities were normalized to the non-silencing control siRNA's fluorescent exposure levels.

Histology

Eyes enucleated on the seventh day after ischemia were immediately fixed in 4% paraformaldehyde (in PBS) for 48 h, transferred to buffered formalin for 24 h and then stored in PBS at 4°C. Eyes were embedded in paraffin, sectioned to 5 μ m and stained with hematoxylin and eosin (H&E). Sections were examined by light microscopy and retinal ganglion cell layer counts quantified as described earlier (Roth et al. 1998; Singh et al. 2001; Junk et al. 2002; Zhang et al. 2002; Dreixler et al. 2009a). Cell counts in the inner nuclear layer (INL) were determined after image capture with Micron 4.5 (Westover Scientific, Mill Creek, WA). Several INL regions of interest (ROI) were selected (approximately 1500 μ m from the optic nerve head) and the numbers of cells per area of the ROIs were manually counted, then averaged. Cells per unit area in the ischemic retinae were compared to the paired normals.

Specific Studies: SB203580 and siRNA on Proteins and ERG

Effects of IPC on p38 activation and downstream signaling were evaluated. Total and phosphorylated p38 were determined 1, 6 and 24 h after IPC. Impact of IPC on p38's ability to signal downstream (i.e., phosphorylate ATF-2) was evaluated using a kinase activity assay. To examine the role of p38 in retinal IPC, inhibition of p38 activation was tested using the relatively specific inhibitor of p38 phosphorylation, SB203580 (24 mM; Calbiochem, Darmstadt, Germany) or PBS (control), injected into the vitreous (4 μ l) of both eyes 15 min before 8 min IPC. We have shown previously that this concentration blocked p38 activation (Roth et al. 2003). Twenty-four h after IPC, 45 min of retinal ischemia was carried out in one eye, as described previously (Dreixler et al. 2008; Dreixler et al. 2009b).

The role of p38 α was specifically examined using RNA interference. Specific siRNA for p38 α (a mixture of the 4 siRNA sequences) or a non-silencing negative control siRNA (all at 3 μ M) were injected into the vitreous of both eyes 6 h before 8 min of IPC. IPC was followed by 45 min of ischemia in one eye 24 h later as described previously (Dreixler et al. 2008; Dreixler et al. 2009a; Dreixler et al. 2009b). ERG was evaluated at baseline before IPC and ischemia and 7 days after ischemia. In addition to function, histological determinations were carried out 7 d after ischemia under these conditions.

Anisomycin, an antibiotic that inhibits protein synthesis, activates MAPK signaling (Barea-Rodriguez et al. 2000; Aakalu et al. 2001). We determined the effects of anisomycin (10 mg/kg, i.p.; Sigma) on p38 activation and downstream signaling. One hour after injection, immunoprecipitated total p38's ability to signal downstream (i.e., phosphorylate ATF-2) was evaluated using the kinase activity assay. After demonstrating the ability of anisomycin to increase p38 signaling, anisomycin was administered 24 h prior to ischemia in the presence of intravitreal SB203580 or PBS and the effects on retinal function 7 d later were also determined (similar to that described above for IPC).

Since adenosine receptors A1 and A2a are involved in initiation of retinal IPC (Li and Roth 1999; Li et al. 2000), the effects of receptor antagonism on p38 phosphorylation due to IPC were determined. The selective adenosine A1 receptor antagonist DPCPX (8-cyclopentyl-1.3-

dipropylxanthine; 4.5 mg/kg in 20% DMSO/80% saline) and selective A2a receptor blocker CSC (8-(3-chlorostyryl) caffeine; 1 mg/kg in 45% 2-hydroxypropyl- β -cyclodextrin/55% saline) were injected (i.p.) 15 min before IPC and p38 phosphorylation determined 6 h later. Previous studies indicated that these doses of agents attenuated IPC (Li and Roth 1999; Li et al. 2000).

In a previous study (Roth et al. 2006), we demonstrated that opening mKATP channels by systemic injection of diazoxide (40 mg/kg i.p.; Louis, MO) mimicked IPC. Effect of mitochondrial KATP (mKATP) channel opening on p38 phosphorylation/IPC was determined. We measured effects of diazoxide injection on total and phosphorylated p38 1, 6 and 24 h later. To examine the role of p38 α downstream from the mKATP channel opening/stimulation, we injected p38 α siRNA or non-silencing siRNA 6 h before diazoxide, created ischemia 24 h later, and evaluated retinal function 7 d later.

Data handling and statistical analysis

For ERG results, the a- and b-waves from ischemic eyes 7 d after ischemia were dually corrected, for day-to-day variation in the normal eye, and for the baseline in the ischemic eye, as previously described (Roth et al. 2006; Dreixler et al. 2008; Dreixler et al. 2009a; Dreixler et al. 2009b). These data were normally distributed as confirmed using normality plots and skewness/kurtosis in Stata version 6.0 (College Station, TX). Thus we compared for each experiment (e.g., SB203580 vs PBS prior to IPC + ischemia) control and experimental retinæ (mean percent changes $\pm$ S.E.M) using the unpaired t test as previously described (Roth et al. 2006; Dreixler et al. 2008; Dreixler et al. 2009a; Dreixler et al. 2009b). For Western blot data, we could not assume a normal distribution and therefore used non-parametric tests. When pairs of eyes were studied in a group (e.g., effect of IPC on phosphorylated p38 levels in normal vs IPC), we referenced densitometric results in the IPC retina as a percentage of that in its paired normal. For examining the time course after IPC (1, 6, and 24 h), Kruskal-Wallis test assessed change in normalized intensity over time after IPC. Mann-Whitney test compared results between two of the groups (1 vs 6 h, and 1 vs 24 h after IPC), and also was used to assess absolute intensity level densitometry data for activation of p38 after systemic injection of anisomycin or diazoxide vs vehicle. A similar approach was used for histological or immunostaining data, which were compared between paired samples using absolute values of the cell counts in the retinal ganglion cell or inner nuclear layers, or the staining intensity in the cell layers or cells.

RESULTS

p38 phosphorylation and downstream signaling following ischemic preconditioning

Phosphorylation of p38 was significantly changed vs the paired normal retinal homogenates over time following IPC ($p < 0.05$ by Kruskal-Wallis, Fig. 1a and 1b). Phosphorylated p38 expression had a small decrease to $88 \pm 3\%$ ($n = 3$) 1 h after IPC stimulus, and then increased to $170 \pm 22\%$ ($n = 3$) by 6 h, and still remained elevated to $126 \pm 9\%$ ($n = 3$) at 24 h; by Mann-Whitney test, the changes at 6 and 24 h were significant compared to 1 h ($p = 0.05$). No significant effects on p38 total protein were observed. The p38 kinase activity assay confirmed that phosphorylated p38 signaling and activation of a downstream target (i.e., phosphorylation of ATF2) increased from $0.7 \times 10^7 \pm 0.2 \times 10^7$ arbitrary units to $6.1 \times 10^7 \pm 4.7 \times 10^7$ units ($p = 0.043$ by Wilcoxon signed rank test; $n = 5$; Fig. 1c) 24 h after IPC.

Effects of blocking p38 downstream signaling using SB203580 on IPC functional protection

Intravitreal injection of the specific antagonist SB203580 at 15 min prior to IPC ($n = 17$) did not affect the a-wave significantly, but significantly decreased b-wave recovery after IPC and ischemia as compared to PBS vehicle control treatment (a-wave $59 \pm 13\%$ vs $77 \pm 11\%$, $p =$

0.33 and b-wave $33 \pm 8\%$ vs $56 \pm 6\%$, $p = 0.03$; Fig. 2a and 2b). Note that SB203580 reduced the b-wave IPC protection to less than the sham IPC levels of $42 \pm 6\%$ seen in our previous studies (Zhang et al. 2002).

Validation of effects of p38 α siRNA

Immunocytochemical analysis of 24 h treatment of PC-12T cells with p38 α siRNA showed a 30% decrease in p38 α protein levels (1586 ± 47 to 1117 ± 20 arbitrary units in non-silencing siRNA-treated controls vs siRNA treated, $p = 0.027$ by Wilcoxon signed rank test; $n = 6$; Fig 3a). p38 α protein trended to decrease *in vivo* 24 h after injection of siRNA in retinal ganglion cell layer from 57 ± 9 mean intensity value units in non-silencing siRNA-injected to 48 ± 9 units in siRNA-injected retinæ ($p = 0.06$, Wilcoxon signed rank test; $n = 4$; Fig 3b). Moreover, p38 activity in whole retinal homogenates by kinase assay also demonstrated a trend with a decrease at 24 h after injection of p38 α siRNA ($1.0 \times 10^7 \pm 0.1 \times 10^7$ arbitrary units) compared to non-silencing siRNA injected retinæ ($0.8 \times 10^7 \pm 0.4 \times 10^6$ units; $p = 0.08$; $n = 5$).

Effects of silencing p38 α using siRNA on IPC functional protection

Although the a-wave was not significantly affected, injection of p38 α siRNA ($n = 9$) significantly reduced the b-wave recovery after IPC and ischemia as compared to a non-silencing control siRNA sequence (a-wave $72 \pm 8\%$ vs $87 \pm 6\%$, $p = 0.16$, and b-wave $47 \pm 4\%$ vs $60 \pm 5\%$, respectively; $p = 0.04$; Fig. 4a and 4b). These results were similar to those with pharmacological blockade of p38 signaling (compare Fig. 2a and 2b).

Histology: Results of siRNA on RGC layer and inner nuclear layer cell counts after IPC then ischemia

p38 α siRNA - treated ischemic retinæ had a significantly decreased percentage of cells in the RGC layer ($46 \pm 7\%$, $n = 3$) as compared to the non-silencing siRNA group ($79 \pm 6\%$, $n = 9$) at 7 d after ischemia ($p = 0.02$ by Mann-Whitney test, Table 1; Fig. 5). The numbers of cells in the retinal inner nuclear layer decreased in the p38 α siRNA-treated ischemic retinæ to $73 \pm 2\%$ versus the normal retinæ; however, the difference between this % decrease and that in the non-silencing siRNA group ($88 \pm 9\%$) was not significant (Table 1).

Pharmacological activation of p38 mimics IPC functional recovery after ischemia

Systemic injection of the p38 activator, anisomycin, significantly increased p38 kinase activity (Fig. 6a). The levels increased to an absolute intensity of 117 ± 16 one h after anisomycin injection compared to 5 ± 1 for vehicle controls ($p = 0.02$ by Mann-Whitney test; $n = 4$). Moreover, we found that anisomycin mimicked IPC via p38 activation. Initial studies indicated that anisomycin (10 mg/kg i.p.) 24 h before ischemia resulted in ERG responses similar to that which occurs post-IPC and ischemia (e.g., a-wave recovery $90 \pm 6\%$ and b-wave recovery $65 \pm 7\%$ of baseline ERG values (data not shown). Intravitreal injection of SB203580, 6 h before anisomycin significantly attenuated the b wave recovery at 7 d after ischemia to $29 \pm 8\%$ ($p = 0.015$; $n = 8$) vs vehicle control ($57 \pm 6\%$; $n = 8$) (Fig. 6b), similar to its effects on IPC (compare to Fig 2). Furthermore, we found that anisomycin did not affect phosphorylation of other effector proteins, in particular, JNK phosphorylation was unchanged 1h after anisomycin (21 ± 2 arbitrary intensity values for anisomycin versus 20 ± 1 for vehicle control; $n = 4$). Therefore, the effects of anisomycin to mimic IPC are via its effects to activate and promote downstream signaling through p38.

Modulation of adenosine and potassium channels in IPC: Role of p38

Injection of the adenosine A1 receptor antagonist DPCPX did not modify phosphorylation/activation of p38 at 6 h after IPC. The 6 h time point was chosen because it was the maximal increase in phosphorylated p38 after IPC (see Fig 1). Phosphorylated p38 6 h after IPC with

15 min prior to IPC treatment with the adenosine A1 receptor antagonist DPCPX was $190 \pm 26\%$ of control normal retina (N.S. compared to IPC alone $170 \pm 22\%$ of control, $n = 3$ for both). Injection of the adenosine A2a receptor antagonist CSC increased phosphorylated p38 levels to $244 \pm 19\%$ of control ($n = 3$; $P = 0.05$ by Mann-Whitney test). Therefore, adenosine receptors are involved in IPC by mechanism(s) other than p38 activation and signaling.

Western blot analysis of phosphorylated p38 following mKATP channel activation using diazoxide indicated that p38 protein phosphorylation/activation was significantly increased versus vehicle control (Fig. 7a). At 1 h after diazoxide injection the phosphorylated p38 expression was 445 ± 37 absolute intensity units ($p = 0.05$ by Mann-Whitney test; $n = 3$) compared to 198 ± 14 for the control; after 6 h it increased to 420 ± 56 ($p = 0.05$ by Mann-Whitney; $n = 3$) versus 175 ± 17 for control. There was a trend to increase after 24 h to 533 ± 85 ($p = 0.12$, $n = 3$) vs 323 ± 52 for the vehicle control. Intravitreal injection of p38 α siRNA 24 h before injection of diazoxide to mimic IPC attenuated the ERG a -wave (Fig. 7b; $44 \pm 8\%$ of normal; $p = 0.016$) and the b-wave ($28 \pm 4\%$ of normal; $p = 0.007$; $n = 11$) recovery after ischemia compared to intravitreal injection of non-silencing siRNA (a-wave $74 \pm 8\%$ of normal and b-wave $53 \pm 6\%$; $n = 7$).

DISCUSSION

Our results confirmed the hypothesis that p38 activation is a necessary component of retinal IPC. We demonstrated that: (1) IPC activated p38, as indicated by its phosphorylation, and p38 kinase downstream signaling can be increased by IPC; (2) inhibition of p38 α and p38 β activation by the antagonist SB203580 attenuated the protective effects of IPC on retinal function after ischemia; (3) silencing or interfering with p38 α RNA using p38 α siRNA also reduced the protective effects of IPC on retinal function and histological protection after ischemia (i.e., similar to blocking p38 downstream signaling); (4) IPC neuroprotection was mimicked by anisomycin that increases p38 kinase activity, and protection of functional recovery following anisomycin was diminished by SB203580 (similar to that due to IPC and demonstrating that p38 is an important mechanism); (5) blockade of adenosine receptors A1 and A2a did not attenuate phosphorylation of p38 by IPC, suggesting that p38 is probably independent or upstream of adenosine effects on IPC; and (6) IPC was mimicked by the mKATP channel activator diazoxide that increases activation of p38, and that siRNA targeted to p38 α blocked the mKATP channel activation-induced protection/improved functional recovery (suggesting that mKATP channel opening is upstream of p38 involvement on IPC).

In our previous studies, we found that p38 phosphorylation increased following ischemia (Roth et al. 2003). In the present study we found that p38 is involved in the neuroprotective effects of IPC. Although a small initial decrease in p38 phosphorylation/activation was observed after 1 h, a large, significant increase was observed at 6 h and remained elevated at 24 h after IPC indicative of p38 activation. We confirmed the activation of p38 by measuring the ability of p38 kinase to phosphorylate the target protein ATF-2, 24 h after IPC (i.e., this was increased by > 2 -fold). This indicates that target proteins of p38 downstream signaling can be expected to be modified by 24 h, the time IPC protective effects are observed in our previous studies (Roth et al. 1998; Dreixler et al. 2008; Dreixler et al. 2009a; Dreixler et al. 2009b).

In order to show the role of p38 in IPC, we used either siRNA targeted to p38 α or the p38 inhibitor, SB203580. Previously we have shown that SB203580 appeared to specifically block p38 with our IPC paradigm and not other MAPKs, JNK or ERK (Roth et al. 2003). Furthermore, it has been shown previously that the predominant p38 isoform in the retina is p38 α , while p38 β was undetectable (Kikuchi et al. 2000). Thus we choose a second specific blockade of p38 by using siRNA targeted to p38 α . Showing that both disparate specific inhibitors of retinal p38 signaling can attenuate IPC protection clearly emphasizes the important role of p38 α in

retinal IPC-induced neuroprotection, although results were not uniformly consistent for a and b-wave, suggesting effects upon specific retinal cell types. Histologically, cell numbers in the retinal ganglion cell layer declined significantly more in the p38 α siRNA-treated IPC retinas than in the non-silencing siRNA group. The effects on cell counts in the inner nuclear layer were not as dramatic, suggesting that most of the effects of p38 α blockade occur in cells in the retinal ganglion layer. We cannot identify however these cells more specifically as retinal ganglion cells or displaced amacrine cells. These results are consistent with our previous experiments on the retinal localization of p38 (Roth et al. 2003).

Anisomycin mimicking of the effects of IPC was demonstrated in these experiments. Although anisomycin can inhibit protein synthesis and activate other proteins/MAPK than p38 (Barea-Rodriguez et al. 2000), attenuation of the IPC-mimicking effects of anisomycin was blocked by SB203580, demonstrating that a specific signaling via p38 is required for this protection, similar to that required for IPC-induced protection. Not only did we confirm that downstream p38 signaling can occur 1h after anisomycin injection, but also that the functional retinal protection by IPC and anisomycin are practically equivalent. Both IPC and anisomycin 24 h prior to ischemia are neuroprotective in this *in vivo* model.

We also demonstrated in our present study that p38 is involved in the protective pathway elicited by the opening of mKATP channels by diazoxide. We have previously shown that diazoxide mimics IPC and that the proposed pathway for protection at least partially overlaps with IPC in that we blocked IPC with 5-HD (5-hydroxydecanoic acid), a specific antagonist of mKATP channels (Roth et al. 2006). In this study, we showed that blockade of p38 α with siRNA attenuated IPC-mimicking by diazoxide, suggesting that p38 functions downstream of mKATP channel activation in IPC. Since blocking p38 reduces IPC as well as the mKATP stimulated neuroprotection, p38 activation is part of the common convergent protective pathway of IPC and IPC-mimicking by diazoxide. Opening of the mKATP channel is also upstream of other signaling pathways in retinal IPC including protein kinases B and C (Dreixler et al. 2008; Dreixler et al. 2009b), so p38 signaling is yet another component related to mKATP channels. It is possible that p38, PKC and PKB are inter-related in producing IPC with the mKATP channel or other mechanisms, but the present study only partially examined the cross-signaling pathways.

Adenosine receptor antagonism did not attenuate p38 activation, suggesting that adenosine receptor stimulation in IPC is apparently upstream of p38 involvement. We have previously found that adenosine is involved in IPC-induced protection (Li et al. 1999; Li et al. 2000), but the present data indicate this does not involve p38 but must be via other pathways such as PKC and Akt. Activation of Akt is downstream of adenosine receptors and the opening of mKATP channels (Dreixler et al. 2009b). All three Akt types exhibit protective mechanisms that block the apoptosis caused by retinal ischemia, indicating an intrinsic redundancy in the Akt pathways *in vivo* that protects against ischemic damage.

The results point out the importance of additional studies that monitor changes in MAPKs, Akt and PKC throughout the post-ischemic period in the presence and absence of previous IPC and their relationships to cellular integrity and function. This includes looking at p38 changes as well as looking at the effects of inhibitors of these pathways during this critical survival period on cellular and functional outcome. Our previous and present studies suggest that multiple pathways are involved in the neuroprotective effects of IPC in the retina.

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References

- Aakalu G, Smith WB, Nguyen N, Jiang C, Schuman EM. Dynamic visualization of local protein synthesis in hippocampal neurons. *Neuron* 2001;30:489–502. [PubMed: 11395009]
- Barea-Rodriguez EJ, Rivera DT, Jaffe DB, Martinez JL Jr. Protein synthesis inhibition blocks the induction of mossy fiber long-term potentiation in vivo. *J Neurosci* 2000;20:8528–8532. [PubMed: 11069960]
- Chang L, Karin M. Mammalian MAP kinase signalling cascades. *Nature* 2001;410:37–40. [PubMed: 11242034]
- Cross TG, Scheel-Toellner D, Henriquez NK, Deacon E, Salmon M, Lord JM. Serine/threonine protein kinases and apoptosis. *Exp Cell Res* 2000;256:34–41. [PubMed: 10739649]
- Dreixler JC, Hagevik S, Hemmert JW, Shaikh AR, Rosenbaum DM, Roth S. Involvement of erythropoietin in retinal ischemic preconditioning. *Anesthesiology* 2009a;110:774–780. [PubMed: 19322943]
- Dreixler JC, Hemmert JW, Shenoy SK, Shen Y, Lee HT, Shaikh AR, Rosenbaum DM, Roth S. The role of Akt/protein kinase B subtypes in retinal ischemic preconditioning. *Exp Eye Res* 2009b;88:512–521. [PubMed: 19084003]
- Dreixler JC, Shaikh AR, Shenoy SK, Shen Y, Roth S. Protein kinase C subtypes and retinal ischemic preconditioning. *Exp Eye Res* 2008;87:300–311. [PubMed: 18722601]
- Farh KK, Grimson A, Jan C, Lewis BP, Johnston WK, Lim LP, Burge CB, Bartel DP. The widespread impact of mammalian MicroRNAs on mRNA repression and evolution. *Science* 2005;310:1817–1821. [PubMed: 16308420]
- Gidday J. Cerebral preconditioning and ischaemic tolerance. *Nat Rev Neurosci* 2006;7:437–448. [PubMed: 16715053]
- Hornung V, Guenther-Biller M, Bourquin C, Ablasser A, Schlee M, Uematsu S, Noronha A, Manoharan M, Akira S, de Fougerolles A, Endres S, Hartmann G. Sequence-specific potent induction of IFN- α by short interfering RNA in plasmacytoid dendritic cells through TLR7. *Nat Med* 2005;11:263–270. [PubMed: 15723075]
- Huesken D, Lange J, Mickanin C, Weiler J, Asselbergs F, Warner J, Meloon B, Engel S, Rosenberg A, Cohen D, Labow M, Reinhardt M, Natt F, Hall J. Design of a genome-wide siRNA library using an artificial neural network. *Nat Biotech* 2005;23:995–1001.
- Irving EA, Bamford M. Role of mitogen- and stress-activated kinases in ischemic injury. *J Cereb Blood Flow Metab* 2002;22:631–647. [PubMed: 12045661]
- Judge AD, Sood V, Shaw JR, Fang D, McClintock K, MacLachlan I. Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA. *Nat Biotech* 2005;23:457–462.
- Junk AK, Mammis A, Savitz SI, Singh M, Roth S, Malhotra S, Rosenbaum PS, Cerami A, Brines M, Rosenbaum DM. Erythropoietin administration protects retinal neurons from acute ischemia-reperfusion injury. *Proc Natl Acad Sci USA* 2002;99:10659–10664. [PubMed: 12130665]
- Kikuchi M, Tenneti L, Lipton SA. Role of p38 mitogen-activated protein kinase in axotomy-induced apoptosis of rat retinal ganglion cells. *J Neurosci* 2000;20:5037–5044. [PubMed: 10864961]
- Kumar S, Boehm J, Lee J. p38 MAP kinases: key signalling molecules as therapeutic targets for inflammatory diseases. *Nat Rev Drug Discov* 2003;2:717–726. [PubMed: 12951578]
- Lee JC, Kumar S, Griswold DE, Underwood DC, Votta BJ, Adams JL. Inhibition of p38 MAP kinase as a therapeutic strategy. *Immunopharmacology* 2000;47:185–201. [PubMed: 10878289]
- Li B, Jennings NM, Rosenbaum PS, Maxwell KM, Roth S. Differential roles of adenosine receptor subtypes in retinal ischemia-reperfusion injury in the rat. *Exp Eye Res* 1999;68:9–17. [PubMed: 9986737]
- Li B, Roth S. Retinal ischemic preconditioning in the rat: requirement for adenosine and repetitive induction. *Invest Ophthalmol Vis Sci* 1999;40:1200–1216. [PubMed: 10235554]

- Li B, Yang C, Rosenbaum DM, Roth S. Signal transduction mechanisms involved in ischemic preconditioning in the rat retina in vivo. *Exp Eye Res* 2000;70:755–765. [PubMed: 10843780]
- Li Y, Roth S, Laser M, Ma JX, Crosson CE. Retinal preconditioning and the induction of heat-shock protein 27. *Invest Ophthalmol Vis Science* 2003;44:1299–1304.
- McLaughlin B, Hartnett KA, Erhardt JA, Legos JJ, White RF, Barone FC, Aizenman E. Caspase 3 activation is essential for neuroprotection in preconditioning. *Proc Natl Acad Sci U S A* 2003;100:715–720. [PubMed: 12522260]
- Ping P, Zhang J, Cao X, Li RC, Kong D, Tang XL, Qiu Y, Manchikalapudi S, Auchampach JA, Black RG, Bolli R. PKC-dependent activation of p44/p42 MAPKs during myocardial ischemia-reperfusion in conscious rabbits. *Am J Physiol* 1999;276:H1468–1481. [PubMed: 10330229]
- Roth S, Dreixler JC, Shaikh AR, Lee KH, Bindokas V. Mitochondrial potassium ATP channels and retinal ischemic preconditioning. *Invest Ophthalmol Vis Sci* 2006;47:2114–2124. [PubMed: 16639023]
- Roth S, Li B, Rosenbaum PS, Gupta H, Goldstein IM, Maxwell KM, Gidday JM. Preconditioning provides complete protection against retinal ischemic injury in rats. *Invest Ophthalmol Vis Sci* 1998;39:775–785.
- Roth S, Shaikh AR, Hennelly MM, Li Q, Bindokas V, Graham CE. Mitogen-activated protein kinases and retinal ischemia. *Invest Ophthalmol Vis Sci* 2003;44:5383–5395. [PubMed: 14638742]
- Singh M, Savitz SI, Hoque R, Rosenbaum PS, Roth S, Rosenbaum DM. Cell-specific caspase expression by different neuronal phenotypes in transient retinal ischemia. *J Neurochem* 2001;77:466–475. [PubMed: 11299309]
- Tanaka H, Yokota H, Jover T, Cappuccio I, Calderone A, Simionescu M, Bennett MV, Zukin RS. Ischemic preconditioning: neuronal survival in the face of caspase-3 activation. *J Neurosci* 2004;24:2750–2759. [PubMed: 15028768]
- Tanaka K. Alteration of second messengers during acute cerebral ischemia-adenylate cyclase, cyclic AMP-dependent protein kinase, and cyclic AMP response element binding protein. *Prog Neurobiol* 2001;65:173–207. [PubMed: 11403878]
- Whitlock NA, Agarwal N, Ma J-X, Crosson CE. Hsp27 upregulation by HIF-1 signaling offers protection against retinal ischemia in rats. *Invest Ophthalmol Vis Sci* 2005;46:1092–1098. [PubMed: 15728570]
- Zhang C, Rosenbaum DM, Shaikh AR, Q L, Rosenbaum PS, Pelham DJ, Roth S. Ischemic preconditioning attenuates apoptosis following retinal ischemia in rats. *Invest Ophthalmol Vis Sci* 2002;43:3059–3066. [PubMed: 12202530]

Figure 1.

IPC changed retinal levels of phosphorylated p38 and increased p38 downstream kinase activity/signaling. **(A)** Quantitation of Western blots for phosphorylated p38. IPC significantly changed phosphorylated p38 levels at 6 and 24 h after IPC as compared to 1 h after IPC in the IPC-treated retinae. **(B)** Representative blots of phosphorylated and total p38 used to quantify data for part A, indicate p38 phosphorylation, equal protein loading and stable total p38 protein levels following IPC. **(C)** Representative Western blot non-radioactive kinase activity assay indicating that IPC induced ATF2 phosphorylation. IPC significantly increased p38 kinase activity and potential for downstream signaling 24 h after IPC.

Figure 2.

SB203580, an inhibitor of phosphorylated p38 phosphorylation of downstream targets, significantly decreased IPC protection. (A) Intravitreal injection of SB203580 prior to IPC resulted in a decreased electroretinography (ERG) b-wave recovery 7 d after ischemia compared to PBS (phosphate-buffered saline) vehicle. PBS response was similar to the protection observed with IPC previously and SB203580 response was similar to non-IPC responses seen previously (see text). ERG a-wave recovery was not significantly affected. (B) Representative ERG b-wave responses obtained from PBS- and SB203580-treated rats used to quantitate data shown in (A).

Figure 3. p38α siRNA specificity. Representative images of (A) *in vitro* immunocytochemical analysis of siRNA-treated PC12-T cultured cells. (B) *In vivo* immunohistochemical analysis of siRNA treated frozen retinal sections. The retinal layers are labeled RGC (retinal ganglion cell layer), INL (inner nuclear layer) and PRL (photoreceptor layer). (C) Western blot analysis of the p38α kinase assay on paired p38α siRNA and non-silencing siRNA injected retinal homogenates.

Figure 4.

p38α siRNA significantly decreased IPC protection of the retina. (A) Intravitreal injection of p38α siRNA prior to IPC decreased ERG b-wave recovery 7 d after ischemia compared to non-silencing siRNA. ERG a-wave recovery was not significantly affected. Thus, p38α siRNA exhibited an effect profile similar to the p38 inhibitor SB203580 (see Fig. 2). (B) Representative ERG traces obtained from non-silencing siRNA- and p38α siRNA -treated rats used to quantitate data shown in (A).

Non-silencing siRNA ischemic retina (day 7) **p38 α siRNA ischemic retina (day 7)** **Figure 5.**

Retinal histopathology (5 μ m paraffin stained sections) following ischemia. Representative images of the ischemic eyes harvested 7 d after ischemia comparing non-silencing siRNA-treated retina (left) to p38 α siRNA-treated retina (right) with IPC 6 h after injection followed by ischemia 24 h later.

Figure 6.

Anisomycin produced a rapid increase in retinal p38 downstream signaling, mimicked IPC when injected systemically 24 h prior to ischemia, and the neuroprotection was attenuated by SB203580. **(A)** Western blot analysis of phosphorylated ATF2 expression showed that anisomycin significantly increased p38 kinase activity as early as 1 h after its administration. Thus, anisomycin exhibited an increase in p38 signaling similar to IPC (see Fig. 1). Representative Western blot from vehicle or anisomycin-treated rats used to quantitate data (on right). **(B)** Intravitreal injection of SB203580 6 h before anisomycin, followed 24 h later by ischemia, significantly decreased the ERG b wave recovery 7 d after ischemia compared to intravitreal PBS (phosphate-buffered saline) + anisomycin. ERG a-wave recovery was not significantly affected. Thus, anisomycin exhibited a p38 dependent profile similar to IPC (see **Figs. 2 & 3**). Representative ERG traces obtained from anisomycin and intravitreal PBS- or anisomycin and intravitreal SB203580-treated rats used to quantitate data (on right).

Figure 7.

Retinal p38 phosphorylation increases following administration of the mitochondrial KATP channel opener diazoxide; also shown is siRNA block of IPC mimicking by diazoxide. **(A)** Western blot demonstrated increased phosphorylation of p38 at 1 and 6 h following diazoxide injection. Thus, diazoxide injection (mimicking IPC) exhibited an early increase in p38 phosphorylation/activation relatively similar to IPC (see Fig. 1). Representative Western blots from 1% DMSO (Vehicle) - or diazoxide-treated rats used to quantitate data (on right). **(B)** Intravitreal p38α siRNA injected 24 h prior to diazoxide, followed by ischemia 24 h later, attenuated the IPC-mimicking of post-ischemic recovery at 7 d after ischemia by diazoxide compared to non-silencing siRNA. Results are similar to SB203580 and p38α siRNA effects on IPC (see **Figs. 2 & 3**) and p38α siRNA on anisomycin (see Fig. 5). Representative baseline and 7 d post-ischemia ERG responses for non-silencing siRNA or p38α siRNA treated rats used in the quantitation of data (on right).

Table 1

Histological determination of the percentage of cells ($\pm$ s.e.m.) in the RGC layer and INL layer. The results in the ischemic eyes were normalized to the opposite normal retinae. Results were compared between groups using Mann-Whitney statistical test. The eyes were harvested 7 d after ischemia

<i>Percentage of cells in the ischemic layer</i>	<i>Non-silencing siRNA 6h before IPC, followed by ischemia 24 h later</i>	<i>p38α siRNA 6h before IPC, followed by ischemia 24 h later</i>	<i>p-value non silencing vs p38 siRNA</i>
RGC layer	79 $\pm$ 6%	46 $\pm$ 7%	0.02
INL layer	88 $\pm$ 9%	73 $\pm$ 2%	0.52