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Mitogen Activated Protein Kinase Phosphatase-1 (MKP-1) in Retinal Ischemic Preconditioning

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Abstract

We previously described the phenomenon of retinal ischemic preconditioning (IPC) and we have shown the role of various signaling proteins in the protective pathways, including the mitogen-activated protein kinase p38. In this study we examined the role in IPC of mitogen-activated protein kinase phosphatase-1 (MKP-1), which inactivates p38. Ischemia was produced by elevation of intraocular pressure above systolic arterial blood pressure in adult Wistar rats. Preconditioning was produced by transient retinal ischemia for 5 min, 24 h prior to ischemia. Small interfering RNA (siRNA) to MKP-1 or a control non-silencing siRNA, was injected into the vitreous 6 h prior to IPC. Recovery was assessed by electroretinography (ERG) and histology. The a- and b-waves, and oscillatory potentials (OPs), measured before and 1 week after ischemia, were then normalized relative to pre-ischemic baseline, and corrected for diurnal variation in the normal non-ischemic eye. The P2, or post-photoreceptor component of the ERG (which reflects function of the rod bipolar cells in the inner retina), was derived using the Hood-Birch model. MKP-1 was localized in specific retinal cells using immunohistochemistry; levels of mitogen-activated protein kinases were measured using Western blotting. Injection of siRNA to MKP-1 significantly attenuated the protective effect of IPC as reflected by decreased recovery of the electroretinogram a- and b-waves and the P2 after ischemia. The injection of siRNA to MKP-1 reduced the number of cells in the retinal ganglion cell and outer nuclear layers after IPC and ischemia. Blockade of MKP-1 by siRNA also increased the activation of p38 at 24 h following IPC. MKP-1 siRNA did not alter the levels of phosphorylated jun N-terminal kinase (JNK) or extracellular signal-regulated kinase (ERK) after IPC. The results suggest the involvement of dual-specificity phosphatase MKP-1 in IPC and that MKP-1 is involved in IPC by regulating levels of activated MAPK p38.

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1. INTRODUCTION

Ischemic preconditioning (IPC) by brief ischemia induced robust tolerance to ischemia in rat retina. (Roth et al. 1998) Among the mechanisms of ischemic tolerance in the retina are altered expression of a variety of genes (Kamphuis et al. 2007), decreased apoptosis (Zhang et al. 2002), and involvement of adenosine (Isenmann et al. 1999), HIF-1 α (Zhu et al. 2007), erythropoietin (Dreixler et al. 2009b), and protein kinases including Akt, PKC, and p38. (Dreixler et al. 2008; Dreixler et al. 2009a; Dreixler et al. 2009c)

Activated mitogen-activated protein kinases (MAPKs) are involved in ischemic injury, and we recently reported that MAPK p38 α is an essential component for IPC in the retina. (Dreixler et al. 2009a; Roth et al. 2003) The MAPK family of proteins consists of three main classes of kinases with extensive cross-talk between them: extracellular-regulated kinase (ERK), p38 MAPK, and c-jun N-terminal kinase (JNK). The ERK pathway is mainly activated by growth factors, promoting cell growth, differentiation, and proliferation. JNK and p38 MAPKs are stress activated kinases critically involved in cell survival. (Chang and Karin 2001) p38 MAPK is generally thought to mediate stress-induced apoptosis in the central nervous system. (Fu 1999; Tian et al. 2000)

Dual-specificity phosphatases (DUSPs) dephosphorylate and inactivate MAPKs by acting simultaneously upon phosphorylated threonine and tyrosine residues. The best characterized is MKP-1 (mitogen-activated protein kinase phosphatase-1, DUSP1), a nuclear phosphatase with a specificity about equal for p38 and JNK, followed by ERK, although with the three-dimensional structure of MKP-1 still not known, specificity *in vivo* has not been well characterized. (Patterson et al. 2009) A variety of factors regulate MKP-1 mRNA levels, including heat shock, hypoxia, arachidonic acid, and other stress conditions, as well as MAPK activity. (Boutros et al. 2008) For example, MKP-1 is induced by MAPK activators and stimulated by the p38 MAPK target ATF2. (Breitwieser et al. 2007) Inhibition of p38 MAPK significantly reduces MKP-1 expression. (Hu et al. 2007) Many studies have shown MKP-1 to be an essential negative regulator of the p38 MAPK pathway. (Wu and Bennett 2005; Grethe and Porn-Ares 2006; Breitwieser et al. 2007; Kondoh and Nishida 2007)

In addition to potentially regulating p38 in ischemic preconditioning, there is other evidence to suggest a role for MKP-1 in IPC. Microarray, Northern, and Western blot analyses confirmed that MKP-1 mRNA and protein levels were up-regulated approximately 8-fold by hypoxia in a pheochromocytoma cell line. Cobalt and deferoxamine also increased MKP-1, suggesting that hypoxia-inducible factor (HIF-1 α) may play a role in the regulation of MKP-1 by hypoxia. (Seta et al. 2001) In hypoxic tumor cells, MKP-1 was identified as a hypoxia-responsive gene. (Laderoute et al. 1999) HIF-1 α has been identified as a mediator of ischemic preconditioning in the retina. (Zhu et al. 2007) In this study we hypothesized, based upon our previous investigation of MAPKs in retinal ischemia and ischemic preconditioning, that MKP-1 is an essential component in the signaling events in ischemic preconditioning that trigger ischemic tolerance. We also examined the relationship between IPC, MKP-1, and p38 in the retina *in vivo*.

2. MATERIALS AND METHODS

2.1. Retinal ischemia and preconditioning

Wistar rats (200–250 gm) purchased from Harlan (Indianapolis, IN) were maintained on a 12 h on/12 h off light cycle. Procedures (Roth et al. 2006; Dreixler et al. 2008) conformed to the ARVO Resolution on the Use of Animals in Research and were approved by our Animal Care Committee.

IPC was produced in rats anesthetized with ketamine (35 mg/kg), and xylazine (5 mg/kg) i.p. Corneal analgesia was with 1–2 drops of 0.5% proparacaine (Alcon). Pupils were dilated with 0.5% tropicamide (Alcon), and cyclomydril (0.2% cyclopentolate HCl and 1% phenylephrine HCl (Alcon). A 3-0 sterile silk suture was passed beyond the eye and pulled through PE-100 tubing to maximal tightness for 5 min while observing the retina under the operating microscope for the absence of perfusion. (Dreixler et al. 2008; Dreixler et al. 2009c) IPC was performed 24 h prior to ischemia.

For retinal ischemia, rats were anesthetized with chloral hydrate, 275 mg/kg i.p., and intraocular pressure (IOP) increased after achieving corneal analgesia to 130 – 135 mm Hg for 55 min using a pressurized bag of sterile ocular irrigating solution (BSS: Alcon, Fort Worth, TX) connected to a 30-g needle positioned in the center of the anterior chamber. The IOP was measured continuously using the needle and pressure tubing via a 3-way stopcock interposed into the line, using an electronic pressure transducer (Hewlett-Packard) zeroed to the level of the eye. IOP exceeded the systolic blood pressure, monitored as non-invasively using tail blood pressure cuff (IITC Life Science; Woodland Hills, CA).

Body temperature was maintained at 36–37 C by a servo-controlled heating blanket (Harvard Apparatus, Natick, MA) to prevent a protective effect on ischemia of hypothermia. (Faberowski et al. 1989) To preclude ischemic tolerance due to hypoxia (Zhu et al. 2007), hemoglobin O₂ saturation was measured with a pulse oximeter (Ohmeda; Louisville, CO) on the rat's tail. Supplemental oxygen, when necessary to maintain O₂ saturation > 94%, was administered using a cannula placed in front of the nares and mouth.

2.2. Electoretinography Before and after Retina Ischemia

Procedures were similar to those we reported previously (Roth et al. 2003; Roth et al. 2006, Dreixler, 2008). Animals were dark-adapted for at least 2 h before recordings. For baseline and post-ischemic (i.e., after 7 days) 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 with 1–2 drops of 0.5% proparacaine (Alcon). Pupils were dilated with 0.5% tropicamide (Alcon), and cyclomydril (0.2% cyclopentolate HCl and 1% phenylephrine HCl (Alcon).

The ERG was recorded at baseline (prior to experiments) and 7 days after ischemia by placing platinum needle electroencephalogram electrodes (Grass, Providence, RI) in contact with the corneal surfaces of both eyes and a reference electrode on the tongue, as we reported previously. (Dreixler et al. 2008; Dreixler et al. 2009a; Dreixler et al. 2009b; Dreixler et al. 2009c) Stimulus-intensity ERG analysis was achieved on a UTAS-E4000 using a full-field Ganzfeld stimulator (LKC Technologies, Gaithersburg, MD), with the rat's head centered 7 inches from the stimulator. The low pass filter was 0.05 Hz and the high pass 500 Hz. Flash intensity varied electronically from $-3.39 \log \text{ cd.s/m}^2$ to $1.89 \log \text{ cd.s/m}^2$. Settings were confirmed by photometry (EG & G Model 550 photometer, Electro-Optics, Boulder, CO). Responses were averaged for 3 to 10 flashes delivered 4 to 60 s apart depending upon flash intensity, with number of flashes decreasing and time between them increasing with flash intensity. Flashes were progressively delivered from the lowest intensity to the highest to prevent possible effect upon dark adaptation, and at least 1 min elapsed between the series of flashes for the three highest intensity settings.

Recorded time, intensity, and amplitude were exported and analyzed in Matlab (Math Works, Natick, MA). Recordings were first baseline-corrected for drift and low frequency noise. Peak a-wave amplitude was calculated as the negative minimum following the light stimulus, and the b wave amplitude as difference between the a-wave and the maximum value recorded thereafter. Oscillatory potentials (OPs) were measured by extracting OP wavelet components using Fast Fourier transformation. The sum of the root mean squares

(Sum RMS) of the amplitudes of the OP wavelets was calculated. Scotopic P2 for the rod bipolar response was calculated using the Lamb and Pugh model. (Bui et al. 2005)

2.3. Histology

Eyes enucleated on the seventh day after ischemia were immediately placed in Davidson's fixative (11% glacial acetic acid, 2% neutral buffered formalin and 32% ethanol in H₂O) for 24 h, then transferred to 70% ethanol for 24 h and stored in phosphate-buffered saline at 4 °C. Eyes were embedded in paraffin, sectioned to 4 µm and stained with hematoxylin and eosin (H&E). Sections were examined by light microscopy and retinal ganglion cell (RGC) layer counts quantitated as described earlier (Roth et al. 1998; Junk et al. 2002; Dreixler et al. 2009b). For determination of cell counts in the inner and outer nuclear layers (INL and ONL), images of retinal sections were captured using Micron (Westover Scientific, Mill Creek, WA). Several cell regions of interest (ROI) were selected (around 1500 µm from the optic nerve bundle) and the numbers of cells were manually counted and corrected for the calculated area of the ROI. These results are expressed as the number of cells per unit area (µm²) × 100.

2.4. RNA interference

Target interfering RNA (siRNA) sequences for MKP-1 (siRNA, Qiagen, Valencia, CA) were: CCC GTT CGG GAC CAA TAT ATT, AAC GAG GCG ATT GAC TTT ATA, CAC GAA CAG TGC CCT GAA CTA, and CTG CTG CAA TTT GAG TCC CAA. The siRNAs were designed using neural-network technology as described previously (Huesken et al. 2005). Using BLAST, we found the siRNAs were 100% homologous to the mRNA sequence of MKP-1. siRNA design was then 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); <http://www1.qiagen.com/Products/GeneSilencing/HPOnGuardsiRNADesign.aspx>). Sequences of the introduced siRNA are uniquely specific to the targeted gene (see reviews for more details; (Dorsett and Tuschl 2004; Genc et al. 2004; Lehner et al. 2004; Mello and Conte 2004). A 2 µl mixture of four siRNAs to MKP-1, or a single negative control, non-silencing siRNA not corresponding to any known rat gene (Qiagen) in RPMI media (Invitrogen, Carlsbad, CA) and RNAiFect transfection reagent (Qiagen) at a final concentration of 3 µM was injected into the mid-vitreous of both eyes of the rats with a microsyringe (Hamilton, Reno, NV) as previously described (Roth et al. 2006).

2.5. Cell Culture and Immunocytochemistry

PC-12 -T cells were used to test knockdown of MKP-1 protein by siRNA. The 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 were differentiated for 48 h with the same culture medium, including nerve growth factor (NGF) 100 ng/ml (Invitrogen). The cells were exposed to 400 nM siRNA in RPMI media with RNAiFect transfection reagent (Qiagen) for 24 h. Expression of the targeted proteins was then examined using immunocytochemical techniques. Cultured PC12-T cells plated on collagen-coated glass coverslips were fixed on ice with 4% paraformaldehyde/4% sucrose solution for 15 min. Cells were washed with ice-cold PBS and stored at 4°C in PBS with 10 mM sodium azide until immunostaining. For staining, cells were incubated in primary antibody, rabbit polyclonal anti-MKP-1 (sc-1199, Santa Cruz Biotechnology: Santa Cruz, CA; 1:50 in PBS with 5% dry milk) overnight at 4 °C. Cells were then exposed to goat anti-rabbit IgG rhodamine-conjugate secondary antibody. (1:500; Jackson Immuno Research, West Grove, PA). Antifade mounting media with DAPI (EMC Biosciences, La Jolla, CA) was applied and coverslips were sealed with nail polish and stored at 4°C. The antibody processing was standardized by utilizing standard antibody

concentrations and antibody exposure times, of both the primary and secondary antibodies, in order to allow for the quantification of fluorescent intensities. (Dreixler et al. 2008; Dreixler et al. 2009c)

2.6. Immunohistochemistry

To examine cellular localization of MKP-1, eyes were removed from euthanized rats and evaluated using immunohistochemistry as previously described (Junk et al. 2002; Roth et al. 2006). Enucleated eyes were fixed at room temperature in 4% paraformaldehyde for 3 h. After removal of the anterior segment, the posterior portion of the eye was post-fixed in the same fixative overnight at 4°C before being placed in 25% sucrose for a second overnight period at 4°C for cryoprotection. Eyecups were embedded in OCT compound (Sakura Finetek, Torrance, CA) and were cut into 10-µm thick cryosections.

Primary antibodies (1:50 concentration) included rabbit polyclonal anti- MKP-1 (Santa Cruz Biotechnology), mouse monoclonal anti-calretinin (Santa Cruz), mouse monoclonal anti-vimentin (Santa Cruz), mouse polyclonal calbindin (Sigma), mouse monoclonal anti-PKCα (Transduction Laboratories, Lexington, KY), and biotin anti-rat Thy-1 (BD Pharmingen; (Roth et al. 2006). Control sections were incubated with non-immune serum. Sections were then exposed to the appropriate secondary antibodies: goat anti-rabbit IgG rhodamine-conjugate (1:500; Jackson ImmunoResearch), goat anti-mouse IgG fluorescein-conjugate (1:500; Southern Biotechnology, Birmingham, AL) or FITC-conjugated egg white avidin (1:500; Jackson ImmunoResearch). Antifade mounting media containing DAPI was applied and sections were cover-slipped.

2.7. Imaging and image analysis

For imaging of the PC12-T cells and the immunostained sections, we utilized a fluorescence microscope (Olympus IX81 inverted microscope), a Fast firewire Retiga EXi chilled CCD camera, and a 40× oil lens. Excitation/dichroic/emission settings were 480/40 nm-505LP-535/30 nm for rhodamine and 530–550 nm – 570DM-590LP for greens (FITC and fluorescein). Quantification of the immunocytochemical fluorescent intensities was performed using NIH ImageJ v.1.33, adapted from our previous methods (Roth et al. 2003). The mean intensity was determined for all single isolated cells in four random fields.

2.8. Western blotting

Procedures were those we used in previous studies (Junk et al. 2002; Zhang et al. 2002). Retinas were rapidly dissected and frozen in liquid N₂, and then crushed with a tissue pulverizer (Beckman, Fullerton, CA) on dry ice. Retinas were solubilized in Milliplex MAP lysis buffer (Millipore, Billerica, MA). Protease inhibitor cocktail (P8340; Sigma) consisting of 4-(2-aminoethyl) benzenesulfonyl fluoride, pepstatin A, bestatin, leupeptin, E-64, and aprotinin was added to prevent protease activity. Samples were centrifuged at 10,000g for 10 min. The supernatant was used for SDS-PAGE and the pellet was discarded. Protein concentration was determined using a modified Bradford assay (Bio-Rad, Hercules, CA).

Equal amounts of retinal protein per lane (40 µg) were diluted with SDS sample buffer and loaded onto gels for SDS-PAGE (4%–20% or 16%; Invitrogen). Proteins were electroblotted to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA) and the efficiency of transfer confirmed by staining the membrane with Ponceau S red (Sigma). Non-specific binding was blocked with 5% nonfat dry milk in Tween-Tris-buffered saline (TTBS). Membranes were incubated at 4°C for varying time periods with primary antibodies (Table 1) that were prepared in 5% nonfat dry milk solution in TTBS.

Anti-rabbit horseradish peroxidase (HRP)-conjugated (goat IgG; Jackson ImmunoResearch) or anti-mouse HRP-conjugated (sheep IgG; Amersham, Buckinghamshire, England) secondary antibodies were applied at 1:20,000. Chemiluminescence was developed with a kit (Super Signal West Pico; Pierce, Rockford, IL). Protein bands were digitally imaged with a commercial system (CCDBIO 16SC Imaging System; Hitachi Genetic Systems/MiraiBio, Alameda, CA) and quantitated by densitometry (Gene Snap and Gene Tools software; Hitachi).

2.9. Studies

The role of MKP-1 was specifically examined using RNA interference. Specific siRNAs for MKP-1 or a negative control siRNA (all at 3 μ M concentrations) were injected into the vitreous of both eyes 6 h before IPC, followed by 55 min of ischemia in one eye 24 h later. Additional experiments were performed to determine if siRNA affected the outcome after ischemia independent of IPC. For these studies, results in two parallel groups were compared. Negative control siRNA or siRNA to MKP-1 was injected, followed 6 h later by sham IPC (suture placed but not tightened), then followed by ischemia 24 h later.

To examine impact of MKP-1 inhibition upon MAPKs in preconditioning, we collected retinas 24 h after IPC with injection of non-silencing siRNA or siRNA to MKP-1 6 h previously. This time point for sampling was chosen based upon the 24 h separation between IPC and ischemia used for the studies of outcome after ischemia.

2.10. Data handling and statistical analysis

ERG data were corrected for diurnal variation in the normal eye and for simultaneous reference to the baseline in the ischemic eye, as previously described (Roth et al. 2006; Dreixler et al. 2008; Dreixler et al. 2009b; Dreixler et al. 2009c). The a- and b-waves, Sum RMS of the OPs, and calculated P2 from ischemic eyes 7 d after ischemia in the groups for comparison were expressed as stimulus-intensity plots (intensity, log cd.s/m² on the x-axis), and % recovery of amplitude on the y-axis. (Dreixler et al. 2008) The data were normally distributed as confirmed using normality plots and skewness/kurtosis in Stata version 10.0 (College Station, TX). The data were analyzed between matched groups using ANOVA and post-hoc unpaired t tests (Stata 10.0). Data were analyzed similarly for Western blots. For histopathological data analysis, we could not assume a normal distribution and therefore used the Mann-Whitney non-parametric test.

3. RESULTS

3.1. MKP-1 cellular localization in the retina

To define the cellular localization of MKP-1 in the retina, frozen sections of rat retina were studied using immunohistochemistry. We co-stained with the following antibodies to specific protein markers for co-localization of MKP-1 in retinal cells: 1) Thy1 for RGCs (Dreixler et al. 2008), 2) vimentin for Müller cells (Verardo et al. 2008), 3) calretinin for amacrine cells (Kielczewski et al. 2005), 4) PKC α for bipolar cells (Roth et al. 2003), and 5) calbindin for horizontal cells. (Hirano et al. 2007; Dreixler et al. 2008) Fig. 1 shows co-localization of MKP-1 with cellular markers in RGCs (**A**), bipolar cells (**B**), displaced amacrine cells in the RGC layer (**C**), horizontal cells (**D**), and Müller cells (**E**). Moreover, co-staining of MKP-1 and DAPI, as seen in **A and C**, further suggest that MKP-1 is located in cells other than RGCs in the retinal ganglion cell layer such as displaced amacrine cells. Control sections for primary antibodies incubated with non-immune serum demonstrated no staining (not shown).

3.2. In Vitro/Cellular validation of MKP-1 siRNA silencing

In PC12-T cells, (Fig. 2) treatment for 24 h with MKP-1 siRNA significantly decreased MKP-1 protein levels to 16 ± 2 mean intensity absolute units as compared to a mean intensity of 47 ± 8 for non-silencing siRNA-treated controls ($p = 0.01$; $n = 6$).

3.3. Blocking MKP-1 with siRNA and IPC

Intravitreal injection of MKP-1 siRNA 6 h prior to IPC ($n = 6$), followed by ischemia 24 h later significantly decreased the a-wave, b-wave, and P2 recovery at 7 days after ischemia, but not the OPs when compared to the non-silencing RNA injected ($n = 7$) control group (Figs. 3A, 4A).

Histological examination of the retinae 7 d after ischemia showed that the number of cells in the RGC layer in the ischemic retinae significantly decreased from 9.9 ± 0.6 for non-silencing siRNA/IPC + ischemia ($p = 0.05$; $n = 6$) to 8.0 ± 0.5 ($n = 6$) in the MKP-1 siRNA/IPC + ischemia ($p = 0.05$; $n = 6$) group (Table 2A). In the latter, the inner and outer nuclear layers were folded and demonstrated inflammatory cell infiltration (Fig. 5). The RGCs in the control non-ischemic eyes were 10.5 ± 0.6 for non-silencing siRNA/IPC + ischemia and 10.7 ± 0.5 in the MKP-1 siRNA/IPC + ischemia group. The number of cells per unit area (μm^2) $\times 100$ for the INL was not significantly changed (Table 2B). The number of cells per unit area (μm^2) $\times 100$ for the INL in the control non-ischemic eyes was 2.4 ± 0.01 for non-silencing siRNA/IPC + ischemia and 2.1 ± 0.01 in the MKP-1 siRNA/IPC + ischemia group. The number of cells/area in the ONL for the ischemic retinae significantly decreased from 5.1 ± 0.2 ($p = 0.05$; $n = 6$) for non-silencing siRNA/IPC + ischemia to 4.4 ± 0.2 ($n = 6$) in MKP-1 siRNA/IPC + ischemia group ($p < 0.05$, Table 2C). The number of cells per unit area (μm^2) $\times 100$ for the ONL in the control non-ischemic eyes was 5.4 ± 0.01 for non-silencing siRNA/IPC + ischemia and 4.8 ± 0.01 in the MKP-1 siRNA/IPC + ischemia group.

Retinal functional recovery in rats receiving intravitreal injection of MKP-1 siRNA 6 h prior to sham IPC ($n = 7$) followed 24 h later by ischemia was compared with rats receiving intravitreal injection of non-silencing siRNA 6 h prior to sham IPC ($n = 6$), then ischemia 24 h later, to determine whether MKP-1 siRNA caused any effect on ischemia. No significant difference was found between the two groups (Fig. 3B, 4B).

Histological examination of the retinae 7 d after ischemia in these groups showed that the number of cells in the RGC layer in the ischemic retinae significantly decreased from 8.6 ± 0.3 for non-silencing siRNA/sham IPC + ischemia ($p = 0.05$; $n = 6$) to 6.8 ± 0.8 ($n = 7$) in the MKP-1 siRNA/sham IPC + ischemia group (Table 3A). The RGCs in the control non-ischemic eyes were 13.8 ± 1.2 for non-silencing siRNA/sham IPC + ischemia and 11.8 ± 0.4 in the MKP-1 siRNA/sham IPC + ischemia group. Retinae in both groups showed folding of the inner and outer retinal layers and inflammatory cell infiltration (Fig. 5). The number of cells per unit area (μm^2) $\times 100$ for the INL (Table 3B) and the ONL (Table 3C) was not significantly changed. The number of cells per unit area (μm^2) $\times 100$ for the INL in the control non-ischemic eyes was 2.6 ± 0.01 for non-silencing siRNA/sham IPC + ischemia and 2.6 ± 0.01 in the MKP-1 siRNA/sham IPC + ischemia group. The number of cells per unit area (μm^2) $\times 100$ for the ONL in the control non-ischemic eyes was 5.7 ± 0.03 for non-silencing siRNA/sham IPC + ischemia and 6.0 ± 0.03 in the MKP-1 siRNA/sham IPC + ischemia group.

3.4. Mechanisms of action of MKP-1 in ischemic preconditioning

The injection of siRNA to block MKP-1 significantly enhanced the phosphorylation of p38 at 24 h after IPC. However, there was no effect upon phosphorylation of other MAPKs, ERK and JNK (Fig. 6).

4. DISCUSSION

The results suggest that MKP-1 is a necessary component in IPC. Blocking MKP-1 production with interfering RNA specific for MKP-1 attenuated the enhanced recovery of the amplitudes of the a-wave, b-wave, and the calculated P2 after ischemia produced by prior IPC. The derived P2, which reflects activity in rod bipolar cells, is a more specific indicator of the activity in the inner nuclear layer, and supports the notion that IPC and ischemia exert their primary effects in the inner retina. (Green and Kapousta-Bruneau 1999) Even though our co-localization experiments (see Fig. 1) did not show MKP-1 in the photoreceptors, the functional protection by MKP-1 seen for the a-wave and photoreceptors may be due to activation of downstream proteins (such as p38) after IPC. Histological results showing decreased cells in the RGC layer in the preconditioned and ischemic MKP-1-treated retinas compared to those treated with non-silencing siRNA support the findings that MKP-1 altered the inner retinal neuroprotective effect of IPC. Blockade of MKP-1 did not alter the functional recovery after ischemia without prior IPC (sham IPC), but did decrease the number of surviving cells in the RGC layer after ischemia. This further suggests that MKP-1 is involved in the retina's endogenous response to ischemia.

Using antibodies directed against targets on specific retinal cells, we found that MKP-1 was present in retinal ganglion cells, amacrine cells in the inner nuclear layer and displaced amacrine cells in the RGC layer, horizontal and bipolar cells, and in Müller cells. The inner nuclear localization of MKP-1 is congruent with the effects of MKP-1 inhibition on inner retinal function after IPC and ischemia. Moreover, MKP-1 localization in this study resembles that which we previously showed for MAPKs in retina. (Roth et al. 2003)

Protein tyrosine phosphatases are a very large family of enzymes that dephosphorylate tyrosine residues. Several of them have recently been shown to be important in retinal physiology and disease including protein tyrosine phosphatase Shp2, and protein-tyrosine phosphatase-1B. (Cai et al. 2010; Rajala et al. 2010) MAPK phosphatases are a subclass of protein tyrosine phosphatases that dephosphorylate phosphotyrosine and phosphothreonine on MAPKs. To date, only one study has examined MAPK phosphatase in retina, where the authors found expression of high levels of the MAPK phosphatase DUSP3 in human retina without identifying a physiological role for this protein in the retina. (Danciger et al. 2001) The exact mechanism of MKP-1 activation is not known, but is believed to consist of a double feedback mechanism involving MAP kinases. Thus the MAPK target p38 has been shown to regulate the induction of MKP-1 at the translational level through MK2 or ATF2. (Breitwieser et al. 2007; Hu et al. 2007) Activated ERK can also alter MKP-1 activity by phosphorylating it and inhibiting ubiquitination to prevent its degradation. (Brondello et al. 1999)

The relationship between MKP-1, the best characterized of the MAPK phosphatases, MAPKs, and preconditioning and ischemia is particularly of interest considering the major role of MAPKs in retinal ischemia and preconditioning. Results of inhibition by siRNA suggest that the neuroprotective effect of preconditioning is significantly compromised when MKP-1 is blocked. One possible explanation of this finding is a change in activation of p38. In this study, we found that blockade of MKP-1 prior to IPC increased levels of phosphorylated p38 at 24 h after IPC, consistent with a role for MKP-1 in regulating p38 activation in the retina. The effect appears to be specific, as other MAPKs and potential targets of MKP-1, ERK and JNK, were not affected. In earlier studies we demonstrated that p38 plays a dual role in preconditioning and ischemia. (Roth et al. 2003; Dreixler et al. 2009a) While p38 is required for preconditioning, increases in p38 activation after ischemia are associated with ischemic injury. (Roth et al. 2003) Excessive activation of p38 in the

face of blockade of MKP-1 may have antagonized the neuroprotective p38 effect in preconditioning.

Little is known about the role of MKP-1 in ischemia. In this study, blockade of MKP-1 did not significantly alter functional recovery after ischemia without prior IPC. But the decrease in cells in the RGC layer suggests an influence upon post-ischemia outcome. Increased expression of MKP-1 has been shown to prevent peptidoglycan-induced increases in tumor necrosis factor (TNF)- α and other pro-apoptotic pathways. (Shepherd et al. 2004) Moreover, atrogin-1, a major atrophy-related E3 ubiquitin ligase, markedly enhanced apoptosis after ischemia in cardiomyocytes via activation of JNK signaling by interacting with and triggering MKP-1 for ubiquitin-mediated degradation. (Xie et al. 2009) These results suggest that increased MKP-1 expression promotes cell survival and is a component of the retina's endogenous protective mechanisms.

In summary, our data suggest that MKP-1 plays a role in ischemic preconditioning, and functions as a modulator of p38 activation. Further studies to determine the mechanisms behind MKP-1 expression in IPC will provide a more complete picture of how neuroprotection is achieved and may lead to advancements in treatments against ischemic neurodegenerative diseases.

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Figure 1.

Co-localization of MKP-1 (red), retinal cell markers (green) and DAPI (blue). Co-localization (as depicted by an orange/yellow color) of MKP-1 with the retinal ganglion cell marker, Thy1 (A), with bipolar cell marker, PKC α (B), with amacrine cell marker, calretinin (C), with horizontal cell marker, calbindin (D), and with Müller cell marker, vimentin (E), is indicated by white arrows. Co-localization of MKP-1 and DAPI alone (showing nuclear localization, pink color) is indicated by white arrowheads. The fluorescent images utilized 40 $\times$ oil magnification. Control sections for primary antibodies incubated with non-immune serum demonstrated no staining (not shown). Scale bars are shown at the bottom of each figure. The figure shows that MKP-1 is present in nuclei throughout the inner retina, and in RGCs, amacrine cells, bipolar and horizontal cells, and Muller cells.

Figure 2. MKP-1 siRNA efficacy was demonstrated in PC12-T cells by immunocytochemistry. At 24 h of incubation with siRNA, there was decreased MKP-1 in MKP-1 siRNA-treated cells (right) as compared to non-silencing control siRNA-treated cells (left).

Figure 3.

Stimulus-Intensity response of the electroretinogram and the effects of siRNA to MKP-1 on preconditioning and ischemia. A. Double normalized (corrected for the non-ischemic eye and for diurnal variation from baseline to 7 days after ischemia) ERG data for a-, b-wave, oscillatory potentials (sum of root mean square, OP RMS) and P2 over a range of flash intensities from -1.02 to 1.40 log cd-s/m² in the IPC experiments. The data were recorded at baseline (prior to preconditioning and ischemia) and at 7 days after ischemia.

Preconditioning was performed 24 h prior to ischemia, and after obtaining baseline recordings. Dashed lines with triangles = non-silencing siRNA + IPC + ischemia. Solid lines with diamonds = MKP-1 siRNA + IPC + ischemia. It can be seen that siRNA to MKP-1 significantly decreased the recovery after ischemia and preconditioning of the a-, b- and P2 waves. B. Double normalized (corrected for the non-ischemic eye and for diurnal variation from baseline to 7 days after ischemia) ERG data for a-, b-wave, OP RMS and P2 over a range of flash intensities from -1.02 to 1.40 log cd-s/m² in the sham IPC experiments. The data were recorded at baseline (prior to sham preconditioning and ischemia) and at 7 days after ischemia. Sham preconditioning was performed 24 h prior to ischemia, after obtaining baseline recordings. There was no significant effect of siRNA to MKP-1 on functional recovery after ischemia without prior preconditioning (sham preconditioning). Overall, results suggest that the inhibition of MKP-1 by siRNA is affecting the IPC alone, and not the response to ischemia. Dashed lines with triangles = non-silencing siRNA + sham IPC + ischemia. Solid lines with diamonds = MKP-1 siRNA + sham IPC + ischemia.

Figure 4.

Representative ERG waveforms showing the neuroprotection by IPC that was attenuated by siRNA to MKP-1, and no effect of siRNA to MKP-1 on ischemia without prior preconditioning. The waveforms are shown at 7 days after ischemia. A. Representative ERG waves for a- and b-waves (top) and OPs (bottom) for non-silencing or MKP-1 siRNA + IPC + ischemia. B. Representative ERG waves for a- and b-waves (top) and OPs (bottom) for non-silencing or MKP-1 siRNA + sham IPC + ischemia.

Figure 5.

Representative histopathological images of hematoxylin and eosin-stained retinæ in 4 µm thick sections for each of the experimental groups. These sections were prepared from retinæ removed from the rats at 7 days following ischemia. Arrows indicate layers demonstrating cell loss. Asterisks denote regions of inflammatory cell infiltration. Retinal cell layers are labeled in a normal retina in the top left image. These deleterious changes are seen in the MKP-1 siRNA + IPC + ischemia and both sham IPC + ischemia groups, but not in non-silencing siRNA + IPC + ischemia. A close-up of an area of each section is depicted below each section in order to better demonstrate cell loss and inflammation. RGC = retinal ganglion cell layer; INL = inner nuclear layer; ONL = outer nuclear layer.

Figure 6.

The effect of MKP-1 siRNA treatment on downstream effectors by Western blot analysis. The results are expressed as: (intensity level of phosphorylated MAPK in IPC retina/ intensity level of phosphorylated MAPK in control retina) divided by (the intensity level of total MAPK in IPC retina/intensity level of total MAPK in control retina) $\times$ 100. MKP-1 siRNA injected into the vitreous 6 h before IPC resulted in an increase in phosphorylated p38 at 24 h after IPC as compared to treatment with non-silencing siRNA (A). Phosphorylated ERK (B) and phosphorylated JNK (C) were not significantly changed. Representative Western blots are depicted on the right. These data suggest that MKP-1 is involved in the activation of p38, but not ERK or JNK, in our experimental paradigm.

Table 1

Antibodies used for Western blotting

Primary Antibody (all are rabbit polyclonal)	Concentration	Incubation time	Manufacturer
ERK	1:1000	Overnight	Cell Signaling (Danvers, MA)
Phospho ERK (Thr202/Tyr204)	1:2000	Overnight	Cell Signaling
JNK	1:1000	Overnight	Cell Signaling
Phospho JNK/SAPK (Thr183/Tyr185)	1:500	Overnight	Cell Signaling
p38	1:1000	Overnight	Cell Signaling
Phospho p38 (Thr180/Tyr182)	1:500	48 h	Promega (Madison, WI)

Table 2

Cell counts in the retina following ischemia in preconditioned eyes

A: Number of cells in retinal ganglion cell (RGC) layer in the ischemic retinae 7 days after ischemia with non-silencing siRNA/IPC compared to the number of cells in the ischemic retinae for MKP1 siRNA/IPC. MKP1 siRNA/IPC produced a significant decrease in the number of cells in the RGC layer.		
	Number of cells in RGC layer	p-value
Non-silencing siRNA/IPC	9.9 ± 0.6	---
MKP1 siRNA/IPC	8.0 ± 0.5	0.05

B: Number of inner nuclear layer (INL) cells/area (μm^2; $\times 100$) in the ischemic retinae 7 days after ischemia with non-silencing siRNA/IPC compared to the number of cells in the ischemic retinae for MKP1 siRNA/IPC. MKP1 siRNA/IPC did not significantly change the number of cells in the INL.		
	Number of INL cells/area	p-value
Non-silencing siRNA/IPC	2.3 ± 0.1	---
MKP1 siRNA/IPC	2.2 ± 0.1	0.42

C: Number of ONL cells/area (μm^2; $\times 100$) in the ischemic retinae 7 days after ischemia with non-silencing siRNA/IPC compared to the number of cells in the ischemic retinae for MKP1 siRNA/IPC. MKP1 siRNA/IPC produced a significant decrease in the number of cells in the ONL.		
	Number of ONL cells/area	p-value
Non-silencing siRNA/IPC	5.1 ± 0.2	---
MKP1 siRNA/IPC	4.4 ± 0.2	0.05

Table 3

Cell counts in the retina following ischemia in sham preconditioned eyes

A: Number of cells in the retinal ganglion cell layer (RGC) in the ischemic retinae 7 days after ischemia with non-silencing siRNA/sham IPC compared to the number of cells in the ischemic retinae for MKP1 siRNA/sham IPC. MKP1 siRNA/sham IPC produced a significant decrease in the number of cells in the RGC layer.		
	Number of cells in RGC layer	p-value
Non-silencing siRNA/Sham IPC	8.6 ± 0.3	---
MKP1 siRNA/Sham IPC	6.8 ± 0.8	0.05

B: Number of INL cells/area (μm^2; $\times 100$) in the ischemic retinae 7 days after ischemia with non-silencing siRNA/sham IPC compared to the number of cells in the ischemic retinae for MKP1 siRNA/sham IPC. MKP1 siRNA/sham IPC produced a non-significant change in number of cells in the INL.		
	Number of INL cells/area	p-value
Non-silencing siRNA/Sham IPC	2.5 ± 0.2	---
MKP1 siRNA/Sham IPC	2.5 ± 0.1	0.83

C: Number of ONL cells/area (μm^2; $\times 100$) in the ischemic retinae 7 days after ischemia with non-silencing siRNA/sham IPC compared to the number of cells in the ischemic retinae for MKP1 siRNA/sham IPC. MKP1 siRNA/sham IPC produced a non-significant change in number of cells in the ONL.		
	Number of ONL cells/area	p-value
Non-silencing siRNA/Sham IPC	5.0 ± 0.2	---
MKP1 siRNA/Sham IPC	5.5 ± 0.1	0.07