Mitogen-Activated Protein Kinases and Retinal Ischemia

Steven Roth,1,2,3 Afzhal R. Shaikh,1 Meghann M. Hennelly,1 Qing Li,1 Vytas Bindokas,2 and Christine E. Graham1

PURPOSE. Mitogen-activated protein kinases (MAPKs), consisting of three major enzymes—extracellular signal-regulated kinase (ERK), p38, and c-jun N-terminal kinase (JNK)—couple cell-surface receptors to critical regulatory targets and gene transcription. We hypothesized that MAPKs are differentially expressed and have distinct functions after retinal ischemia.

METHODS. Rats were subjected to retinal ischemia by elevation of intraocular pressure. Changes in MAPK expression were examined by Western blot of whole retinal homogenates and by immunohistochemistry of retinal cryosections. Phosphorylated (activated) ERK, p38, and JNK proteins were localized by fluorescent double labeling. The functional significance of activated MAPKs was assessed using pharmacological antagonists. Specific MAPK blockade was documented by kinase assay and immunohistochemistry for phosphorylated target proteins. The outcome after ischemia was examined with electroretinography (ERG), by measuring retinal cell layer thickness in paraffin-embedded sections, and by TUNEL staining on retinal cryosections. Data were analyzed using ANOVA and post hoc t-test, with P < 0.05 considered significant.

RESULTS. Expression of phosphorylated JNK and p38 increased significantly after ischemia and followed a specific time course, beginning at 1 hour, and persisting up to 1 week later. JNK and p38 were expressed in the nuclei of ganglion and amacrine cells, the outer plexiform layer, the nerve fiber layer, and the axonal terminals of bipolar cells. Phosphorylated ERK was expressed in Müller cells, peaking at 1 to 6 hours after ischemia. Blocking activation of p38 or ERK significantly improved recovery of the ERG b-wave after ischemia, dramatically decreasing thinning of the inner nuclear layers, and decreased the percentage of TUNEL-positive cells.

CONCLUSIONS. The MAPKs each demonstrate a specific cellular distribution after ischemia, and ERK and p38 are linked to apoptosis. Blockade of p38 or ERK provides significant protection from ischemic damage, suggesting a novel therapeutic role for MAPK inhibition in neuroprotection.

Materials and Methods

Ischemia Methodology

Our procedures22 conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Animal Care Committee at the University of Chicago. In brief, Sprague-Dawley rats (200–250 g) purchased from HarlanSprague-Dawley (Indianapolis, IN) were maintained on a 12-hour light-dark cycle and were dark adapted for at least 2 hours before experiments. Before ischemia was induced, animals were anesthetized with intraperitoneal

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chloral hydrate (450 mg/kg). For baseline and postischemia follow-up electroretinograms (ERGs), rats were injected intraperitoneally with ketamine (53 mg/kg; Parke-Davis, Morris Plains, NJ), and xylazine (5 mg/kg; Bayer Animal Health, Shawnee Mission, KS). Corneal analgesia was achieved using 1 to 2 drops of 0.5% proparacaine (Allergan, Hombilguieros, Puerto Rico). Pupillary dilation was maintained using 0.5% tropicamide (Alcon, Humacao, Puerto Rico) and cyclopleydr (0.2% cyclopentolate HCl and 1% phenylephrine HCl; Alcon, Fort Worth, TX). Body temperature was maintained at 36.5°C to 37.0°C using a servocontrolled heating blanket (Harvard Apparatus, Natick, MA).

The intraocular pressure (IOP) was increased to 110 mm Hg for 45 or 60 minutes, by using an elevated 500-µL plastic container of sterile normal saline (Baxter, North Chicago, IL), connected to a 27-gauge needle placed in the anterior chamber of the eye. The opposite eye of each animal served as a nonsichronic control.

**Immunoblot Analysis**

Procedures were the same as those used in previous studies.22,23 To study phosphorylation of ERK, JNK, and p38, retinas were rapidly dissected from euthanatized rats and frozen in liquid N2, and then crushed with a tissue pulverizer (Beckman Instruments, Fullerton, CA) on dry ice, and solubilized in 9 M urea, 4% Nonidet P-40, and 2% 2-mercaptoethanol, at pH 9.5. Protease inhibitor cocktail (P8340; Sigma-Aldrich, St. Louis, MO) consisting of 4-(2-aminoethyl) benzenesulfonyl fluoride, pepstatin A, bestatin, leupeptin, E-64, and aprotinin, was added to prevent protease activity. Samples were centrifuged for 10 minutes at 14,000g. The supernatant was used for SDS-PAGE and the pellet discarded. Protein concentration was determined with 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% gradient; Invitrogen, San Diego, CA). Proteins were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore, Bedford, MA) and the efficiency of transfer was confirmed by staining the membrane with ponceau S red (Sigma-Aldrich). Gel retention was assessed by staining with Coomassie blue (Pierce, Rockford, IL). Non-specific binding was blocked with 5% nonfat dry milk in Tween-Tris-buffered saline (TTBS). Membranes were incubated overnight at 4°C with anti-phospho-p44/p42 MAPK (p-ERK, rabbit polyclonal, Thr202/Tyr204; 1:2000, Cell Signaling Technology; Beverly, MA), anti-diphosphorylated JNK (rabbit polyclonal, Thr183/Tyr185, 1:1000; Cell Signaling Technology), and anti-diphosphorylated p38 (rabbit polyclonal, pTgYp; 1:2000; Promega, Madison, WI). Anti-diphosphorylated p38 was prepared in TTBS with 5% nonfat milk, and anti-phospho-p44/p12 MAPK and anti-diphosphorylated JNK were prepared in TTBS with 5% BSA.

Anti-rhodopsin (monoclonal mouse, clone Rho1D2, 1:1500) was used to verify equal loading of protein. The antibody was a gift from Robert Molday (University of British Columbia, Victoria, British Columbia, Canada). Appropriate horseradish peroxidase (HRP)–conjugated secondary antibodies, which were anti-rabbit (goat IgG; Jackson ImmunoResearch, West Grove, PA), or anti-mouse (sheep IgG; Amersham, Arlington Heights, IL) were applied at concentrations of 1:20,000. Chemiluminescence was developed with a kit (Super Signal West Pico, Pierce). Protein bands were digitally imaged with a commercial system (CCD BIO 16SC Imaging System; Hitachi Genetic Systems/MiraBló, Alameda, CA).

**Electroretinography**

Procedures used in our laboratory have been described in detail.24–26 In brief, responses to 10-µs white-light flashes from a Ganzfeld (Nicolet, Madison, WI) were recorded on a data-acquisition system (Spirit 486 System; Nicolet). Data are the average of three flashes delivered at least 2 minutes apart. The ERG wave amplitudes 3 and 7 days after ischemia were measured and reported as a percentage of the baseline, nonsichronic wave amplitude.

**Histopathology**

The eyes were enucleated on the seventh day after ischemia and immediately placed in Davidson’s fixative. The posterior portion of the eye was separated carefully to avoid retinal detachment, and then embedded in paraffin. Sections 5 µm thick were stained with hematoxylin and cosin (H&E) and examined by light microscopy. Retinal cell layer thickness was quantitated as described by us earlier.22–24,27

**Immunohistochemistry**

Enucleated eyes were fixed at room temperature in 4% paraformaldehyde for 2 hours. After removal of the anterior segment, the posterior portion of the eye was postfixed in the same fixative for 4 hours before being placed in 30% sucrose overnight at 4°C for cryoprotection. Eye cups were embedded in OCT compound (Sakura Finetec, Torrance, CA) and were cut into 10-µm-thick cryosections. For the peroxidase (3,3′-diaminobenzidine; DAB) staining procedure, sections were incubated overnight at 4°C in 1:2000, 1:1000, and 1:1000 dilutions, respectively, of primary antibody: anti-phospho-p42/p44 MAPK, anti-phaspho-JNK, and anti-phospho-p38. Cleaved caspase-3 antibody (rabbit polyclonal, Asp175; Cell Signaling Technology) was applied at 1:500. Anti-phospho-ATF-2 (rabbit polyclonal, Thr71; Cell Signaling Technology) and anti-phospho-c-jun (rabbit polyclonal, Ser63; Cell Signaling Technology) were used at concentrations of 1:1000. After sections were washed in PBS, they were immunostained with an avidin-biotin complex kit (Vectastain Elite ABC; Vector Laboratories, Burlingame, CA). Sections were incubated with universal biotinylated secondary antibody (Vector), and a Dako (Carpinteria, CA) liquid DAB kit was used to develop the brown color.

To investigate the cell types expressing the phosphorylated ERK, JNK, and p38, a double-labeling procedure using mouse monoclonal antibodies was performed in a manner similar to that in our previous studies.27 Antibodies included anti-syntaxin (clone HPC-1, 1:500; Sigma-Aldrich), anti-Thy-1 (1:50; BD PharMingen, San Diego, CA), anti-calbindin (1:500, Sigma-Aldrich), or anti-glial fibrillary acidic protein (GFAP; Alexa Fluor 488 conjugate, 1:50; Molecular Probes, Eugene, OR). Nuclei were identified with a green nucleic acid stain (1 µM) in dimethyl sulfoxide (DMSO; Sytox; Molecular Probes). Sections were exposed to fluoresein (fluoresein conjugated avidin, 1:500), Jackson ImmunoResearch, goat anti-mouse IgG FITC conjugate, 1:200; Southern Biotechnology, Birmingham, AL) or rhodamine (anti-rabbit IgG rhodamine conjugate, 1:200; Jackson ImmunoResearch) secondary antibody.

<table>
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<tr>
<th>Table 1. Cell Counts for Phosphorylated JNK and p38 (P-JNK and P-p38) after 60 Minutes of Ischemia</th>
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<tr>
<td><strong>P-JNK Cell Count</strong></td>
</tr>
<tr>
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<tr>
<td>0</td>
</tr>
<tr>
<td>1</td>
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<tr>
<td>6</td>
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<tr>
<td>24</td>
</tr>
<tr>
<td>72</td>
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<tr>
<td>168</td>
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See Figure 1 for explanation. Counts are expressed as the number of immunopositive cells per 40 × field (mean ± SEM). There was a significant increase in P-JNK and p38 activation after ischemia. *P < 0.05; **P < 0.005; #P < 0.001.
antibody for 2 hours and then examined by fluorescence microscopy (Axioplan; Carl Zeiss Meditec, Thornwood, NY) with a charge-coupled device (CCD) camera (FxHQ; Photometrics). Images were then deconvolved on computer (Open Laboratory; ImproVision, Lexington, MA).

**TUNEL Staining**

TdT-mediated dUTP-biotin nick-end labeling (TUNEL) staining was performed on 10-μm-thick frozen retinal sections with a TdT-fluorescein fragmentation detection kit (FragEL DNA; Oncogene, La Jolla, CA), with procedures modified from Gavrieli et al. and as we described previously. TUNEL-positive cell nuclei were visualized as a green color; the total cell population was identified as a blue color using DAPI stain incorporated into the coverslip mounting medium. TUNEL-positive cells were considered apoptotic if they were undergoing cellular shrinkage and chromatin condensation based on observations by microscopy.

**Image Analysis**

Positive immunostaining cells on retinal sections were counted in five adjacent 40× fields starting within 50 μm of the optic nerve head, advancing progressively toward the periphery. The number of positive cells was expressed as the mean ± SEM per high-power field in the ganglion cell (GCL) and inner (INL) and outer nuclear (ONL) layers. TUNEL-positive and DAPI-stained cells were counted using a protocol we developed in Image J software (available at http://rsb.info.nih.gov/}

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**Figure 1.** The time course of changes in expression of phosphorylated ERK, JNK, and p38 is shown in 10-μm-thick retinal cryosections stained with DAB and photographed under light microscopy. Times on the right indicate the duration of ischemia. Times at the top indicate elapsed time after ischemia. ERK: phosphorylated ERK was evident in Müller cells (arrowbeads) and in astrocytic projections (arrows) for up to 24 hours after ischemia. JNK: phosphorylated JNK was present in RGCs (arrowbeads). The peak increase in phosphorylated ERK and JNK was at 1 to 6 hours after ischemia. Phosphorylated JNK was still present in the RGCs 24 hours after ischemia. p38: phosphorylated p38 was expressed in RGCs (arrowbeads) and in the INL (arrows). Its expression increased up to and including 168 hours after ischemia. Magnification: ×40; insets: ×100.
adequately inhibitory dose. solubilization for parenteral use and hence the capacity to achieve an injection was chosen rather than intravitreal because of greater ease of inhibited phosphorylation of ERK in rat whole retinal homogenates based on preliminary experiments in which we determined that it

FIGURE 2. Western blot analysis of retinal homogenates collected up to 168 hours after ischemia. Each lane contains protein from three pooled rat retinas. Phosphorylated p38, ERK, and JNK increased between 1 to 24 hours after ischemia. The results with anti-rhodopsin confirmed equal loading of proteins. N, normal.

Effects of Inhibition of MAPKs

The inhibitors used in this study were U0126 for ERK (Promega), SP600125 (JNK Inhibitor 2, JNKII) and JNK Inhibitor I (JNKI1) for JNK (Tocris, St. Louis, MO, and Calbiochem, San Diego, CA, respectively), and SB203580 HCl for p38 (Calbiochem). The highly specific MEK inhibitor U0126 noncompetitively inhibits activation of ERK by interfering with the adenosine triphosphate (ATP) and ERK substrate binding of MAPK kinase (MEK). It has approximately 100-fold greater affinity for MEK than the other commonly used MEK inhibitor, PD098059,5-55 U0126 (26 mM in 1% DMSO) was injected intraperitoneally (2000 μg/kg), 1 hour before ischemia. This dose was chosen based on preliminary experiments in which we determined that it inhibited phosphorylation of ERK in rat whole retinal homogenates from control and ischemic-perfused eyes (Fig. 10). Intraperitoneal injection was chosen rather than intravitreal because of greater ease of solubilization for parenteral use and hence the capacity to achieve an adequately inhibitory dose.

SB203580 HCl is a highly specific, cell-permeable and water-soluble inhibitor of p38.55 We injected into the vitreous 2 μL of a 24-mM solution, 15 minutes before and immediately after ischemia ended, resulting in an estimated vitreous concentration of 160 μM after each injection. JNKI1 is a cell-permeable, biologically active peptide that blocks phosphorylation of c-Jun. It contains the minimal 20-amino-acid inhibitory domain of islet-brain, the rat and human homologue of Jun-interacting protein (JIP-1). JIP-1, which is present in rat retina, blocks the activation of JNK by preventing its nuclear translocation.25

The peptide sequence (H-Gly-Arg-Lys-Lys-Arg-Lys-Arg-Arg-Arg-Pro-Pro-Arg-Pro-Lys-Arg-Pro-Thr-Thr-Leu-Leu-Phe-Pro-Val-Pro-Arg-Ser-Gln-Asp-Thr-NH2) is critical for interaction with JNK.59 To allow cell entry, the peptide is linked to the 10-amino-acid HIV-TAT48-57 as a carrier peptide with two proline residues as spacer. JNKI1 was dissolved in H2O (1 mg in 250 μL), and 2 μL of this solution was injected into the vitreous 15 minutes before and immediately after ischemia, resulting in an estimated vitreous concentration of 68 μM after each injection.

SP600125 is a cell-permeable, reversible, and highly specific inhibitor of JNK.57 We prepared SP600125 as a 100-mM stock solution in DMSO and injected 2 μL of a 1-nM solution (1% DMSO) before and after ischemia, resulting in an estimated vitreous concentration of 65 μM after each injection. In preliminary experiments, we confirmed the effectiveness of the JNK inhibitors in blocking the activity of JNK enzyme in vitro (JNKα1/SAPK1c, active; Upstate Biotechnology, Lake Placid, NY).

The nonischemic eye received the same injection of agent or vehicle. Ischemic, untreated eyes in the control group were injected with vehicle. Kinase assays and immunohistochemistry for phospho-c-Jun (target for activated JNK) and phospho-ATF-2 (target for activated p38) were used to confirm the presence of blockade of JNK and p38, respectively.

Kinase Assay

JNK activity was measured in whole retinal homogenates by nonradioactive assay after immunoprecipitation. We added 1 μg of JNK1 monoclonal mouse IgG1 primary antibody (BD PharMingen) and 30 μL of red protein G affinity gel (Ezview; Sigma-Aldrich) to 500 μL of retinal protein and incubated the mixture overnight with gentle rocking at 4°C. The retinal samples were generated by pooling three retinas from animals treated the same and were collected 1 hour after ischemia (the same time points were used in the ERK and p38 activity assays). This

FIGURE 3. Postischemic retinas were double-labeled with a nuclear stain (Sytox; Molecular Probes) and anti-phospho-JNK or anti-phospho-p38 and examined by fluorescence microscopy to determine whether JNK and p38 are present in the cell nucleus. (A, green) Nuclear staining in the GCL, INL, and ONL. Double-labeling of Sytox and phospho-JNK (D, yellow, arrows) indicated that JNK was present in the nuclei in the GCL and double-labeling with phospho-p38 and Sytox (E, yellow) showed p38 in nuclei of the GCL (arrows) and INL (arrowheads). Magnification, ×40.
FIGURE 4. Postischemic retinas were double-labeled with the ganglion cell marker anti-Thy-1, and anti-phospho-JNK or anti-phospho-p38. Thy-1 (green) specifically stained the membrane of RGCs. Phosphorylated JNK and p38 colocalized (yellow) in the RGCs (top right, arrows) and in a region closer to the optic nerve (region 2), in nerve fiber (NF) bundles (✱) and in axonal projections in the IPL (arrowheads). Phosphorylated p38 was present in RGCs (lower right, yellow, arrows) and in nerve fibers (✱). Magnification, ×40.

FIGURE 5. Postischemic retinas were double-labeled with (D) anti-phospho-JNK or (H) p38, and (A) anti-HPC to detect amacrine cells, (B) anti-PKC to detect bipolar cells, and (C) anti-calbindin to detect horizontal cells. (E, I) JNK and p38 colocalized with HPC in amacrine cells in the INL and with displaced amacrine cells in the GCL (yellow, arrows), as well as with nerve fibers in the INL (lower arrowhead). (F, J) JNK and p38 were present in axonal terminals of bipolar cells (yellow, arrows). (G, K) Scattered cells double-labeling for calbindin and JNK or p38 were present, some of which may have been horizontal cells (K, lower arrow). Magnification, ×40.
time point was chosen based on results showing increased expression of the proteins by Western blot analysis and immunohistochemistry. The samples were then centrifuged for 30 seconds, the supernatant removed, and the pellet washed twice with 1/100 lysis buffer (20 mM Tris [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na3VO4, and 1 μg/mL leupeptin) and twice with 1/100 kinase buffer (25 mM Tris [pH 7.5], 5 mM β-glycerolphosphate, 2 mM dithiothreitol (DTT), 0.1 mM Na3VO4, and 10 mM MgCl2). The pellet was resuspended in 50 μL of 1× kinase buffer supplemented with 100 μM ATP (Cell Signaling Technology) and 2 μL of c-Jun beads (Cell Signaling Technology), and the reaction solution was incubated at 30°C for 30 minutes. The reaction was terminated by adding 12.5 μL 6× SDS buffer, and the samples were heated at 95°C to 100°C for 5 minutes. SDS-PAGE and Western blot analyses were performed. The primary antibody used for the Western blot was an anti-phospho-c-Jun rabbit polyclonal antibody (Ser 63; 1:500 dilution in TTBS with 5% BSA; Cell Signaling Technology).

To determine p38 activity, a nonradioactive immunoprecipitation assay kit (Cell Signaling Technology) was used with an immobilized phospho-p38 MAPK (Thr180/Tyr182) monoclonal antibody (20 μL added to 300 μg protein; Cell Signaling Technology). After immunoprecipitation overnight at 4°C, lysis and kinase buffer washes, the pellet was resuspended in 50 μL of 1× kinase buffer supplemented with 200 μM ATP and 2 μg ATF-2 fusion protein. The reaction solution was incubated for 30 minutes at 30°C and then terminated with 12.5 μL 6× SDS buffer. For the Western blot analysis, a polyclonal rabbit phospho-ATF-2 (Thr71) antibody was used (1:500 dilution in TTBS with 5% BSA; Cell Signaling Technology).

Because U0126 inhibits MEK activity and MEK is immediately upstream of ERK, the effect of U0126 as an inhibitor of MEK was determined using Western blot analysis with a polyclonal rabbit anti-phospho-ERK primary antibody (1:2000 in TTBS with 5% BSA; Cell Signaling Technology).

Studies

For Western blot analysis and immunohistochemistry, retinas were collected 1, 6, 24, 72, and 168 hours after ischemia. Expression of the MAPKs was examined after 45 and 60 minutes of ischemia. For inhibitor, functional, and histologic studies, 60 minutes of ischemia was used, because this duration of ischemia produces more severe functional and histologic impairment. For in vitro assays of kinase activity the retinas were collected at 1 or 6 hours after ischemia or after injection of the inhibitors. To test the impact of the inhibitors of MAPKs on the outcome after ischemia, the ERG was measured at 3 and 7 days after ischemia, the retinal sections were prepared for histology at 7 days after ischemia, or immunohistochemistry was performed up to 7 days after ischemia.

Statistics

Both ERG, histologic, and Western blot data were analyzed as previously described, with ANOVA and post hoc t-test on computer soft-
the mean /H11006 P and 0.005, respectively) and 24 and 72 (both ischemia, apparently in Mu INL, inner plexiform layer (IPL), and GCL at 1 hour after ischemia (Table 1). ERK phosphorylation was evident in the GCL (Fig. 1). Phosphorylated p38 was evident in RGCs at 1 to 72 hours after ischemia. In the INL, increased expression of phosphorylated p38 was seen until 168 hours after ischemia, with the greatest changes at 1 and 6 hours (P < 0.005, Table 1 and Fig. 1). The results for 45 and 60 minutes of ischemia were similar for all the MAPKs. Quantitative data are shown only for 60 minutes of ischemia, and 60 minutes of ischemia was used in all the subsequent studies. Western blot analysis of whole retinal homogenates showed changes parallel to those detected with immunohistochemistry (Fig. 2).

Double-labeling immunohistochemistry was used to determine the retinal cell types expressing phosphorylated MAPKs after ischemia. Phosphorylated JNK was primarily present in nuclei of cells in the GCL, whereas phosphorylated p38 nuclear staining was found in the GCL and staining was particularly dense in the INL (Fig. 3). These results are very similar to the distribution of the proteins in Figure 1. Double-labeling with the ganglion cell marker Thy-1 showed that phosphorylated JNK and p38 were expressed in ganglion cells, the nerve fiber layer, and axons in the IPL (Fig. 4). Staining with anti-HPC demonstrated particularly dense colocalization of p38 in the INL, in axons in the IPL, and in cells that were apparently displaced amacrine cells in the GCL. This distribution of HPC is consistent with our previous studies, using the same antibody. For phosphorylated JNK a similar pattern of colocalization with HPC was present (Fig. 5). There was no double-labeling of phosphorylated ERK with these markers, although ERK was clearly localized to Müller cell bodies, end plates, and astrocytic processes (Fig. 6). With the bipolar cell marker anti-PKCa, phosphorylated JNK and p38 were both colocalized in bipolar cell axons and terminals (Fig. 5).

Expression of MAPKs in the Retina

There was little expression of phosphorylated ERK and JNK in the normal retina, and minimal staining for p38 was detected in the GCL (Table 1). ERK phosphorylation was evident in the INL, inner plexiform layer (IPL), and GCL at 1 hour after ischemia, apparently in Müller cell bodies and in astrocytic processes. Beyond 1 and 6 hours, staining was largely seen in Müller cell bodies. Only faint immunoreactivity remained by 72 and 168 hours (Fig. 1). Phosphorylated JNK was evident in retinal ganglion cells (RGCs) at 1 to 24 hours after ischemia, with cell counts increasing significantly at 1 and 6 (P < 0.001 and 0.005, respectively) and 24 and 72 (both P < 0.05) hours after ischemia. In the INL, increased expression of phosphorylated JNK was seen at 1 and 6 hours (P < 0.05; Table 1, Fig. 1). Phosphorylated p38 was evident in RGCs at 1 to 72 hours after ischemia, with cell counts increasing at 1 to 24 hours (P < 0.001) and significant increases at 72 hours as well (P < 0.005). In the INL, increased expression of phosphorylated p38 was seen until 168 hours after ischemia, with the greatest changes at 1 and 6 hours (P < 0.005, Table 1 and Fig. 1).

The results for 45 and 60 minutes of ischemia were similar for all the MAPKs. Quantitative data are shown only for 60 minutes of ischemia, and 60 minutes of ischemia was used in all the subsequent studies. Western blot analysis of whole retinal homogenates showed changes parallel to those detected with immunohistochemistry (Fig. 2).

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TUNEL Staining

As demonstrated previously, TUNEL-positive cells after ischemia were present in the GCL, INL, and ONL. Consistent with our previous studies, cleaved caspase 3 staining was also present in these regions (data not shown). Phosphorylated ERK, p38, and JNK colocalized with some of these TUNEL-positive cells with ischemia in the GCL and ONL were attenuated by treatment with U0126 or SB203580. Because there was no effect of the JNK inhibitors on outcome after ischemia (Figs. 8, 9) no attempt was made to study changes in TUNEL positivity with blockade of JNK. *P < 0.05 versus normal; **P < 0.002 versus normal; #P < 0.05 versus ischemic/vehicle treated.

| TUNEL Staining | Table 2. Percentage of TUNEL-positive Cells in Retinas Treated with U0126 (to Inhibit ERK) or SB203580 (to Inhibit p38) |

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<th>GCL</th>
<th>INL</th>
<th>ONL</th>
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<tr>
<td>Normal</td>
<td>0.8 ± 0.6</td>
<td>0.05 ± 0.05</td>
<td>0.05 ± 0.03</td>
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<tr>
<td>Vehicle/ischemia</td>
<td>14.0 ± 4.1**</td>
<td>14.1 ± 2.9**</td>
<td>7.3 ± 2.4**</td>
</tr>
<tr>
<td>SB203580/ischemia</td>
<td>1.8 ± 1.4#</td>
<td>12.2 ± 3.3**</td>
<td>0.14 ± 0.06#</td>
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<tr>
<td>U0126/ischemia</td>
<td>3.6 ± 1.9#</td>
<td>9.1 ± 3.9</td>
<td>0.24 ± 0.15#</td>
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Retinal cryosections 10 μm thick at 24 hours after ischemia were stained for TUNEL-positive cells and for the total cell population with DAPI. Slides were examined by fluorescence microscopy and image analysis software was used to determine the percentage of TUNEL-positive cells. Significant increases in percentage of TUNEL-positive cells with ischemia in the GCL and ONL were attenuated by treatment with U0126 or SB203580. Because there was no effect of the JNK inhibitors on outcome after ischemia (Figs. 8, 9) no attempt was made to study changes in TUNEL positivity with blockade of JNK.
positive cells in the INL, ONL, and GCL (Fig. 7). Colabeling of ERK and TUNEL was found in some Müller cell bodies (Fig. 7B). There were significant increases in the percentage of TUNEL-positive cells in the GCL ($P < 0.002$), INL ($P < 0.05$), and ONL ($P < 0.002$) compared with nonischemic normal retina 24 hours after ischemia. Pharmacological blockade of ERK with U0126 or p38 with SB203580, before and/or immediately after ischemia, significantly decreased TUNEL positivity in the GCL ($P < 0.05$) and ONL ($P < 0.05$) compared with results in the vehicle-treated ischemic retina (Table 2).

**Functional Effects of Blocking MAPKs**

Pharmacological blockade of ERK and p38 enhanced recovery of the ERG b-wave at 7 days after ischemia nearly twofold ($P < 0.005$ and 0.05 versus vehicle treated, respectively, Fig. 8). The only significant effect on the a-wave in the study was a decreased recovery at 7 days in retinas treated with JNKI2 (SP600125, $P < 0.05$). The two agents used to block JNK activity had no effect on recovery of the b-wave after ischemia and did not prevent retinal damage (Figs. 8, 9). There was a trend toward worsening recovery in retinas treated with the
JNK inhibitor SP600125 (Fig. 8). There was a significant reduction in overall or inner retinal thickness after ischemia in the vehicle-treated group ($P < 0.0001$ and $P < 0.0002$, respectively) and in the groups treated with either JNK inhibitor ($P < 0.0001$ for inner retinal thickness in both). In contrast, blockade of ERK with U0126 or p38 with SB203580 completely prevented the thinning of the retina.

Western blot analysis for phosphorylated ERK confirmed blockade of the immediately upstream MEK by U0126, and no effect on ERK activation by the p38 inhibitor (Fig. 10). Kinase assays showed that JNK and p38 inhibitors prevented phosphorylation of their specific substrates (Fig. 10). Immunohistochemistry for phospho-c-Jun and phospho-ATF confirmed blockade of JNK and p38 by the two JNK inhibitors and SB203580, respectively, whereas JNK inhibition did not alter ischemia-induced expression of phospho-ATF, and p38 blockade did not alter ischemia-induced expression of phospho-c-Jun (Fig. 11).

**DISCUSSION**

In the present study, expression of the MAPKs ERK, JNK, and p38 demonstrated a specific time course and localization after retinal ischemia. Blockade of ERK and p38, but not of JNK, activity resulted in significant improvement in retinal function and prevention of histologic injury and apoptosis after ischemia, showing significant functional involvement of these MAPKs in cell death after ischemia in the retina.

Earlier studies demonstrated that cell death after retinal ischemia occurs by both apoptosis and necrosis. The mechanisms of apoptosis after retinal ischemia have not been completely elucidated, but involve activation of caspases, endonucleases, bax/bcl-2, inducible nitric oxide synthase, and p53. The MAPKs, modulators of transcription, affect the rate of apoptosis through mechanisms that probably modify gene expression. However, it has not been determined whether MAPKs have a functional role in retinal ischemia or in the induction of apoptosis after ischemia. In a previous study, we found that, after ischemia, activated MAPKs were expressed in whole retinal homogenates.

Brain-derived neurotrophic factor (BDNF) and bright light capable of preconditioning the retina against the damage that follows prolonged exposure to light stimulate the expression of ERK in Müller cells. These findings suggest a role for ERK in neuroprotective signaling. p38 was expressed in RGCs after axotomy, and blockade of p38 improved RGC survival and prevented NMDA-induced cell death. Apart from our earlier study of expression of JNK after ischemia and precon-
There have been no reports of the functional significance of JNK activation in the retina. We found that activated ERK in Müller cells was expressed in the early hours after retinal ischemia, with peak expression at 1 to 6 hours after ischemia. Blockade of the upstream ERK activator, MEK, by injection of U0126 before ischemia, significantly improved retinal function and completely prevented histologic damage. TUNEL-positive staining in the GCL and in the photoreceptors was significantly decreased after U0126.

These results suggest that the activation of ERK is a major signaling component of the retinal response to ischemia, and that ERK is involved in cell death by signals originating in retinal Müller cells that are evidently transmitted to nearby RGCs and photoreceptors.

ERK is activated by mitogens, and after translocation to the nucleus, stimulates transcription factors involved in proliferation and differentiation. Seemingly, production of such survival signals would protect cells from ischemic damage. Conversely, ERK activation occurs after ischemia in brain, heart, and kidney, and in many studies, blocking ERK activation ameliorated ischemic injury. The mechanisms responsible for cell death after ERK activation have not been determined. One possibility is that ERK phosphorylates synapsin I, a phosphoprotein in nerve terminals that maintains synaptic vesicle contact with actin. When phosphorylated, the vesicles dissociate and release neurotransmitters. Excessive release of glutamate and aspartate leads to neuronal damage after ischemia. MEK inhibitors block oxidative glutamate toxicity in cortical neurons.

Inhibition of ERK as a protection for retinal cells against ischemic injury and apoptosis is consistent with the results of several studies showing improved outcome after cerebral ischemia or traumatic brain injury. However, the result contradicts a recent study in which intravitreal injection of U0126 decreased ERK expression and improved RGC survival after transient clamping of the central retinal artery. Either the difference in ischemia models is a factor, or U0126 was protective in our study by blockade of other enzymes. Dose–response experiments have demonstrated that the dose of U0126 that we used attenuates, but does not completely eliminate, the increased activation of ERK after ischemia. Moreover, none of the other MAPKs we studied (p38 and JNK) were affected by U0126 in our study, as demonstrated by kinase assays and immunohistochemistry for target substrate proteins. U0126 blocks activation of AP-1, a transcription factor downstream of Elk-1 and ERK, JNK, and p38 activation. U0126 also alters glutamate release from synaptosomes, and inactivates ERK5, which is increased by oxidative stress. Therefore, an effect on molecules other than ERK cannot be excluded as a mechanism of its neuroprotective effect in our study.

p38 is activated after stress stimuli such as UV irradiation, cytokines, and NMDA or after ischemia. Activation of p38 in our experiments occurred within 1 to 6 hours of ischemia, preceding the peak of apoptotic and necrotic injury that occurs 24 hours after ischemia. This time course resembled the activation of p38 in the retina after injection of NMDA, but activation occurred earlier than that in the retina after optic nerve transection. However, after ischemia, in-
creases in phosphorylated p38 expression were still present 168 hours later. We found that p38 was widely expressed in the inner retina, both in cells and nerve fibers, and in the nuclei of cells staining positive for phosphorylated p38. Therefore, p38 is positioned as a key mediator of retinal cell death after ischemia. This hypothesis was confirmed by the findings that both retinal function and histology were preserved after ischemia when p38 was inhibited. Of interest, blockade of either ERK or p38 completely prevented retinal histologic damage after ischemia. The result suggests that the two pathways converge to a common downstream mediator or mediators that cause cell death after ischemia in the retina. Because of the specific effects of the inhibitors shown in our study, it is unlikely that the results are explainable by nonspecific blockade of MAPKs.

Because the dose of SB203580 used in our study did not block activation of the other MAPKs JNK and ERK, as shown by kinase assays and immunohistochemistry for target substrate proteins, its effect appears to be the result of specific inhibition of p38. The downstream genes that are affected by p38 activation in the retina are not yet known. Our study showed that p38 was present in TUNEL-positive cells and that the percentage of TUNEL positivity decreased after ischemia when p38 was inhibited. Of interest, blockade of either ERK or p38 completely prevented retinal histologic damage after ischemia. The result suggests that the two pathways converge to a common downstream mediator or mediators that cause cell death after ischemia in the retina. Because of the specific effects of the inhibitors shown in our study, it is unlikely that the results are explainable by nonspecific blockade of MAPKs. The pathway controls the activity of many transcription factors, including ATF-2, CHOP/GADD153, CREBs, ELK-1, Ets-1, MAX, MEF-2, NF-κB, HSF, and SAP-1. Processes that may be influenced by activation of p38 include the production of arachidonate metabolites, cytokine signaling, generation of nitric oxide, and cytoskeletal dynamics. Many of these pathways have been implicated in cell death after ischemia and are possible mechanisms for the neuroprotective effect of inhibiting p38.

Compared with studies of ERK and p38, there have been few studies in which the significance of JNK in cell death was
investigated. Both in vitro and in vivo evidence support a role for JNK in cell death, including apoptosis in a number of different cell types including neurons. Although there is some evidence that JNK may upregulate certain DNA repair genes in response to stress, thus enhancing cell survival. JNK phosphorylates transcription factors c-jun, ELK-2, p53, and others, although little is known about the mechanisms of cell death induced through JNK. In contrast to our results with ERK and p38 and despite the presence of phosphorylated JNK and activation of phospho-c-jun in the retina, we found no effect of the inhibition of JNK on the outcome after retinal ischemia. SP600125, an anthrapyrazolone-reversible, ATP-competitive inhibitor of JNK, and a cell-permeable peptide containing the inhibitory domain of islet-brain protein (JNK1) both blocked JNK-induced phosphorylation of c-Jun. We conclude that JNK does not play a significant role in retinal cell death after ischemia.

The time course of activation of ERK and p38 in the retina after ischemia demonstrates some similarities to that after axotomy or intravitreal injection of NMDA. In the rat retina, nearly 80% of RGCs were lost by 10 days after axotomy. In contrast, ERK was activated as early as 1 day later, and remained activated at 7 days. After intravitreal injection of NMDA, more than 80% of RGCs were dead within 24 hours, whereas p38 was activated in RGCs within 1 hour of injection and then remained elevated for 12 hours. TUNEL-positive cells were evident within 6 hours of NMDA injection. In our model, TUNEL staining peaked 18 to 24 hours after ischemia, whereas ERK and p38 were activated within 1 hour of ischemia. Therefore, our results are similar to those of the other models of retinal injury (axotomy and NMDA injection), that ERK and p38 activation preceded the onset of apoptotic cell death.

In summary, we showed that the phosphorylation of the MAPks ERK, JNK, and p38 has a specific time course and cellular localization after ischemia. Both ERK and p38 activation are associated with evidence of apoptosis-related gene expression in the inner and outer retina. Specific pharmacological blockade of ERK and p38, but not JNK, significantly improved retinal recovery after ischemia. These results suggest that ERK and p38 inhibition show promise as a clinically relevant treatment of retinal ischemic injury.

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References