Calpain, Not Caspase, Is the Causative Protease for Hypoxic Damage in Cultured Monkey Retinal Cells

Emi Nakajima,^{1,2} Katherine B. Hammond,¹ Jennifer L. Rosales,¹ Thomas R. Shearer,² and Mitsuyoshi Azuma^{1,2}

PURPOSE. Cell death occurring in human retina during AMD, high IOP, and diabetic retinopathy could be caused by activation of calpain or caspase proteolytic enzymes. The purpose of the present study was to determine whether calpains and/or caspase-3 were involved in cell death during retinal hypoxia in a monkey model.

METHODS. Dissociated monkey retinal cells were cultured for two weeks and subjected to 24-hour hypoxia/24-hour reoxygenation. TUNEL staining and immunostaining for Müller and photoreceptor markers were used to detect which retinal cell types were damaged.

RESULTS. Culturing dissociated monkey retina cells for two weeks resulted in proliferation of Müller cells and maintenance of some rod and cone photoreceptor cells, as identified by vimentin, recoverin, and rhodopsin immunocytochemical staining. Hypoxia/reoxygenation increased the number of cells staining positive for TUNEL. Immunoblotting showed that the calpain-specific 145 kDa α -spectrin breakdown product (SBDP) increased in hypoxic cells, but no caspase-specific 120 kDa α -spectrin breakdown product was detected. TUNEL staining and proteolysis were significantly reduced in the retinal cells treated with 10 and 100 μ M calpain inhibitor SNJ-1945. Caspase inhibitor, z-VAD, did not inhibit cell damage from hypoxia/reoxygenation. Intact pro-caspase-3 was in fact cleaved by activated calpain during hypoxia/reoxygenation to pre 29 kDa caspase-3 and 24 kDa inactive fragments. No 17 and 12 kDa fragments, which form the active caspase-3 heterodimer, were detected. Calpain-induced cleavage of caspase was inhibited by SNJ-1945.

CONCLUSIONS. Calpain, not caspase-3, was involved in hypoxic damage in cultured monkey retinal cells. (*Invest Ophthalmol Vis Sci.* 2011;52:7059–7067) DOI:10.1167/iovs.11-7497

Retinal cell death from ischemia occurs in millions of patients due to conditions such as age-related macular degeneration (AMD), high intraocular pressure (IOP) from glaucoma, and diabetic retinopathy. However, ischemia-induced cell death in retina is not yet well studied,¹ while it has been widely

studied in cerebral ischemia. In ischemic retina, the decreased blood flow from the choroid or retinal arteries can lead to blindness due to rapid cell death in the ganglion, rod, cone, and retinal pigment epithelial cells. Müller cells, a type of glial cell in retina, may be somewhat less susceptible. They are important, however, because Müller cells are thought to protect retinal neurons from various insults² and are involved in the control of angiogenesis, regulation of retinal blood flow,³ and differentiation into new photoreceptor cells to replace damaged cells.^{4,5} Although ischemia alone can produce tissue injury, it is well known that exposure of ischemic tissues to oxygen on reperfusion can also be an important cause of tissue damage.⁶ For example, retinal cells were damaged during the reperfusion period in a rat in vivo ischemia-reperfusion model.⁷ Thus, the present experiments investigated retinal cell damage under hypoxia alone as well as after reoxygenation after hypoxia.

The molecular pathways causing cell death are complex, and often are cell- and inducer-specific. Activation of calpains (by increased intracellular calcium) and/or caspase-3 (cleavage by initiator caspases 8 and 9)⁸ are frequently involved as executioner proteases for such substrates as cytoskeletal proteins and poly (ADP-ribose) polymerase, respectively.

Previous Studies with Calpain in Retinal Ischemia

Our in vivo studies with rats suggest that calpain isoforms play an important role in retinal ganglion cell death induced by ischemia-reperfusion⁷ and by acute ocular hypertension,⁹ which were ameliorated by using calpain inhibitors, SJA6017 and SNJ-1945 respectively. Calpain activation and degradation of known calpain substrates have also been observed during retinal cell damage in rat and monkey whole tissue culture models of hypoxia.^{10–12}

Previous Studies with Caspase in Ischemia

Caspase-3, a key executioner of apoptosis, is known to play an important role in neuron cell death in cerebral ischemia.¹³ However, the involvement of calpain and/or caspase-3-induced proteolysis in specific types of retinal cells during ischemia in primate primary cell culture has not been studied. Such studies, especially in primate models, are essential for developing drugs to prevent cell death in human retinal diseases.

SNJ-1945 is a third-generation, soluble, orally-available inhibitor, with improved specificity for calpains 1 and 2.¹ Several groups reported that calpain inhibition by SNJ-1945 protected against cell damage, such as from N-methyl-D-aspartic acid (NMDA),^{14,15} thapsigargin,¹⁶ and N-methyl-N-nitrosourea¹⁷ treatments; light-induced degeneration¹⁸; heart,^{19,20} and cerebral ischemia²¹; and acute ocular hypertension.⁹ However SNJ-1945 has not been tested in nonhuman primate retina under ischemic conditions. Thus, the purpose of the present hypoxia/reoxygenation study was to determine whether calpains

From the ¹Senju Laboratory of Ocular Sciences, Senju Pharmaceutical Corporation Limited, Beaverton, Oregon; and ²Department of Integrative Biosciences, Oregon Health & Science University, Portland, Oregon.

Supported in part by NIH Grant RR00163 to the Oregon National Primate Research Center.

Submitted for publication March 3, 2011; revised May 24, June 22 and 27, and July 5, 2011; accepted July 7, 2011.

Disclosure: E. Nakajima, Senju Pharmaceutical Co., Ltd. (E); K.B. Hammond, None; J.L. Rosales, None; T.R. Shearer, Senju Pharmaceutical Co., Ltd. (C); M. Azuma, Senju Pharmaceutical Co., Ltd. (E)

Corresponding author: Mitsuyoshi Azuma, Senju Pharmaceutical Corporation Limited, 20000 NW Walker Rd., Suite JM508, Beaverton, OR 97006; azumam@ohsu.edu.

and/or caspase-3 cause Müller and photoreceptor cell death using cultured mixed retinal cells from monkey.

METHODS

Eyes from monkeys were obtained from experiments unrelated to the present studies. These monkeys had not undergone systemic hypoxia or ischemia before death. Experimental animals were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and with the Guiding Principles in the Care and Use of Animals (DHEW Publication, NIH 80-23). Eye globes were obtained from 21 rhesus monkeys (*Macaca mulatta*) ranging in age from 0 to 15 years from the Oregon National Primate Research Center (Beaverton, OR). The average time between death and dissection was less than one hour.

Plated Cell Culture

Establishment of Primary Plate Cultures. Retinas from two monkey eyes per animal were dissected in Hanks' balanced salt solution (HBSS) and washed for 15 minutes with 2 mg collagenase A/mL (Roche Applied Science, Indianapolis, IN). The remaining tissue was treated with papain from a dissociation kit (Worthington Biochemical Corporation, Lakewood, NJ), following the manufacturer's protocol. Cells were grown in high glucose (25 mM) Dulbecco's modified Eagle's medium (DMEM) medium (Invitrogen Corporation, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; PAA Laboratories, Inc., Dartmouth, MA), 2 mM glutamine (final concentration; Invitrogen) and 1% penicillin/streptomycin (Invitrogen) on poly-L-lysine/laminin coated, 6-well plates for immunoblot studies, or on 96-well plates for TUNEL and immunocytochemistry (Becton Dickinson, Franklin Lakes, NJ). High glucose DMEM was needed to maintain cell cultures for up to 14 days; 1×10^5 cells/cm² were cultured at 37°C in humidified 95% air/5% CO₂.

Media for Treatment Groups. Nearly confluent cultures, containing 81% Müller and 19% photoreceptor cells (Fig. 1) were then precultured overnight in DMEM without FBS plus 5.5 mM glucose (physiological glucose levels) and B-27 for normoxic conditions, or DMEM without FBS plus 0.5 mM glucose and B-27 for hypoxic conditions. B-27 was used to support cells after FBS was completely removed because B-27 contains vitamins, essential fatty acids, hormones, and antioxidants suitable for testing the efficacy of neuroprotectants when a serum-free medium is required.²² Low glucose was used in the hypoxic groups because reduced glucose is a contributing factor in many ischemic injuries.^{23,24}

Hypoxia was imposed by culturing the plates in a gas-generating pouch system with indicator (GasPack EZ Anaerobe pouch system; Becton Dickinson)²⁵ for 1 or 2 days, followed by 1 day of normoxia. When used, calpain inhibitor SNJ-1945 at 1, 10, or 100 μ M; or pan-caspase inhibitor (z-VAD-fmk; EMD Chemicals, Inc., Gibbstown, NJ) at 100 μ M was added 1 hour before hypoxia. Each inhibitor was dissolved in dimethyl sulfoxide (DMSO) as a 100 mM stock. All the normoxia and hypoxia groups contained the highest concentration of DMSO used in all groups; DMSO at this level had no toxic effects. Effectiveness of the pan-caspase inhibitor z-VAD was confirmed by treating cultured monkey retinal cells with 1 μ M staurosporine.²⁶ This caused caspase-3 activation and production of the caspase-3 dependent 120 kDa α -spectrin breakdown product. These events were inhibited by 100 μ M z-VAD (data not shown).

Suspension Cell Culture

Suspension cultures were established to more closely mimic the higher ratios of photoreceptor to Müller cells found in vivo. Retinal cells were dissociated using the methods described above. Cells at 8×10^5 cells per tube (5 mL, Falcon round-bottom; Becton Dickinson) were cultured in suspension at 37°C in humidified 95% air/5% CO₂ for 24 hours, using DMEM (Invitrogen) with 5.5 mM glucose and B-27 for normoxic conditions, or DMEM with 0.5 mM glucose and B-27 for hypoxic

FIGURE 1. Immunofluorescence microscopy of dissociated retinal cells cultured under normoxic conditions for 1, 7, and 14 days showing: (A) rods (yellow, anti-rhodopsin plus anti-recoverin) and cones (red, anti-recoverin); (B) Müller cells (green, anti-vimentin); and (C) counts of cell types expressed as mean $\pm$ SEM ($n = 3$).

conditions. Hypoxia was imposed by incubating the tubes in a pouch (GasPack EZ; Becton Dickinson)²⁵ for 18 hours, followed by 6 hours of normoxia. When used, calpain inhibitor SNJ-1945 at 100 μ M was added 1 hour before hypoxia. Samples at time 0 were also saved to measure cell numbers at the initiation of each experiment.

Immunocytochemistry

Cells were fixed for 20 minutes at room temperature in 10% buffered formalin. The fixed cells were incubated for 30 minutes in blocking solution containing 10% FBS and 0.1% Triton X-100. Cells were then incubated for 2 hours at room temperature with antibodies to rhodopsin (specific rod cell marker²⁷; Millipore Corporation, Billerica, MA), recoverin (rod and cone cell marker²⁸; Abcam Inc., Cambridge, MA), and vimentin (Müller cell marker²⁹; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in blocking solution. After washing with PBS three times, antibody bound cell marker proteins were visualized by incubation for one hour at room temperature with fluorescence-conjugated secondary antibodies (Alexa Fluor 488, 546 or 633; Invitrogen). After washing with PBS three times, the cells were observed with a fluorescence microscope (Axiovert 200; Zeiss MicroImaging, Thornwood, NY) and digitized with a CCD camera (AxioCam MRM; Zeiss MicroImaging). Rods and cones could be distinguished because rods are stained by both recoverin and rhodopsin antibodies, while cones are stained only by recoverin antibody.

TUNEL Staining

Apoptosis in cultured cells was determined using the terminal deoxynucleotidyltransferase (TdT)-mediated biotin-16-dUTP nick-end labeling (TUNEL) technique (Cell Death Detection Kit; Roche). The fixed cells were stained with TUNEL reagent following the recommendations of the manufacturer.

Cell Counting and Statistical Analysis

For quantitative measurement; recoverin-, rhodopsin-, vimentin-, TUNEL-, and DAPI-positive cells were counted in five rectangular areas ($434 \times 325 \mu\text{m}$) in each well and averaged. Percentage of TUNEL-positive cells in three wells from three different monkeys was calculated as (TUNEL-positive nuclei/DAPI-positive nuclei) $\times$ 100 and expressed as an average. Dunnett's *t*-test (JMP 8.0.1 statistical software; SAS Institute Inc., Cary, NC) was used to evaluate the differences between hypoxia and inhibitor groups.

Immunoblotting and Zymography

Total cellular proteins were extracted by sonication in buffer containing 20 mM Tris (pH 7.5), 5 mM EGTA, 5 mM EDTA, and 2 mM dithioerythritol. Protein concentrations were measured (BCA assay; Thermo Fisher Scientific Inc., Rockford, IL) using bovine serum albumin as standards. For immunoblotting, equal amounts of protein were loaded into each lane and run (4%–12% NuPAGE gels; Invitrogen) with MES or MOPS buffer and then transferred to polyvinylidene fluoride (PVDF) membrane by electro transferring at 100 V for 1 hour. The membranes were probed with antibodies to calpain 1 (Thermo Fisher Scientific Inc.), calpain 2 (Sigma Aldrich Corp., St. Louis, MO), α -spectrin (Affiniti Research Products Limited, Mamhead Castle, UK), rhodopsin (clone Rho 1D4; Millipore), m-opsin (Abcam), vimentin (Santa Cruz Biotechnology), caspase-3 (Santa Cruz Biotechnology), and β -actin (Sigma). Binding of secondary antibodies, conjugated to alkaline phosphatase or to horseradish peroxidase, was visualized with BCIP/NBT (Bio-Rad Laboratories, Hercules, CA) or by chemiluminescence (Amersham ECL Plus; GE Health Care Biosciences, Piscataway, NJ). Western blot images were captured (FluorChem FC2 imager; Cell Biosciences, Inc., Santa Clara, CA). Band intensities were measured with ImageJ 1.40 software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>). To compensate for variability of staining between membranes, the densities of the bands were normalized to the density of β -actin.

Statistical analyses of the data were performed by Dunnett's *t*-test (JMP 8.0.1 software; SAS Institute Inc.).

Casein zymography was performed according to the method of Raser et al.³⁰ Cells were sonicated in buffer containing 20 mM Tris (pH 7.5), 5 mM EGTA, 5 mM EDTA, 2 mM dithioerythritol, and 100 μ M SNJ-1945 (reversible inhibitor). Ten percent native gels, copolymerized with 0.1% casein were prerun with no samples in buffer containing 25 mM Tris (pH 8.3), 192 mM glycine, 1 mM EGTA, and 1 mM dithiothreitol for 15 minutes at 4°C. Twenty μ g of retinal soluble proteins were then loaded and run. After electrophoresis, the gels were incubated with slow shaking overnight at room temperature in 20 mM Tris (pH 7.4), 10 mM dithiothreitol, and 2 mM CaCl_2 . Gels were stained with a Coomassie G-250 stain (SimplyBlue Safe stain, Invitrogen) for 1 hour, and destained with distilled water for 1 hour. For presentation, the gel images were taken (FluorChem FC2 imager; Cell Biosciences, Inc.) and digital images were inverted to visualize dark areas of caseinolysis caused by calpains. Electrophoresis for immunoblotting from the native PAGE gels was performed with the same conditions as above, except the gels did not contain casein; and transfer conditions were the same as from standard SDS-PAGE gels.

At least three independent experiments from different cultures were conducted for all studies in this report.

Proteolysis of Pro-caspase-3 by Calpain

To investigate proteolysis of pro-caspase-3 by calpain, 20 units recombinant inactive human pro-caspase-3 (pET28; Enzo Life Sciences International, Inc., Plymouth Meeting, PA) per mL were incubated at 37°C for 1 hour with 60 units purified calpain 1 from human erythrocytes or purified calpain 2 from porcine kidney (EMD Chemicals Inc.) per mL and 2.5 mM calcium in 50 mM Tris buffer (pH 7.6). Hydrolyzed fragments of caspase-3 were detected by immunoblotting with anti-caspase-3 antibody. Active human recombinant caspase-3 (BioVision, Inc., Mountain View, CA) was used as a reference for the migration positions of the 12- and 17-kDa bands for activated caspase-3. All samples were loaded with 0.025 units of caspase-3 per lane. According to the manufacturer, the recombinant inactive pro-caspase-3 contained an extra 21 amino acid N-terminal sequence, containing the pET28a leader, 6 $\times$ histidine (HIS) tag, thrombin cleavage site, and an extra Met from an *NdeI* cut site. This extra tag may explain the slower rate of migration of the inactive pro-caspase-3 compared with intact band of active caspase-3 (see Fig. 7B).

RESULTS

Changes in Cell Populations during Primary Culture of Dissociated Monkey Retina

Rods (yellow, Fig. 1A), cones (red), and Müller cells (green, Fig. 1B) were observed in monkey retinal cells cultured on poly-L-lysine/laminin coated-plates. Rods and cones retained their round shape for 14 days. In contrast, the cell bodies of Müller cells started expanding 3 days after initiating culture. During prolonged culture, the number of Müller cells markedly increased (Fig. 1C) and were nearly confluent after 14 days of culture, with a photoreceptor to Müller cell ratio of approximately 1:4. Ganglion, amacrine, and horizontal cells were observed only during the early time periods of the cell culture (data not shown). Bipolar cells were observed throughout the 14 days of culture (data not shown). Because our experiments were directed toward modeling processes relevant to ischemic retina, hypoxia/reoxygenation studies below focused on Müller and photoreceptor cells.

Changes in Retinal Cell Morphology and Cytology Due to Hypoxia and Reoxygenation

Rod and cone numbers were reduced 1 day after hypoxia (green, Fig. 2A). Reoxygenation further damaged the photore-

FIGURE 2. Immunofluorescence microscopy of dissociated retinal cells cultured under progressively severe hypoxic conditions and quadruple-labeled for cell markers: (A) rods and cones (green, anti-recoverin) and Müller cells (red, anti-vimentin); (B) TUNEL-positive (green) apoptotic cells; (C) image of TUNEL-positive apoptotic cells (green) overlaying image of DAPI-stained nuclei (blue); (D) Phase-contrast micrographs in the stained areas above. (E) Immunoblots for calpain substrate α -spectrin (lane 1) 1 day normal, (lane 2) 1 day hypoxia, (lane 3) 1 day hypoxia/1 day reoxygenation, (lane 4) 2 day hypoxia, (lane 5) 2 day hypoxia/1 day reoxygenation, showing α -spectrin at 280 kDa (filled arrowhead), break down products at 150 (gray arrowhead), and 145 kDa (open arrowhead); (F) Immunoblot for β -actin at 42 kDa as a loading control. Immunofluorescence microscopy and immunoblots are representative of those performed 3 times. Normal groups at days 2 and 3 day were omitted because they showed no changes.

ceptor cells so completely that only debris resembling small dots remained, and the larger flat Müller cells shrunk (red, Fig. 2A). Similar morphologic changes were observed after 2 days of hypoxia and 1 day of reoxygenation. Hypoxia and reoxygenation also increased the presence TUNEL-positive cells (green, Fig. 2B); total cell numbers decreased (blue, DAPI-positive cells Fig. 2C); and the nuclei became smaller and rounded in shape. Some of the remaining photoreceptor and Müller cells were TUNEL-positive at 1 day hypoxia (cyan, Fig. 2C). Because reoxygenation damaged the photoreceptor cells completely, most of the remaining TUNEL-positive cells were thought to be Müller cells (cyan, Fig. 2C). Although we did not count the cell numbers for separate photoreceptor and Müller cells in hypoxia, staining for photoreceptor marker (recoverin) and for Müller cell marker (vimentin) was consistent in three different monkeys. The phase-contrast images of the same areas at each time point confirmed the extensive damage to the retinal cells caused by increasing hypoxia (Fig. 2D). Alpha-spectrin is a well known substrate for calpains and the spectrin breakdown product (SBDP) at 145 kDa is uniquely produced only by calpain.³¹ In cultured retinal cells, hypoxia and reoxygenation caused loss of the intact 280 kDa α -spectrin (Fig. 2E, lanes 2–5), leading to accumulation of SBDP's at 150 kDa (gray arrowhead) and at 145 kDa (open arrowhead, calpain-specific) in a time-dependent cell damage.

Calpain Inhibitor SNJ-1945 Reduces Cellular Damage from Hypoxia/Reoxygenation

Loss of rods (yellow, Fig. 3A); loss of cones (green, Fig. 3A); loss and shrinkage of Müller cells (red, Fig. 3C); accumulation of apoptotic cells (green, Fig. 3B); and the presence of apopto-

tic nuclei (cyan, Fig. 3C) were partially prevented by calpain inhibitor SNJ-1945 (lanes 3 vs. 2), but not by pan-caspase inhibitor z-VAD (lane 4). SNJ-1945 significantly reduced apoptosis in a dose dependent manner (Fig. 3D). Normoxic cells contained only $0.2 \pm 0.1\%$ TUNEL-positive cells; whereas $60.6 \pm 1.9\%$ of the cells after 1 day hypoxia/1 day reoxygenation were TUNEL-positive, and 100 and 10 μM SNJ-1945 significantly reduced these numbers to $37.5 \pm 3.5\%$ and $51.9 \pm 1.7\%$, respectively (Fig. 3D). The recovery of TUNEL-positive cells by SNJ-1945 could also be due to an increased number of surviving cells. In contrast, 100 μM pan-caspase inhibitor z-VAD had no significant effect against induction of apoptotic cells.

Proteolysis of Retinal Cell Protein Markers

The density of protein bands on immunoblots for rhodopsin (rod cell marker, Fig. 4A), m-opsin (cone cell marker, Fig. 4B), and vimentin (Müller cell marker and well known substrate for calpains,³² Fig. 4C) were decreased or fragmented in retinal cells cultured under hypoxia/reoxygenation. Rhodopsin showed two bands in our sample at approximately 48 and 52 kDa, while the calculated molecular weight for human rhodopsin is 39 kDa. Staining of the bands was blocked by a peptide of an epitope recognized by the rhodopsin antibody (PEP-174; Thermo Fisher Scientific Inc.), confirming that the 48- and 52-kDa bands were derived from rhodopsin (data not shown).

Densitometric analysis of photoreceptor cell markers—both of the rhodopsin bands and the opsin band, showed significant partial inhibition of proteolysis by 100 μM calpain inhibitor SNJ-1945 (lane 3), while caspase inhibitor had no significant effect (lane 6).

FIGURE 3. Immunofluorescence microscopy of hypoxic retinal cells at 16 days of culture with inhibitors: (lane 3) calpain inhibitor SNJ-1945, or (lane 4) pan-caspase inhibitor z-VAD. (A) Rods (yellow; anti-rhodopsin plus anti-recoverin) and cones (green; anti-recoverin), (B) TUNEL-positive cells (green). (C) Merged images of TUNEL-positive cells (green), DAPI stained nuclei (blue), and Müller cells (red; anti-vimentin) resulting in cyan (arrowheads) labeling of apoptotic cells. (D) Percentages of TUNEL-positive cells. Data are mean $\pm$ SEM ($n = 3$). * $P < 0.05$ relative to hypoxia/reoxygenation (Dunnett's t -test).

Measures of Calpain Activation

In cultured retinal cells, hypoxia/reoxygenation caused loss of the calpain substrate, α -spectrin at 280 kDa (Fig. 5A, lane 2), leading to accumulation of SBDP's at 150 kDa (gray arrowhead) and at 145 kDa (open arrowhead, calpain-specific). Ten and 100 μ M SNJ, but not z-VAD, significantly reduced 145 kDa SBDP in hypoxic cells (Fig. 5A, bar graph). The 120 kDa, caspase-3-specific SBDP band was not observed (Fig. 5A), further indicating that caspase-3 was not involved in this hypoxia/reoxygenation model.

Autolysis is associated with the activation of calpains.³³ The intact 80 kDa catalytic subunit of low, calcium-requiring calpain 1 (also termed μ -calpain, requiring micromolar levels of calcium for activation) decreased after hypoxia/reoxygenation; and the active, autolytic fragments at 78 and 76 kDa appeared (Fig. 5B, lane 2). Calpain inhibitor SNJ-1945 at 100 μ M inhibited the decrease of intact 80 kDa band and inhibited the appearance of 78 and 76 kDa bands, whereas caspase inhibitor z-VAD did not prevent these events. This suggested that the calpain cascade is independent of the caspase cell death cascade.

The intensity of the intact band for the 80 kDa catalytic subunit of high calcium-requiring calpain 2 (also termed m-calpain, requiring near-millimolar levels of calcium for activation) was slightly decreased under hypoxia/reoxygenation; probably as a result of activation followed by autodegradation (Fig. 5C, lane 2). Because the active, N-terminal autolyzed form of calpain 2 migrates the same as intact 80 kDa calpain 2 on SDS-PAGE,^{12,34} the 80 kDa band in lane 2 of Figure 5C probably contained both forms of calpain 2. We were also not able to detect further autolyzed calpain 2 band at 43 kDa. This was the same as a previous report using monkey retina tissue cultured

under hypoxia.¹² These data may indicate that calpain 2 is not as fully activated compared with calpain 1 in hypoxic monkey retina.

To further verify calpain 2 activation, casein zymography on a native PAGE was performed, because active intact and autolyzed calpain 2 separate in the absence of SDS. Casein zymography measures calpain activities remaining in the samples. The separating PAGE gel containing casein was first run in buffer containing excess EGTA to chelate calcium and reversibly inhibit calpains. Then, the gel was post-incubated with high calcium buffer to over titrate EGTA and to activate remaining calpains followed by proteolysis of casein. Indeed, the band for remaining active calpain 2 markedly decreased under hypoxia/reoxygenation (Fig. 5D, lane 2), supporting that calpain 2 is activated and auto-degraded. Calpain inhibitor SNJ-1945 at 100 μ M also partially inhibited the decrease in remaining active calpain 2 (lane 3), but calpain 1 did not appear to be protected well by SNJ-1945 (Fig. 5D) compared with immunoblot (Fig. 5B); zymography may be of lower sensitivity compared with the immunoblotting. The electrophoretic migration positions of calpain 1 and 2 were confirmed by immunoblotting from the native gels without casein with antibodies against each calpain (Fig. 5E).

Retinal Cells in Suspension Culture

A limitation of the culture plate study above was that the relative number of photoreceptor cells was lower than in vivo. This was addressed by culturing dissociated monkey retinal cells in short-term suspension cultures. Immunocytochemistry on monkey retinal cells cultured for only 24 hours in suspension showed higher concentrations of photoreceptor cells (red, Fig. 6A, 24h Normoxia) compared with Müller cells

FIGURE 4. Immunoblots for cell-type marker proteins in retinal cells cultured under hypoxic conditions: (lane 2) alone; (lanes 3–5) plus calpain inhibitor; or (lane 6) plus caspase inhibitor. (A) Rhodopsin in rod cells; (B) m-opsin in cone cells; (C) vimentin in Müller cells. Bar graphs indicate band intensity normalized to β -actin loading control, expressed as mean $\pm$ SEM ($n = 3$ to 6). * $P < 0.05$, ** $P < 0.01$, all relative to hypoxia/reoxygenation (lane 2), using Dunnett's t -test.

(green). Rods (yellow, Fig. 6B, 24h Normoxia) were the major photoreceptor cells compared with cones (green). Eighteen hours of hypoxia followed by 6 hours of reoxygenation caused shrinkage of Müller cells (Fig. 6A, Vehicle) and reduced the numbers of rods and cones (Fig. 6B, Vehicle), while calpain inhibitor SNJ-1945 showed a moderate but reproducible ability to prevent photoreceptor loss (Fig. 6B, +100 μ M SNJ).

Hypoxia/reoxygenation significantly increased TUNEL positive cells (Fig. 6C, +Vehicle) to $39.0 \pm 6.1\%$, while SNJ significantly reduced the number of TUNEL positive cells (Fig. 6C, +100 μ M SNJ) to $19.9 \pm 4.1\%$. These apoptotic changes were localized to nuclei of hypoxic retinal cells as shown by the increased cyan staining (Fig. 6C, arrowheads). SNJ reduced the number of apoptotic nuclei, and some of these apoptotic cells were located over red-stained rods and cones.

The observation that hypoxia/reoxygenation-induced activation of calpain in the suspension cultured retinal cells was further confirmed by noting the decrease of intact calpain 1 and the presence of autolytic fragments of calpain 1 (Fig. 6D, lane 3, 78 and 76 kDa), autolytic activation of calpain 2, production of the calpain-specific 145 kDa α -spectrin breakdown product, and moderate inhibition by SNJ of the markers for calpain activation (Fig. 6D, lane 4). Thus, although this short 24-hour, photoreceptor-rich suspension culture showed only minimal activation of calpain, hypoxia/reoxygenation caused calpain-induced proteolysis and apoptotic death of the rods and cones, which was partially inhibited by SNJ-1945, as was observed in the 14-day plate cultures.

Calpain Degrades, but Does Not Activate Caspase-3 during Hypoxia/Reoxygenation

In hypoxic retinal cells, endogenous pro-caspase-3 was proteolyzed to inactive 29- and 24-kDa fragments (Fig. 7A, lane 2); this degradation was inhibited significantly by SNJ-1945 in a dose-dependent manner (Fig. 7A, bar graph, lanes 3–5), but not inhibited by caspase inhibitor (Fig. 7A, bar graph, lane 6). To confirm calpain dependent cleavage of caspase-3, inactive pro-caspase-3 (inact. proCasp3) was incubated in vitro with high concentrations of either active calpain 1 (calp 1) or calpain 2 (calp 2) with or without calpain inhibitor, SNJ-1945. Pro-caspase-3 was degraded by calpain 2 to similar inactive 29- and 24-kDa forms (Fig. 7B, lane 5) as observed in hypoxic cells. Calpain 1 also produced the 29 kDa band, but production of the 24 kDa band

FIGURE 5. Immunoblots of marker proteins indicating calpain activation in hypoxic retinal cells cultured: (lane 2) alone; (lanes 3–5) plus calpain inhibitor; or (lane 6) plus caspase inhibitor. (A) Intact α -spectrin at 280 kDa (filled arrowhead) and breakdown products at 150 kDa (gray arrowhead) and the calpain-specific fragment at 145 kDa (open arrowhead). Below: density of α -spectrin 145 kDa band normalized to the β -actin loading control, expressed as mean $\pm$ SEM ($n = 3$ to 6) * $P < 0.05$, ** $P < 0.01$, all relative to hypoxia/reoxygenation (lane 2), using Dunnett's t -test. (B) Intact calpain 1 catalytic subunit at 80 kDa (black arrowhead) and fragments at 78 kDa (gray arrowhead) and 76 kDa (open arrowhead) observed after autolytic activation. (C) Intact calpain 2 catalytic subunit at 80 kDa (black arrowhead). Autolyzed, active form of calpain 2 shows the same migration as intact 80 kDa calpain 2 on SDS-PAGE.^{11,32} (D) Casein zymogram (image inverted) showing remaining active calpains in the cultured retinal samples (20 μ g per lane). Black arrowhead indicates active calpain 1, and gray arrowhead indicates active calpain 2. The left two lanes are purified human erythrocyte calpain 1 (CL1) and porcine kidney calpain 2 (CL2) used as standards and positive controls. (E) Immunoblots of calpain 1 and calpain 2 from native PAGE gel used to confirm migration positions of active calpains 1 and 2 in the cultured retinal samples (20 μ g per lane). The left two lanes are purified human erythrocyte calpain 1 (CL1) and porcine kidney calpain 2 (CL2) used as standards and positive controls.

FIGURE 6. Immunofluorescence microscopy of monkey retina cells in suspension culture after 24 hours of hypoxia. (A) Vimentin (green) and rods and cones (red; anti-recoverin), (B) rods (yellow; anti-rhodopsin and anti-recoverin) and cones (green; anti-recoverin), (C) merged images of TUNEL-positive cells (green), DAPI-stained nuclei (blue), and rods and cones (red; anti-recoverin) resulting in cyan (arrowheads) labeling of apoptotic cells (same areas as in B). Percentages of TUNEL-positive cells are shown below the images. Data are mean $\pm$ SEM ($n = 3$). * $P < 0.05$ relative to hypoxia/reoxygenation (Dunnett's t -test). (D) Immunoblots for marker proteins for calpain activation: calpain 1, calpain 2, α -spectrin; β -actin as loading control.

by calpain 1 was minimal (Fig. 7B, lane 3). Formation of these fragments was inhibited by SNJ-1945 (Fig. 7B, lanes 4 and 6), also indicating that production of both 24 and 29 kDa caspase-3 fragments are calpain dependent, similar to previous experiments with neuroblastoma cells³⁵ and rat forebrain hemispheres.³⁶ According to the previous study,³⁶ only when exogenous, activated caspase-3 was included in calpain 2-treated group, caspase-3 was degraded to the 17 kDa active form by the calpain-dependent 29 kDa fragment. Because our system did not contain active caspase-3, the 29 kDa fragment stayed inactive (Figs. 7A and 7B). Caspase-3 was apparently more susceptible to calpain 2 hydrolysis than calpain 1 under our experimental conditions as in previous reports.^{35,36} The 33 kDa form shown was probably formed by removal of the extra amino acids added during the synthesis of recombinant pro-caspase-3 (see Methods). In contrast, activated pro-caspase-3 was degraded to 17 and 12 kDa forms (Fig. 7B, lane 1), which are known to be active.³⁷ These results suggest that calpains are responsible for proteolysis of pro-caspase-3 in hypoxic retinas, but this proteolysis did not cause activation of pro-caspase-3, only degradation.

DISCUSSION

The major finding of the present investigation was that hypoxia/reoxygenation in cultured monkey cells caused TUNEL-pos-

itive apoptotic cell death by activation of calpains, not caspase-3. Calpain-induced proteolysis was part of the executioner mechanism in three different types of retinal cells: rods, cones, and Müller cells; and the proteolysis could be partially inhibited by calpain inhibitor, SNJ-1945.

Müller and Photoreceptor Cell Damage under Hypoxia/Reoxygenation

Photoreceptor cells lost their shape completely under hypoxia/reoxygenation whereas Müller cells retained their cell shape but were shrunk (Figs. 2A and 6A). Along with their high rates of oxidative metabolism (photoreceptors use 3 to 4 times more oxygen than other retinal and central nervous system neurons³⁸), our results showed that photoreceptor cells are more susceptible to hypoxia/reoxygenation. When photoreceptor neurons are lost, the visual system collapses, leading to blindness. In contrast, glial cells are reported to be more resistant to ischemia, anoxia, and hypoglycemia.^{39,40} Müller cells survive most retinal injuries, where they are important in

CASPASE AS SUBSTRATE FOR CALPAINS

FIGURE 7. (A) Immunoblot for endogenous pro-caspase-3 in hypoxic retinal cells treated without (lane 2) and with enzyme inhibitors (lanes 3–6). Intact pro-caspase-3 is indicated at 33 kDa (filled arrowhead) along with calpain-dependent breakdown products at 29 (gray arrowhead) and 24 kDa (open arrowhead). Below: density of caspase-3 bands at 29 and 24 kDa normalized to β -actin loading control, expressed as mean $\pm$ SEM ($n = 3$ to 9), ** $P < 0.01$ all relative to the hypoxia/reoxygenation group (lane 2) using Dunnett's t -test. (B) Immunoblots for pro-caspase 3 after in vitro incubation of: (lane 1) activated recombinant caspase-3 showing loss of intact pro-caspase-3 at 34 kDa, production of known active caspase-3 fragments at 17 and 12 kDa (arrows), and absence of inactive 29 and 24 kDa fragments. Other lanes show: (lane 2) incubation of inactive recombinant pro-caspase-3 alone; (lane 3) plus purified active calpain 1; (lane 4) plus active calpain 1 and 100 μ M calpain inhibitor; (lane 5) plus purified active calpain 2; or (lane 6) plus active calpain 2 and 100 μ M calpain inhibitor.

recovery from pathogenic events. Inhibition of Müller cell damage would therefore be important because they are radial glial cells, passing through the entire retina and contacting neuron soma and their processes. Müller cell functions include: formation and maintenance of the blood-retinal barrier (BRB); supplying retinal neurons with nutrients; maintaining ion, water and pH homeostasis; releasing neurotransmitters (D-serine, glutamate, and ATP); providing neurotransmitter precursors to neurons; converting all-*trans*-retinal into 11-*cis*-retinol for uptake by cone receptors; and phagocytizing degenerating retinal cells.³ Importantly, Müller cells from diseased adult rat retina dedifferentiated into multipotent progenitor cells which then differentiated into a number of retinal cell types, including photoreceptor cells.⁴ Furthermore, immortalized Müller stem cells from adult human eyes were shown to exhibit neural stem cell properties when they were grafted into rat eyes.⁵ Thus, protection of Müller cells, as well as photoreceptor cells, by a calpain inhibitor as observed in the present study, would potentially allow for the Müller cells to differentiate into new retinal neurons and replace those lost during hypoxia/reoxygenation.

Possible Mechanisms Linking Hypoxia/Reoxygenation to Activation of Calpains in Retina

The many mechanisms in retina responding to hypoxia include HIF-induced overproduction of VEGF (disrupting the blood-retinal barrier), production of inflammatory cytokines, enhanced NO production (enhanced free radical production along with vasodilation), enhanced extracellular glutamate resulting in increased calcium flux into the cytoplasm, mitochondrial dysfunction and decreased oxygen-dependent energy production (with loss of ATPase-linked calcium channels), and release of calcium into the cytoplasm from compromised endoplasmic reticulum (ER) and mitochondria.⁴¹ A common messenger in many of these pathways is increased cytoplasmic, free calcium. Constant or transiently elevated levels of intracellular free calcium, or calpain activation, have been observed in many models of retinal hypoxia or retinal degeneration including RGC-5 cells exposed to ionomycin, rat retinas cultured in 95% N₂/5% CO₂, monkey retinas cultured in 95% N₂/5% CO₂, human and monkey retinal soluble lysate exposed to 2.5 mM calcium, rats with central artery occlusion, rats with intraocular pressure elevated by infusion with hydrostatic saline, oxidative stress in retinal microvascular endothelial cells, NMDA-induced excitotoxicity in rat retina, monkey experimental ocular hypertension induced by laser photocoagulation of the trabecular meshwork, and in WBN/Kob rats and rd1 mice with photoreceptor degeneration.¹

The predominant calpains observed in monkey retina are calpains 1 and 2, which are activated by elevated levels of intracellular free calcium.¹ The binding of up to 10 calcium ions causes: (1) dissociation of the endogenous calpain inhibitor calpastatin (CS) from the inactive CS/calpain complex; (2) movement of the active site residues cysteine, histidine, and asparagine closer to form a deep, functional proteolytic pocket; and (3) autolysis of the N-terminus of the catalytic subunit. We observed autolysis of calpain 1 and calpain 2 in the hypoxic retinas in the present study (Figs. 5B–5E), indicating that intracellular calcium levels were high enough to activate calpain 1 and calpain 2. The *in vitro* activation requirement of calpain 1 is approximately 10 to 50 μ M and that of calpain 2 is approximately 500 μ M, but is likely lower within cells due to activator mechanisms, such as binding with phospholipids, concentration of calcium with calpain at subcellular sites, and even increased susceptibility to activation by high frequency oscillations in calcium.⁴²

Mechanisms of Calpain-Induced Proteolysis Leading to Cell Death

The present study noted that calpain-induced proteolysis caused the breakdown of rhodopsin in rods, m-opsin in cones, vimentin in Müller cells, α -spectrin to the calpain-specific 145 kDa SBDP, and caspase-3 to inactive fragments. Even simple incubation of calcium with homogenates of monkey retinal proteins has been shown to cause changes in 15 different protein spots, including α -enolase, β -tubulin, and HSP70.¹² The substrate specificity of calpain is based on the availability of unstructured regions rather than strict amino acid sequences, although they are reproducible at specific sites for each protein. Thus, cell death induced by calpain is likely the result of multiple attacks on many different proteins at several subcellular locations.

In our study, pan-caspase inhibitor was ineffective. Therefore, calpain-induced proteolysis is probably a major executioner, but crosstalk and parallel activation of other cell death mechanisms are very likely. For example, the importance of caspase-independent cell death pathways has been well documented.⁴³ Mitochondria release factors such as apoptosis-inducible factor (AIF) is well studied in caspase-independent pathways. AIF is translocated to the nucleus and causes chromatin condensation and apoptosis. These effects of AIF are not inhibited by caspase inhibitors. In fact, a recent study using *in vivo* and *in vitro* models of retinitis pigmentosa showed that elevated calcium leads to calpain-induced proteolysis and the release of AIF from the inner membrane of the mitochondria and caspase 12 from the ER. These factors were translocated to the nucleus, leading to apoptotic death in the rod photoreceptors.⁴⁴ As in the present studies, calpain inhibition blocked apoptosis.

Calpain Inhibition

Despite the lack, as yet, of a totally defined mechanism for hypoxia/calpain-induced retinal cell death, the clinically relevant finding in this study was that calpain inhibitor SNJ-1945 significantly inhibited apoptotic cell death under hypoxia/reoxygenation in a dose-dependent manner using primate retinal cells (Fig. 3D). Proteolysis of photoreceptor markers rhodopsin and opsin, and proteolysis of Müller cell marker vimentin, were inhibited by SNJ-1945 (Fig. 4). Calpain inhibitor SNJ-1945 may contribute to valuable neuroprotection against human retinal diseases, not only by a direct protection of neuronal cells but also by allowing differentiation of new retinal neurons from Müller-derived stem cells. Two hypotheses regarding the mechanism producing AMD are: (1) initial atrophy of retinal pigment epithelial cells causes secondary loss of the choriocapillaris and photoreceptor degeneration, and (2) initial dysfunction of the choroidal vascular causes secondary loss of the RPE and photoreceptor degeneration.⁴⁵ Hypoxia/hypoxemia is a likely common protagonist, and early intervention would be expected to produce better clinical results. Although the present *in vitro* study was of short duration, the data further support the contention that SNJ-1945 might be most efficacious if administered in grade 3 and 4 AMD patients when soft drusen are forming.⁴⁶

Note that treatment with SNJ-1945 in the present study was before the initiation of hypoxic insult, which is unlikely in clinical situations. We have not yet tested posttreatment with SNJ-1945, but Koumura et al. reported that SNJ-1945 offered neuroprotection against acute cerebral ischemia even 6 hours after middle cerebral artery occlusion in mice.²¹ The present observations suggest that SNJ-1945 may be a candidate for such testing during the treatment phase for retinal ischemic conditions.

References

1. Azuma M, Shearer TR. The role of calcium-activated protease calpain in experimental retinal pathology. *Surv Ophthalmol*. 2008; 53:150–163.
2. Kawasaki A, Otori Y, Barnstable CJ. Muller cell protection of rat retinal ganglion cells from glutamate and nitric oxide neurotoxicity. *Invest Ophthalmol Vis Sci*. 2000;41:3444–3450.
3. Bringmann A, Pannicke T, Grosche J, et al. Muller cells in the healthy and diseased retina. *Prog Retin Eye Res*. 2006;25:397–424.
4. Das AV, Mallya KB, Zhao X, et al. Neural stem cell properties of Müller glia in the mammalian retina: regulation by Notch and Wnt signaling. *Dev Biol*. 2006;299:283–302.
5. Lawrence JM, Singhal S, Bhatia B, et al. MIO-M1 cells and similar Muller glial cell lines derived from adult human retina exhibit neural stem cell characteristics. *Stem Cells*. 2007;25:2033–2043.
6. Granger DN, Korthuis RJ. Physiologic mechanisms of postischemic tissue injury. *Annu Rev Physiol*. 1995;57:311–332.
7. Sakamoto Y, Nakajima T, Fukiage C, et al. Involvement of calpain isoforms in ischemia-reperfusion injury in rat retina. *Curr Eye Res*. 2000;21:571–580.
8. Thornberry NA, Lazebnik Y. Caspases: enemies within. *Science*. 1998;281:1312–1316.
9. Oka T, Walkup RD, Tamada Y, et al. Amelioration of retinal degeneration and proteolysis in acute ocular hypertensive rats by calpain inhibitor ((1S)-1-(((1S)-1-benzyl-3-cyclopropylamino-2,3-di-oxopropyl) amino)carbonyl)-3-methylbutyl)carbamate 5-methoxy-3-oxapentyl ester. *Neuroscience*. 2006;141:2139–2145.
10. Tamada Y, Fukiage C, Daibo S, Yoshida Y, Azuma M, Shearer TR. Involvement of calpain in hypoxia-induced damage in rat retina in vitro. *Comp Biochem Physiol B Biochem Mol Biol*. 2002;131:221–225.
11. Tamada Y, Nakajima E, Nakajima T, Shearer TR, Azuma M. Proteolysis of neuronal cytoskeletal proteins by calpain contributes to rat retinal cell death induced by hypoxia. *Brain Res*. 2005;1050:148–155.
12. Nakajima E, David LL, Bystrom C, Shearer TR, Azuma M. Calpain-specific proteolysis in primate retina: contribution of calpains in cell death. *Invest Ophthalmol Vis Sci*. 2006;47:5469–5475.
13. Broughton BR, Reutens DC, Sobey CG. Apoptotic mechanisms after cerebral ischemia. *Stroke*. 2009;40:e331–e339.
14. Liu MC, Kobeissy F, Zheng W, Zhang Z, Hayes RL, Wang KK. Dual vulnerability of tau to calpains and caspase-3 proteolysis under neurotoxic and neurodegenerative conditions. *ASN Neuro*. 2011;3:e00051.
15. Shimazawa M, Suemori S, Inokuchi Y, et al. A novel calpain inhibitor, ((1S)-1-(((1S)-1-benzyl-3-cyclopropylamino-2,3-di-oxopropyl)amino) carbonyl)-3-methylbutyl)carbamate 5-methoxy-3-oxapentyl ester (SNJ-1945), reduces murine retinal cell death in vitro and in vivo. *J Pharmacol Exp Ther*. 2010;332:380–387.
16. Martinez JA, Zhang Z, Svetlov SI, Hayes RL, Wang KK, Larner SF. Calpain and caspase processing of caspase-12 contribute to the ER stress-induced cell death pathway in differentiated PC12 cells. *Apoptosis*. 2010;15:1480–1493.
17. Oka T, Nakajima T, Tamada Y, Shearer TR, Azuma M. Contribution of calpains to photoreceptor cell death in N-methyl-N-nitrosourea-treated rats. *Exp Neurol*. 2007;204:39–48.
18. Imai S, Shimazawa M, Nakanishi T, Tsuruma K, Hara H. Calpain inhibitor protects cells against light-induced retinal degeneration. *J Pharmacol Exp Ther*. 2010;335:645–652.
19. Yoshikawa Y, Zhang GX, Obata K, et al. A cardioprotective agent of a novel calpain inhibitor, SNJ-1945, exerts beta1 actions on left ventricular mechanical work and energetics. *Am J Physiol Heart Circ Physiol*. 2010;299:H396–H401.
20. Yoshikawa Y, Zhang GX, Obata K, et al. Cardioprotective effects of a novel calpain inhibitor SNJ-1945 for reperfusion injury after cardioplegic cardiac arrest. *Am J Physiol Heart Circ Physiol*. 2010; 298:H643–H651.
21. Koumura A, Nonaka Y, Hyakkoku K, et al. A novel calpain inhibitor, ((1S)-1-(((1S)-1-benzyl-3-cyclopropylamino-2,3-di-oxopropyl)amino) carbonyl)-3-methylbutyl) carbamate 5-methoxy-3-oxapentyl ester, protects neuronal cells from cerebral ischemia-induced damage in mice. *Neuroscience*. 2008;157:309–318.
22. Ricart KC, Fiszman ML. Hydrogen peroxide-induced neurotoxicity in cultured cortical cells grown in serum-free and serum-containing media. *Neurochem Res*. 2001;26:801–808.
23. Sims NR, Muyderman H. Mitochondria, oxidative metabolism and cell death in stroke. *Biochim Biophys Acta*. 2010;1802:80–91.
24. Shi H, Liu K. Effects of glucose concentration on redox status in rat primary cortical neurons under hypoxia. *Neurosci Lett*. 2006;410: 57–61.
25. Park KJ, Kim YJ, Choi EJ, et al. Expression pattern of the thioredoxin system in human endothelial progenitor cells and endothelial cells under hypoxic injury. *Korean Circ J*. 2010;40:651–658.
26. Yu AC, Liu RY, Zhang Y, et al. Glial cell line-derived neurotrophic factor protects astrocytes from staurosporine- and ischemia-induced apoptosis. *J Neurosci Res*. 2007;85:3457–3464.
27. Laabich A, Vissvesvaran GP, Lieu KL, et al. Protective effect of crocin against blue light- and white light-mediated photoreceptor cell death in bovine and primate retinal primary cell culture. *Invest Ophthalmol Vis Sci*. 2006;47:3156–3163.
28. Hendrickson A, Bumsted-O'Brien K, Natoli R, Ramamurthy V, Possin D, Provis J. Rod photoreceptor differentiation in fetal and infant human retina. *Exp Eye Res*. 2008;87:415–426.
29. Lewis GP, Fisher SK. Muller cell outgrowth after retinal detachment: association with cone photoreceptors. *Invest Ophthalmol Vis Sci*. 2000;41:1542–1545.
30. Raser K, Posner A, Wang K. Casein zymography: a method to study mu-calpain, m-calpain, and their inhibitory agents. *Arch Biochem Biophys*. 1995;319:211–216.
31. Zhang Z, Larner SF, Liu MC, Zheng W, Hayes RL, Wang KK. Multiple alphaII-spectrin breakdown products distinguish calpain and caspase dominated necrotic and apoptotic cell death pathways. *Apoptosis*. 2009;14:1289–1298.
32. Matsushima H, David LL, Hiraoka T, Clark JI. Loss of cytoskeletal proteins and lens cell opacification in the selenite cataract model. *Exp Eye Res*. 1997;64:387–395.
33. Kitagaki H, Tomioka S, Yoshizawa T, et al. Autolysis of calpain large subunit inducing irreversible dissociation of stoichiometric heterodimer of calpain. *Biosci Biotechnol Biochem*. 2000;64:689–695.
34. Azuma M, Fukiage C, David LL, Shearer TR. Activation of calpain in lens: a review and proposed mechanism. *Exp Eye Res*. 1997;64: 529–538.
35. McGinnis KM, Gnegy ME, Park YH, Mukerjee N, Wang KK. Procaspase-3 and poly(ADP)ribose polymerase (PARP) are calpain substrates. *Biochem Biophys Res Commun*. 1999;263:94–99.
36. Blomgren K, Zhu C, Wang X, et al. Synergistic activation of caspase-3 by m-calpain after neonatal hypoxia-ischemia: a mechanism of "pathological apoptosis"? *J Biol Chem*. 2001;276:10191–10198.
37. Nicholson DW, Ali A, Thornberry NA, et al. Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature*. 1995;376:37–43.
38. Braun R, Linsenmeier R, Goldstick T. Oxygen consumption in the inner and outer retina of the cat. *Invest Ophthalmol Vis Sci*. 1995;36:542–554.
39. Silver IA, Deas J, Erecinska M. Ion homeostasis in brain cells: differences in intracellular ion responses to energy limitation between cultured neurons and glial cells. *Neuroscience*. 1997;78: 589–601.
40. Stone J, Maslim J, Valter-Kocsi K, et al. Mechanisms of photoreceptor death and survival in mammalian retina. *Prog Retin Eye Res*. 1999;18:689–735.
41. Kaur C, Foulds WS, Ling EA. Hypoxia-ischemia and retinal ganglion cell damage. *Clin Ophthalmol*. 2008;2:879–889.
42. Friedrich P. The intriguing Ca²⁺ requirement of calpain activation. *Biochem Biophys Res Commun*. 2004;323:1131–1133.
43. Cregan SP, Dawson VL, Slack RS. Role of AIF in caspase-dependent and caspase-independent cell death. *Oncogene*. 2004;23:2785–2796.
44. Sanges D, Comitato A, Tammara R, Marigo V. Apoptosis in retinal degeneration involves cross-talk between apoptosis-inducing factor (AIF) and caspase-12 and is blocked by calpain inhibitors. *Proc Natl Acad Sci U S A*. 2006;103:17366–17371.
45. McLeod DS, Grebe R, Bhutto I, Merges C, Baba T, Luty GA. Relationship between RPE and choriocapillaris in age-related macular degeneration. *Invest Ophthalmol Vis Sci*. 2009;50:4982–4991.
46. Azuma MCK, Fujii A, Shearer TR. Patient selection criteria for pilot studies on amelioration of non-neovascular age-related macular degeneration. *J Ocul Pharmacol Ther*. 2010;26:367–371.