Calpain Activation in Experimental Glaucoma

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PURPOSE. Glaucoma is a neurodegenerative disease in which elevated intraocular pressure (IOP) leads to progressive loss of retinal ganglion cells (RGCs) and blindness. Calcium dyshomeostasis has been suggested to play a role in the pathologic events that lead to RGC loss, though the details of these events are not well understood. Calcium-induced activation of calpain has been shown to contribute to neuronal death in a wide variety of neurodegenerative diseases. The authors hypothesize that similar events occur in glaucoma.

METHODS. The authors used a well-established rat model of experimental glaucoma. Retinal tissues were harvested after 5 or 10 days of elevated IOP and were subjected to immunoblot analysis, immunoprecipitation, and MALDI-ProTOF/MS peptide fingerprint mapping. Immunohistochemistry was used to localize calpain activation.

RESULTS. The authors present four independent lines of evidence that calpain is activated in experimental glaucoma. First, they showed that a 55-kDa autocatalytic active form of calpain is detected on immunoblot analysis. Second, they demonstrated the cleavage of two well-established calpain substrates, spectrin and calcineurin, only in eyes with elevated IOP. Third, they used MALDI-ProTOF to analyze cleaved calcineurin and immunoblot analysis of spectrin cleavage products and showed that both substrates were cleaved by calpain in experimental glaucoma. Fourth, they used immunohistochemistry to show that calpain-mediated spectrin cleavage occurs in RGCs under conditions of elevated IOP.

CONCLUSIONS. These data support the hypothesis that calpain is activated under conditions of elevated intraocular pressure and provide further details of the pathologic events leading to RGC loss in glaucoma. (*Invest Ophthalmol Vis Sci.* 2010;51:3049–3054) DOI:10.1167/iov.09-4364

Glaucoma is the leading cause of irreversible blindness globally,¹ and intraocular pressure (IOP) elevation is the biggest risk factor for glaucomatous visual loss.² The pathologic hallmark of glaucoma is atrophy of the optic nerve associated with retinal ganglion cell (RGC) death. Research on the mechanisms of RGC death indicates that RGCs die by apoptosis in human and experimental glaucoma.^{3–6} Current glaucoma therapy is directed at lowering IOP through surgical or pharmacologic approaches. These pressure-lowering approaches do not directly target the causes of RGC death and dysfunction.

Recent studies suggest that calcium dyshomeostasis may contribute to glaucomatous damage,^{7–11} and one calcium-responsive enzyme, calcineurin, appears to be activated in experimental glaucoma.¹² We now examine whether another calcium-activated enzyme, calpain, is also activated in the retina in experimental animals with increased IOP.

Calpains are ubiquitously expressed calcium-dependent cysteine proteases. The two major forms are μ -calpain and m-calpain, which respond to micromolar and millimolar concentrations of Ca^{2+} , respectively,¹³ and undergo autocatalytic cleavage on activation.^{14,15} Calpain activation has been implicated in a number of neurodegenerative processes, including Alzheimer's disease, Parkinsonism, and Huntington's disease.^{11,16} In the eye, calpain activation has been shown in retinal ischemia-reperfusion injury,¹⁷ after axotomy,¹⁸ in retinal detachment,¹⁹ and after excitotoxic injury.^{20,21}

There are multiple substrates for activated calpain, including cytoskeletal proteins such as spectrin¹⁵ and regulatory proteins such as calcineurin, a Ca^{2+} calmodulin-dependent protein phosphatase.²² Spectrin is an extensively studied 240-kDa cytoskeletal protein whose degradation is an early marker of neurodegeneration. Caspase cleavage yields a 120-kDa spectrin fragment, whereas calpain-mediated proteolysis of spectrin yields a 145-kDa fragment.¹⁵ Calcineurin has been demonstrated in several in vivo neuronal injury models to be cleaved by calpain,^{22,23} and we have previously demonstrated that calcineurin is cleaved in experimental glaucoma.¹² However, multiple proteases, including caspases, are also activated during cell death cascades. In some cases, including spectrin and calcineurin, substrates can be cleaved by more than one protease.^{15,24} The goal of this study was to investigate whether calpain is activated in experimental glaucoma and, if so, to distinguish whether calpain or other proteases such as caspases are responsible for the cleavage of substrates in a rat model of elevated IOP. Based on the data presented in this article, we conclude that calpain is activated in experimental glaucoma and suggest that it is an upstream therapeutic target for neuroprotection of RGCs in glaucoma.

MATERIALS AND METHODS

Animals

All experiments were performed in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Adult male Brown Norway rats (weight range, 300–450 g; Charles River Laboratories, Boston, MA) were used in this study.

Experimentally Induced Glaucoma

Unilateral elevation of IOP was produced by injecting hypertonic saline into aqueous veins, as originally described by Morrison.²⁵ Briefly, hypertonic 1.9 M saline was injected into limbal aqueous humor-collecting veins of the left eye (OS). The right eye (OD) served as control. If IOP was not elevated within 2 weeks, reinjection was performed in a different episcleral vein. A maximum of three injections were performed. Rats were killed after 5 or 10 days of elevated IOP for retinal protein analysis.

Intraocular Pressure Determination

All IOP measurements were performed in conscious, gently restrained animals between 10 am and 2 pm to minimize variability in IOP. After

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Supported by National Institutes of Health Grant R01EY13399 and Core Grant P30EY014104, Research to Prevent Blindness, and the Massachusetts Lions Eye Research Fund.

Submitted for publication July 23, 2009; revised December 17, 2009; accepted January 4, 2010.

Disclosure: **W. Huang**, None; **J. Fileta**, None; **I. Rawe**, None; **J. Qu**, None; **C.L. Grosskreutz**, None

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applying 1 drop of 0.5% proparacaine local anesthesia, IOP was measured with a tonometer (TonoPen XL; Medtronic Ophthalmics, Jacksonville, FL). Fifteen readings were taken for each eye and were averaged. Baseline IOP was obtained on three consecutive days before the first saline injection and three times per week thereafter. We subjected the animals to a 5- or 10-day period of elevated pressure exposure. After IOP had been elevated for 10 days, rats were killed with CO₂ asphyxiation, their eyes were rapidly removed, and the eyecups or retinas were collected for protein isolation for immunoprecipitation or for Western blot analysis. As a measure for IOP exposure, we integrated IOP with time, which takes into account both the length and the degree of IOP exposure. Integrated IOP was calculated as the area under the time-pressure curve (experimental eye/control eye), beginning with the day of the first saline injection.

Tissue Preparation

For immunoblot analysis, retinas were homogenized and lysed with buffer containing 1 mM EDTA/EGTA/dithiothreitol, 10 mM HEPES pH 7.6, 0.5% surfactant (Igepal; Sigma-Aldrich, St. Louis, MO), 42 mM KCl, 5 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, and 1 tablet of protease inhibitors (Complete Mini; Roche Diagnostics GmbH, Mannheim, Germany) per 10 mL buffer. Samples were incubated for 15 minutes on ice and then were centrifuged at 21,000 rpm at 4°C for 30 minutes, and the supernatant was stored at -80°C. The protein concentration of the supernatant from each retina was determined using the reagents (DC Protein Assay; Bio-Rad Laboratories, Hercules, CA).

For immunohistochemistry, eyes were enucleated, and the anterior segments were removed. Eyecups were fixed with 4% paraformaldehyde in PBS overnight, cryoprotected with serial sucrose dilutions, frozen in optimal cutting temperature compound (Sakura Finetek USA, Inc., Torrance, CA), and cut into 16-μm sections using a cryostat.

Back-Labeling of RGCs

Rats were deeply anesthetized with a mixture of acepromazine maleate (1.5 mg/kg), xylazine (7.5 mg/kg), and ketamine (75 mg/kg) (all from Webster Veterinary Supply, Sterling, MA). Anesthetized rats were put in a stereotaxic apparatus (Kopf Instruments, Tujunga, CA), and the skull was exposed and leveled by use of the lambda and bregma sutures as landmarks. Craniotomy was performed, and an injector was lowered into the superior colliculus 5.9 mm posterior to the bregma, 1.7 mm lateral to the midline, and 4.6 mm ventral to the skull surface. Two microliters of a 4% fluorogold (Fluorochrome LLC, Denver, CO) solution in PBS with 1.5% dimethyl sulfoxide was injected over 10 minutes. A second injection was performed on the ipsilateral side 7 mm posterior to the bregma, 1.2 mm lateral to the midline, and 3.9 mm ventral to the skull surface. Similarly, two injections were performed on the contralateral side of the brain. The skin was then sutured, and antibiotic ointment was applied. Seven days were allowed for retrograde transport of fluorogold (Fluorochrome LLC) before further experimental interventions.

Western Blot Analysis

Proteins were separated on SDS-PAGE gels (10%–20% Tris-HCl Ready-Gels; Bio-Rad Laboratories). Twelve micrograms of total retinal protein was loaded per lane, transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA), and blocked with 5% nonfat dry milk in 0.1% TBST. The primary antibodies used were calpain (1:1000; Sigma, St. Louis, MO), spectrin (1:2000; Chemicon, Temecula, CA), and calcineurin (1:250; BD Transduction Laboratories, Lexington, KY). Secondary antibodies were rabbit peroxidase-conjugated (1:10,000; Jackson ImmunoResearch, West Grove, PA) and mouse peroxidase-conjugated (1:10,000; Jackson ImmunoResearch). After overnight incubation at 4°C, membranes were washed with TBST and incubated for 1 hour in secondary antibody at room temperature. Labeled protein was detected using ECL Plus (Amersham Pharmacia Biotech, Piscataway, NJ). Membranes were exposed to specialized film (HyperFilm; Amersham Biosciences, Chicago, IL). α-Tubulin (1:2000; Abcam, Cambridge, UK) was used as a loading

control. Densitometry was carried out using imaging software (ImageQuant 1.2; Molecular Dynamics, Inc., Sunnyvale, CA).

Immunohistochemistry

Retinal sections were treated with 0.3% H₂O₂ and 0.3% normal goat serum (Invitrogen, Eugene, OR) in PBS for 10 minutes, blocked for nonspecific binding of the antibodies with 4% normal goat serum and 0.3% Triton X-100 in PBS for 1 hour, and blocked for nonspecific binding of avidin and biotin with the avidin/biotin blocking kit (Vector Laboratories, Inc., Burlingame, CA). After blocking, the sections were incubated in primary antibody ab38, which recognizes calpain-cleaved α-spectrin (1:5000, rabbit polyclonal; a generous gift from David R. Lynch, University of Pennsylvania, Philadelphia) at 4°C overnight. Then the sections were incubated in biotinylated goat anti-rabbit antibody (1:1000; Vector Laboratories, Inc.) at room temperature for 1 hour and were incubated in avidin-biotin-peroxidase complex (ABC kit; Vector Laboratories, Inc.) at room temperature for 1 hour. Immunostaining was visualized by incubating the sections in diaminobenzidine and hydrogen peroxide (DAB kit; Vector Laboratories, Inc.) for 2 minutes. Results were confirmed in three additional animals.

For immunofluorescence, sections were blocked with 4% normal goat serum and 0.3% TritonX-100 in PBS at room temperature for 1 hour, incubated in primary antibody ab38 (1:5000) at 4°C overnight, and incubated in Alexa-594 conjugated goat anti-rabbit antibody (1:250) at room temperature for 1 hour.

All images were taken with a microscope (BX51; Olympus, Albertslund, Denmark) and a digital camera (DP70; Olympus) and were processed with image editing software (Photoshop CS; Adobe Systems Inc., San Jose, CA).

Immunoprecipitation

An initial preclearing step of incubating 50 μL prepared protein A-Sepharose beads (Upstate Biotechnology, Lake Placid, NY) with 500 μL retina lysate for 1 hour at 4°C was performed. Immunoprecipitation was carried out using a commercial kit (Seize Primary Immunoprecipitation Kits; Pierce, Rockford, IL). In accordance with the manufacturer's protocol, anti-calcineurin antibody (1:250; BD Transduction Laboratories) was directly and covalently linked to the resin. The immobilized antibody resin was then incubated overnight at 4°C with retinal lysates to capture the target protein. After a final wash, the captured protein was eluted and added to 5× sample buffer. Samples were heated at 95°C to 100°C for 5 minutes and loaded on a 10% to 20% gradient gel for electrophoresis.

Proteomics

After electrophoresis, protein bands were identified on the gel by staining with Coomassie Blue R-250. Bands were cut out for in-gel trypsin digestion. Gel pieces were placed in a 96-well microtiter plate (ZipPlate; Millipore, Billerica, MA) and processed as described according to the manufacturer's protocol. In brief, the gel band was washed in 25 mM ammonium bicarbonate/5% acetonitrile for 30 minutes and was destained twice with ammonium bicarbonate/50% acetonitrile for 30 minutes. Gel bands were then dehydrated with 100% acetonitrile for 15 minutes, rehydrated in 15 μL of 25 mM ammonium bicarbonate containing 100 ng trypsin (Trypsin Gold; Promega, Madison, WI), and incubated at 30°C overnight. The C₁₈ resin of the 96-well microtiter plate (ZipPlate; Millipore) was then activated with 9 μL acetonitrile for 15 minutes at 37°C. Peptides were washed out of the gel plug with 190 μL of 0.1% trifluoroacetic acid (TFA) for 30 minutes and were bound to the C₁₈ resin by low vacuum followed by washing twice with 100 μL TFA under high vacuum. Peptides were then directly eluted onto a disposable MALDI target plate (PerkinElmer, Shelton, CT) by direct vacuum elution with matrix α-cyano-4-hydroxy cinnamic acid (α-CHCA) in 50% acetonitrile/50% TFA. Matrix was air-dried, allowing matrix crystals to form. The MALDI plate was then loaded into a pro-TOF 2000 MALDI-TOF (PerkinElmer), and data were acquired with a mass range of 750 to 4500 Da. The resultant data were then searched on a local copy of NCBI (National Center for Biotechnology Informa-

FIGURE 1. Calpain cleavage in experimental glaucoma. (A) Western blots showing a 55-kDa autocatalytic calpain fragment in eyes with elevated IOP at 5 and 10 days. (B) Summary data show a statistically significant increase in the 55-kDa autocatalytic calpain fragment after 5 and 10 days of elevated IOP (mean $\pm$ SEM). * $P < 0.05$.

tion, www.ncbi.nih.gov) protein database with the ProFound search engine (Rockefeller University, New York, NY)

RESULTS

IOP History

Before any intervention, the average IOP was 18.25 ± 0.15 mm Hg (mean $\pm$ SD) for the control and 18.87 ± 0.81 mm Hg for

the experimental eyes ($n = 12$). Control eye IOP remained unchanged throughout the experiment. Average peak IOPs for 5-day control and experimental glaucoma eyes were 18.8 ± 1.5 mm Hg and 42.5 ± 1.5 mm Hg; for 10-day control and experimental glaucoma eyes, they were 18.16 ± 0.84 and 42.21 ± 1.8 mm Hg, respectively. The average integrated pressure-time difference between control and experimental eyes was 152.3 ± 18.3 mm Hg-days for 5 days and 332.28 ± 51.43 mm Hg per day for 10 days.

Calpain Cleavage with Elevated IOP

We first asked whether calpain is activated in eyes with high IOP. Calpain exists as a proenzyme activated by elevations of intracellular calcium that induce autocatalytic cleavage to yield an active 55-kDa product.¹⁵ We performed Western blot analysis with an antibody that recognizes the 55-kDa autolytic fragment of calpain (Sigma, St. Louis). We found that the 55-kDa fragment is present in half the eyes with high IOP after 5 days ($n = 6$) and in all the eyes with high IOP after 10 days ($n = 6$; Fig. 1A). This 55-kDa autolytic fragment was significantly increased in eyes with high IOP compared with their fellow control eyes (Student's *t*-test: 5 days, $P < 0.05$; 10 days, $P < 0.01$; Fig. 1B). In eyes with high IOP, the increase in the 55-kDa autolytic fragment was significantly greater after 10 than after 5 days. The amount of 55-kDa product on Western blot did not correlate with the degree of increased IOP among the 10-day animals, possibly because of the relatively small variation in degree of elevated IOP in these animals.

Calpain cleavage strongly suggests calpain activation but does not directly address whether activated calpain cleaves specific substrates. Indeed, calpain activity is tightly regulated with mechanisms, including calpastatin, potentially blocking the proteolytic activity of cleaved calpain. We therefore examined whether two known substrates of calpain were cleaved in experimental glaucoma and further examined these cleavage fragments.

FIGURE 2. Elevated IOP leads to cleavage of spectrin. (A) Western blot analysis shows spectrin cleavage products at 120 kDa and 145 kDa in eyes after 5 and 10 days of elevated IOP. (B–F) Localization of calpain-cleaved spectrin in the retina. Immunohistochemistry showing elevated level of calpain-cleaved α -spectrin in the GCL, IPL, and INL in the glaucomatous eye (C) compared with the control eye (B). Omitting primary antibody eliminated all staining (D). (E, F) High-magnification images showing calpain-cleaved α -spectrin in the RGC cytoplasm (arrow) and primary dendrite (arrowhead) in the glaucomatous eye. (E) Immunostaining of calpain-cleaved α -spectrin (ab38). (F) Fluorogold (FG) retrograde-labeled RGC. (G) Merge of (E) and (F). Scale bars: 50 μ m (D); 10 μ m (G). GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer.

FIGURE 3. MALDI-ProTOF/MS peptide fingerprint mapping of calcineurin cleavage site. (A) Representative immunoblot analysis of retinal protein from an animal with experimental glaucoma showing cleaved calcineurin only in the eye with elevated IOP. (B) Immunoblot of glaucomatous retinal protein isolated by immunoprecipitation with a calcineurin antibody. Arrow: cleaved calcineurin band. (C) MALDI-ProTOF/MS of fragments from the tryptic digest of cleaved calcineurin isolated from the gel shown in (B). (D) MALDI-ProTOF/MS results for glaucomatous retinal proteins (full-length and cleaved calcineurin) isolated from the gel shown in (B). Cleaved calcineurin is truncated in the region of the arrow that indicates a predicted calpain cleavage site. This truncated form of calcineurin lacks the autoinhibitory domain (*underlined*). The full-length calcineurin band includes the c-terminal autoinhibitory domain. BSA, bovine serum albumin; IP, immunoprecipitation.

Spectrin Cleavage with Elevated IOP

Immunoblot analysis was performed using an antibody that recognizes both the 120-kDa and the 145-kDa fragments of cleaved spectrin (Fig. 2A). After 5 days of elevated IOP, increases in both the 120- and the 145-kDa bands, corresponding to caspase and calpain cleavage of spectrin, were found in 3 of 5 eyes. After 10 days, both the 120-kDa and the 145-kDa bands were increased in 4 of 6 animals (Fig. 2A). Taken together, 8 of 11 eyes with high IOP had an increase in the 145-kDa band (Fisher's exact test, $P < 0.05$), and 7 of 11 eyes with high IOP had an increase in the 120-kDa band (not significant).

To determine the cell types responsible for the observed calpain activation, we used an antibody that specifically recognizes calpain-cleaved spectrin (ab38, a generous gift from David Lynch at the University of Pennsylvania). We found that calpain-cleaved spectrin immunoreactivity was increased in the retina in experimental glaucoma (Fig. 2C), with especially prominent staining in the RGC layer of glaucomatous eyes (Fig. 2G).

MALDI-ProTOF/MS Peptide Fingerprint Mapping of Calcineurin Cleavage Site

We previously reported that calcineurin was cleaved in half the animals with high IOP at 5 days and in all the animals with high IOP after 10 days.¹² Figure 3A shows a representative immunoblot from a rat with 10 days of experimental glaucoma, illustrating calcineurin cleavage in the eye with high IOP, yielding a band between 45 and 50 kDa. Both calpain and caspase 3 have been demonstrated to cleave calcineurin-yielding products with slightly different molecular weights. Because it is difficult to definitively distinguish between the sizes of the caspase and calpain cleavage products of calcineurin by immunoblot analysis, we performed proteomic analysis of full-length and cleaved calcineurin from eyes with experimental glaucoma.

We performed experiments in which peptide fingerprint mapping was used to characterize the cleaved form of calcineurin after 10 days of elevated IOP. Comparison of full-length sequence calcineurin (*Rattus norvegicus*, Swiss-Prot:

FIGURE 4. Hypothesized calcium-activated events and calpain activation under conditions of elevated IOP.

locus P2BA_RAT, accession no. P63329) with the known substrate specificity of calpain²⁶ predicts a calpain cleavage site at amino acid 459. The full-length and cleaved calcineurin from retinas of rats with experimental glaucoma was isolated by immunoprecipitation and then separated by SDS gel electrophoresis (Fig. 3B). Bovine serum albumin (66 kDa) was used as a molecular marker. Two bands were identified on the gel with molecular weights at 60 kDa and ~45 to 50 kDa. We isolated and submitted both bands from the gel for trypsin digestion and MALDI-ProTOF/MS analysis. MALDI-ProTOF/MS analysis confirmed that both bands were calcineurin (Fig. 3C). We detected trypsin fragments demonstrating cleavage at approximately amino acid 459 from the ~45- to 50-kDa band (Fig. 3D). This cleavage site was comparable to the calpain cleavage site reported after excitotoxic brain injury²² in mice and in the failing human heart²⁷ with a calculated molecular weight of 50 kDa in our rat model. Interestingly, as in the other injury paradigms, the autoinhibitory domain of calcineurin was lost in the cleaved fragment (Fig. 3D, underlined sequence). Importantly, the presence of tryptic fragments from calcineurin c-terminal to the reported caspase 3 cleavage site at amino acid 385/386 demonstrated that calpain, not caspase 3, is responsible for cleaving calcineurin in experimental glaucoma.

DISCUSSION

We present four independent lines of evidence that calpain is activated in experimental glaucoma: (1) an autocatalytic cleaved form of calpain is detected on Western blot only in eyes with elevated IOP; (2) two well-established substrates of activated calpain, spectrin and calcineurin, are cleaved only in the retinas of rats with experimental glaucoma; (3) immunohistochemical analysis of control and glaucomatous retinas demonstrates increased staining of calpain-cleaved spectrin in experimental glaucoma and shows specifically that this increase occurs in RGCs; (4) distinguishing proteolytic products by size for spectrin, and by MALDI-ProTOF/MS analysis of cleaved calcineurin, reveals that these substrates are cleaved at predicted calpain cleavage sites.

Calpains exist as proenzymes in resting cells and are activated by calcium and autolytically cleaved.¹⁵ Autolysis of the N-terminal region of domain I of calpain leads to the generation of two active proteases of 78 and 76 kDa, respectively. Addi-

tional autocatalytic processing of domain IV of this protease leads to the generation of a 55-kDa active form.¹⁵ Our data show that the 55-kDa fragment was detected in half the eyes with high IOP after 5 days and in all the eyes with high IOP after 10 days. Interestingly, we previously reported that calcineurin cleavage is not seen in all eyes at 5 days but is seen in all eyes at 10 days,¹² supporting our hypothesis that calpain is activated under conditions of high IOP and cleaves calcineurin in experimental glaucoma.

Spectrin is a cytoskeletal calmodulin-binding protein that is cleaved during apoptosis by calpain and caspases¹⁵ to form distinct products. Calpain cleavage of spectrin results in a 145-kDa fragment, and caspase cleavage produces a 120-kDa product.¹⁵ Here we report increases in both the 120-kDa and the 145-kDa spectrin breakdown products, suggesting that both calpain and the caspases are active in experimental glaucoma. These data are consistent with our previous data showing that both caspase 8 and 9 are activated in experimental glaucoma⁴ and with previous reports showing caspase 3 activation.⁵ Calcineurin is a known substrate of calpain²² and caspase 3²⁴; we next asked which protease was acting to cleave calcineurin in experimental glaucoma.

The actions of recombinant caspase 3 (and, to a lesser extent, caspase 7) on purified calcineurin protein showed that a ~45 kDa truncated calcineurin is formed.²⁴ By comparing the known specificity of calpain²⁶ with the rat calcineurin sequence (*R. norvegicus*, Swiss-Prot: locus P2BA_RAT, accession no. P63329), we predicted a cleavage site at amino acid 459 that would yield an ~50-kDa product. MALDI-TOF/MS has been used as a sensitive analytical approach to determine the site of calcineurin cleavage after excitotoxic brain injury in mice and in the failing human heart and has implicated calpain as the responsible protease.^{22,27} By examining the tryptic fragments of full-length and cleaved calcineurin from rats with 10 days of elevated IOP with MALDI-ProTOF, we identified a probable cleavage site at amino acid 459 in calcineurin, yielding a cleaved calcineurin fragment with a calculated molecular weight of ~50 kDa. This cleavage site is comparable to the calpain cleavage site reported after excitotoxic brain injury in mice²² and in the failing human heart.²⁷ No smaller fragment corresponding to the caspase cleavage site at amino acid 385 was seen in retinas of rats with experimental glaucoma. Our results point to calpain as the protease responsible for calcineurin cleavage under conditions of high IOP.

Two other models of ocular hypertension have been examined from the point of view of spectrin cleavage and caspase/calpain activation. An acute elevation of IOP to 120 mm Hg for 1 hour led to apparent calpain-mediated cleavage of spectrin at 1 week but no clear evidence of caspase cleavage of spectrin.^{28,29} In this model, the IOP exceeded the perfusion pressure of the eye and might have modeled the combined effects of ischemia and elevated IOP. By contrast, 5 to 12 weeks of a more modest elevation of IOP revealed an elevation of the 120-kDa caspase-3-mediated spectrin breakdown product with no change from control in the 145-kDa band.³⁰ Our animals had an IOP range between the very high IOP model of Oka et al.^{28,29} and the more chronic model of Tahzib et al.,³⁰ and our data show increases in both the 145-kDa and 120-kDa bands, consistent with activation of both calpain and caspase 3.

Calpains have been implicated in several other models of axonal or neuronal injury. Calpain activation has been shown after optic nerve stretch *in vivo*³¹ and in growth hormone-mediated apoptosis of RGCs *in vitro*.³² Calpain activation has also been demonstrated in other neurodegenerative diseases such as Alzheimer's disease, Parkinsonism, and spinal cord injury.^{11,16,31} The data presented in this article support the addition of glaucoma to this list of diseases involving calpain activation.

There are a number of different points in death cascades at which cross-talk between caspases and calpain have been described. For example, activation/cleavage of caspase 3 by calpain has been reported after hypoxia,³³ and caspase 3 has been demonstrated to cleave the calpain inhibitor, calpastatin, further activating calpain.³⁴ Activation of both the caspase and the calpain pathways was shown to occur in parallel during photoreceptor apoptosis.^{35,36} We hypothesize that similar events may be present in RGCs during apoptosis in glaucoma, with clear evidence showing the activation of both caspase⁴ and calpain (Figs. 1–3). Our current data emphasize, however, that calpain activation is upstream of calcineurin cleavage, which has been implicated in neuronal death, and we suggest that it also likely mediates the cleavage of additional substrates. We hypothesize that calpain activation may amplify calcineurin activation by creating a nonregulatable form of the enzyme, which in turn may have multiple downstream consequences, including dephosphorylation of the proapoptotic protein pBad (Fig. 4). Further studies will be needed to characterize these pathways, which could lead to therapeutic strategies of combinations of inhibitors targeting these early molecular events in glaucomatous neurodegeneration.

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