Isolevuglandin-Modified Proteins, Including Elevated Levels of Inactive Calpain-1, Accumulate in Glaucomatous Trabecular Meshwork†

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ABSTRACT: We report that protein adducts of iso[4]levuglandin E_2 (iso[4]LGE_2), a highly reactive product of free radical-induced lipid oxidation, accumulate in human glaucomatous trabecular meshwork (TM) but not in controls. Reactive oxygen species play a pathogenic role in primary open angle glaucoma by fostering changes that reduce permeability of the TM tissue and consequently impede aqueous humor outflow resulting in elevated intraocular pressure. IsoLGs covalently modify proteins and are especially effective in causing protein−protein cross-linking. We found elevated levels of calpain-1 in glaucomatous TM. However, calpain activity in glaucomatous TM is only about 50% of that in controls. This paradox is explicable by the fact that modification by isoLGs renders calpain-1 inactive. Thus, treatment of calpain-1 with iso[4]LGE_2 in vitro results in covalent modification, inactivation, the formation of high molecular weight aggregates (as determined by Western and dynamic light scattering analyses), and resistance to proteasomal digestion. Iso[4]LGE_2-modified calpain-1 undergoes ubiquitination, and its loading impairs the cellular proteasome activity, consistent with competitive inhibition and formation of suicidal high molecular weight aggregates. These data suggest that interference with proteasomal activity, owing to protein modification by isoLGs, could contribute to glaucoma pathophysiology by decreasing the ability of the TM to modulate outflow resistance.

Glaucomas are a group of irreversible blinding neurodegenerative diseases that are late onset and progressive in nature. They are designated primary, when they occur without a known cause, or secondary, when onset can be attributed to any other illness or injury. Worldwide about 70 million people suffer from glaucoma (*1*). Primary open angle glaucoma (POAG[1]) is characterized by changes that reduce permeability of the trabecular meshwork (TM) tissue and consequently impede aqueous humor outflow resulting in elevated intraocular pressure (IOP). Mounting evidence supports the hypothesis that reactive oxygen species (ROS) play a pathogenic role in POAG (*2*). Elevated levels of hydrogen peroxide in the aqueous humor promote TM degeneration and outflow resistance. Protection against the deleterious effects of ROS should be provided by the antioxidant activities of superoxide dismutase and glutathione peroxidase that are elevated in the aqueous humor of glaucoma patients. However, a deletion of the gene encoding for glutathione-S-transferase M1 may predispose POAG patients to ROS-induced oxidative injury (*3*). Oxidative damage of DNA, indicated by levels of 8-hydroxy-2′-deoxyguanosine, and oxidative damage of lipids, indicated by levels of conjugated dienes, are significantly increased in the TM of glaucoma patients compared to controls (*3*−*5*). The IOP and the severity of visual-field defects in glaucoma patients are positively correlated with levels of oxidative DNA damage in the TM (*3*, *5*). A possible role for oxidative protein damage in the etiology of POAG is suggested by a marked decline of proteasome activity in TM cells resulting from exposure to chronic oxidative stress (*6*). Proteasome failure could contribute to glaucoma pathophysiology by decreasing the ability of the TM to modulate outflow resistance.

Calpains are ubiquitous calcium-dependent cysteine proteases that exist in two major forms, μ-calpain and m-calpain, in all tissues and belong to a superfamily of 14 cysteine proteases. Activation of calpains in vitro requires the presence of calcium at micromolar quantities for μ-calpain and millimolar quantities for m-calpain at neutral pH (*7*, *8*). Both forms possess a large subunit of about 80 kDa and a small subunit of about 30 kDa. Increased calpain activity has been found in several neurological disorders, such as spinal cord injury, ischemic brain injuries, stroke, and have been implicated in several neurodegenerative disorders such as Alzheimer's disease and multiple sclerosis (*8*). Autocatalysis of calpain, activated by calcium, converts the 80 kDa subunit to a 76 kDa form (*9*). Differences in calpain-1 processing have been found in some disease conditions. For example,

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[1] Abbreviations: DLS, dynamic light scattering; GAPDH, glyceraldehydes phosphate dehydrogenase; HNE, 4-hydroxynonenal; IOP, intraocular pressure; LG, levuglandin; pAb, polyclonal antibody; PBS, phosphate-buffered saline; POAG, primary open angle glaucoma; RGC, retinal ganglion cell; Rh, hydrodynamic or Stokes radius; ROS, reactive oxygen species; TM, trabecular meshwork.

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in Alzheimer's disease, altered ratios of 80 kDa to 76 kDa bands were observed (*9*).

We now report the molecular level identification of lipid-derived oxidative protein modifications that are elevated in glaucomatous TM and that interfere with the housekeeping and/or regulatory functions of proteases, i.e., calpain-1 and the proteasome (vide infra). Because of their extraordinary proclivity to covalently modify and cross-link proteins (*10*, *11*), it seemed reasonable to speculate that the generation of isolevuglandins (isoLGs) by free radical-induced oxidation of phospholipid-esterified arachidonic acid impairs aqueous outflow in glaucomatous TM by producing oxidative modifications of TM proteins resulting in their aggregation and accumulation. Furthermore, protein modification by isoLGs has been shown not only to inhibit their degradation by the proteasome but also to poison this pivotal cytosolic protease (*12*).

Our previous proteomic and Western analyses of optic nerve (*13*) showed an increased presence of the thioprotease calpain-1 in glaucomatous versus normal optic nerve (unpublished observations), consistent with the upregulation of calpain-1 in damaged neuronal tissue as found in other neurodegenerative models (*8*, *14*, *15*). In contrast, preliminary proteomic analysis (*16*) seemed to show a decreased presence of calpain-1 in glaucomatous versus normal TM. We now present evidence demonstrating that modification of calpain-1 by isoLGs occurs in glaucomatous TM and results in decreased activity of this protease in glaucomatous compared with normal TM even though the level of protein showing calpain-1 immunoreactivity is far greater in the glaucomatous versus normal TM. This paradox is explicable by the fact that modification by isoLGs renders calpain-1 inactive (vide infra). Although calpain-1 is upregulated in glaucomatous versus normal optic nerve, no appreciable modification by isoLGs was detected in this tissue.

MATERIALS AND METHODS

Tissue Procurement. Donor eyes from normal (control) and POAG cadavers were enucleated within 16 h of death and obtained from the National Disease Research Interchange, Philadelphia, PA and the Cleveland Eye Bank, Cleveland, Ohio. Acceptable eyes were those that had some available medical and ophthalmic histories and lacked major CNS disorder (Supporting Information Table ST1). Control eyes were from normal donors that lacked optic neuropathy and any history of eye diseases. Glaucomatous and age-matched (±4 years) normal eyes from donors between 50 and 90 years of age were used in this study. Research was conducted following the tenets of the Declaration of Helsinki.

Western Analyses. Proteins were extracted from tissues (TM or optic nerve) by homogenization in 100 mM Tris−Cl buffer pH 7.5 containing 5 mM dithiotheritol, 1 mM $SnCl_2$, 50 mM $NaHPO_4$, 1 mM diethylene-triaminepentaacetic acid, 100 mM butylated hydroxy toluene and 0.5% SDS. Insoluble material was removed by centrifugation (8000*g* for 5 min), and soluble protein was quantified by the Bradford assay. Western blot analyses were performed with 10 μg of protein extract, 4−20% gradient gels (Invitrogen Inc., CA), electroblotting to PVDF membrane, and probing with polyclonal (Abcam Inc., Cambridge, MA) and monoclonal calpain-1 antibody (Sigma Chemical Co., St. Louis, MO; catalog no. C267) or rabbit polyclonal antibody against iso-[4]LGE_2-protein adducts moiety (*17*). In addition, blots were also probed with calpain-2 large subunit antibody (Cell Signaling Technologies, Boston, MA; catalog no. 2539) and calpain-1 small subunit antibody (Calbiochem, San Diego, CA; catalog no. 208730). For all analyses commercially available 4−20% SDS−polyacrylamide gels (Invitrogen Inc., Carlsbad, CA) were used unless stated otherwise. For quantitative Western analyses, antimouse and antirabbit secondary antibodies linked to IR-dyes (700 or 800 nm) were used on an Odyssey infrared imaging system according to the manufacturer (Li-Cor Biosciences, Lincoln, NB).

Synthesis of Iso[4]LGE$_2$. Iso[4]LGE_2 (5-acetyl-6-formyl-9-hydroxy-7(*E*), 11(*Z*)-heptadecadienoic acid), a highly oxidized lipid, is generated during autoxidation of arachidonic acid or arachidonate esters. Synthesis of iso[4]LGE_2 was carried out using diethyl-(2-oxopropyl)-phosphonate, 4-iodobutyrate, isopropylidene-L-glyceraldehyde, and a vinylstannane as starting materials, exploiting 1,4-addition of a vinyl nucleophile to a γ-alkoxy enone as the key carbon−carbon bond-forming step following methodology described previously (*17*).

Assay of Calpain-1 Activity. Calpain-1 assay was performed with 10 μg of tissue extracts or recombinant control calpain-1 using a calpain activity assay kit (Biovision Inc., Mountainview, CA) following recommended protocols. The flourometric assay is based on detecting the cleavage of a calpain substrate, Ac-LLY-AFC, that fluoresces at 505 nm, whereas the cleaved substrate fluoresces at 400 nm. The fluorescence was detected using Spectra Max Gemini spectrofluorometer (Molecular Devices, Sunnyvale, CA) and Softmax Pro 4.0 software. In TM tissue extracts the activity was also determined in presence of 5 mM Calpeptin (*18*) (*Z*-Leu-norleucinal; Alexis Corporation, Lausen, Switzerland, catalog no. 260-014-M005).

Purification of Autocatalytic Calpain-1 Fragments. Purified calpain-1 from human erythrocytes was purchased from Calbiochem EMD BioSciences (San Diego, CA) and stored at −80 °C. About 10 μL of 1 mM $CaCl_2$ solution was added to purified calpain-1 (per 100 μg of protein) to initiate autocatalysis. Autocatalytic products were purified using either column chromatography or gel electrophoresis and band excision. Column chromatography utilized an established gel exclusion procedure with a 25 mL column of Sephacryl S-200. Alternatively, protein bands (generated from 100 to 150 μg of purified calpain-1) were excised after SDS−PAGE separation, homogenized, and repeatedly extracted using four 5 μL increments of water containing 50 mM NaCl (maintained at 65 °C). The protein was recovered by centrifugation at 12 K rpm for 10 min. Purified protein fragments were stored at 4 °C for future experiments.

In Vitro Protein Modification by Iso[4]LGE$_2$. Purified calpain-1 and its fragments were subjected to modification using iso[4]LGE_2 prepared by total synthesis as described above. The individual fragments were subjected to varying concentrations of iso[4]LGE_2 and allowed to rock overnight at room temperature in phosphate-buffered saline (PBS) in a final volume of 50 μL. Modified proteins were precipitated over 10 min by adding 200 μL of acetone at 0−4 °C, centrifuged at 12 K rpm for 5 min, and dried using a speed vac for 10 min. The modified proteins were washed and

finally resuspended in 15 μL of PBS and used for experiments or SDS−PAGE separation.

In Vitro Ubiquitination and Proteasome 26S Treatment. In vitro ubiquitination was performed using an S100 HeLa conjugation kit and mammalian purified stable 26S proteasome fraction competent for degradation of preformed ubiquitin−protein conjugates. All reagents except mentioned otherwise were procured from Boston Biochemicals, Cambridge, MA.

Plugged Gel Variable Field Electrophoresis. Samples were embedded in polyacrylamide−agarose plugs using an 8% polyacrylamide mix so that the final acrylamide concentration is 4%. Polymerization was initiated with the addition of 10% v/v ammonium persulphate, and the entire solution was loaded onto the wells of commercial precast gels; the plugs also contained a final 0.5% (w/v) of agarose (molecular biology grade; Sigma Chemical Co., St. Louis, MO). Gels were subjected to an initial voltage of 200 V (30 mA) for 1 min and subsequently alternating pulses of 200 and 80 V for 1 min for five cycles followed by constant 80 V for 90 min.

TM Cell Culture and Inhibition of Proteasomal Activity. Primary TM cells were derived from donor human eyes following methods described previously (*16*). About 3000 primary TM cells were subjected to proteasomal inhibition using epoxomicin (Peptides International, catalog no. 4381-v); an equal amount of cells were used as control grown under identical conditions. Briefly, the cells were grown for 24 h at 37 °C and subjected to inhibition with 0.04 μmol (final concentration) epoxomicin (*19*) for 6 h. Cells were lysed by brief sonication, and 10 μg of total protein was subjected to ELISA analysis. The proteins were incubated on plates (BD Falcon plate 353912) at 37 °C for 1 h. The supernatant was discarded, and plates were blocked with 1% ovalbumin in 1× PBS, 300 μL/well, washed and subsequently incubated with primary calpain-1 antibody (1:500 dilution), washed and incubated with secondary antibody (1:2000 dilution) coupled with alkaline phosphatase for 1 h. The plates were washed and added with 100 μL/well of 1 mg/mL phosphatase substrate in diethanolamine buffer pH 7.5. The absorbance was measured at 405 nm on a Synergt HT plate reader (Bio-Tek Instruments, Inc., Winooski, VA).

Dynamic Light Scattering. Dynamic light scattering was performed using about 1 μg of protein mixture in a 12 μL holding cuvette using a Proteinsolutions microsampler (MS and LSR) on a Proteinsolutions DynaPro light scattering device (Wyatt Technology Corporation, Santa Barbara, CA). Online acquisition and data analysis was performed using Dynamics software (version 5.26.02) on an Optiplex GX110 computer connected to a microsampler.

RNA Isolation and Northern Analysis. Total RNA from optic nerve was isolated using TRIZOL with modification of the protocol recommended by the supplier (Invitrogen Inc., Carlsbad, CA), subjected to Northern blotting using standard protocols (*20*).

Probing Translation with Polyadenylated RNA-Depleted Extracts. Assays were performed probing translation of calpain-1 upon addition of purified polyA calpain-1 and GAPDH mRNAs to tissue extracts (TM or optic nerve) depleted of total mRNA, calpain-1, and GAPDH proteins. Only tissues derived from eyes that underwent enucleation within 6 h (or less) of donor death were used. The eyes were kept on ice in Optisol GS medium until the extracts were prepared (maximum storage period, 6 h). Surgical and other instruments and solutions used in the extract preparation were precooled to 4 °C. All buffers and reagents were prepared in DEPC water. Tissue extracts were prepared by mincing the tissue into small pieces with a sharp knife and placed in a minimal volume of 100 mM Tris−Cl buffer pH 7.5 containing 50 mM NaCl and 0.01% genapol and RNase inhibitor (RNAsecure; Ambion Inc., Austin, TX). The tissues were homogenized 10−20 times with a hand held homogenizer using 0.5 mL of RNase free pestle (K749521-0590; Kontes Glass Company, Vineland, NJ). Extracts were depleted of poly A RNA with oligo dT-cellulose matrix (BioWorld, Dublin, OH), then depleted of calpain-1 and GAPDH proteins using mAb and pAb to calpain-1 and GAPDH, respectively, conjugated to protein A sepharose beads. Tissue extracts containing about 90−100 μg of protein in 100 μL of the above buffer were first passed five times through the oligodT column (matrix volume 100 μL). The eluates were then passed five times through each immunoaffinity column (matrix volume 50 μL). About 70 μg of total protein so obtained from each donor was used for each analysis. Polyadenylated RNA for calpain-1 and GAPDH were generated using MEGAscript high-yield transcription kit and polyadenylation kit (Ambion Inc., Austin, TX) and following the recommended protocol and purified using a oligodT column (50 μL). For this purpose, plasmid constructs for calpain-1 (MHS1011-61083) and GAPDH (MHS1011-60742) were procured from Openbiosystems Inc., Huntsville, AL. About 10 μL (∼0.1 μg) of oligodT-purified poly A RNA of calpain-1 and GAPDH prepared as described above was added to poly A RNA and protein-depleted tissue extracts (per 50 μg of extract) and incubated at 37 °C for 90−120 min. Prior to addition of RNA 1 μg of trehelose (per 50 μg of protein) was added to each extract. ^{35}S-labeled methionine (540 Ci/mmol; MP Biomedical Inc., CA) was used to detect the translated product. Two identical gels (SDS−PAGE), one with ^{35}S-labeled and the other with cold methionine, were subjected to simultaneous side-by-side electrophoresis with Seeblue Plus2 prestained protein markers (Invitrogen Inc., CA). The cold methionine gel was blotted to a PVDF membrane and (Sigma Chemical Co., St. Louis, MO) probed with calpain-1 and GAPDH antibodies to determine the identity of protein bands in the corresponding radiolabeled SDS−PAGE. Detection utilized mouse mAb anticalpain-1 and rabbit pAb anti-GAPDH secondary antibodies. The radioactive protein bands corresponding to antibody-detected counterparts in cold methionine gels were excised and subjected to counting in a scintillation counter (TRI-CARB, 1900CA).

RESULTS

Iso[4]LGE$_2$-Modified Proteins Accumulate in Glaucomatous TM in vivo. Our previous proteomic analyses of the TM detected several proteases in normal but not in glaucomatous TM (*16*). However, to detect these proteins by mass spectrometry we relied on tryptic digestion and identification of the derived tryptic peptides. We considered the possibility that proteases might elude detection if oxidized lipids covalently modify them, causing their aggregation and impairing their tryptic digestion. In the present study, we

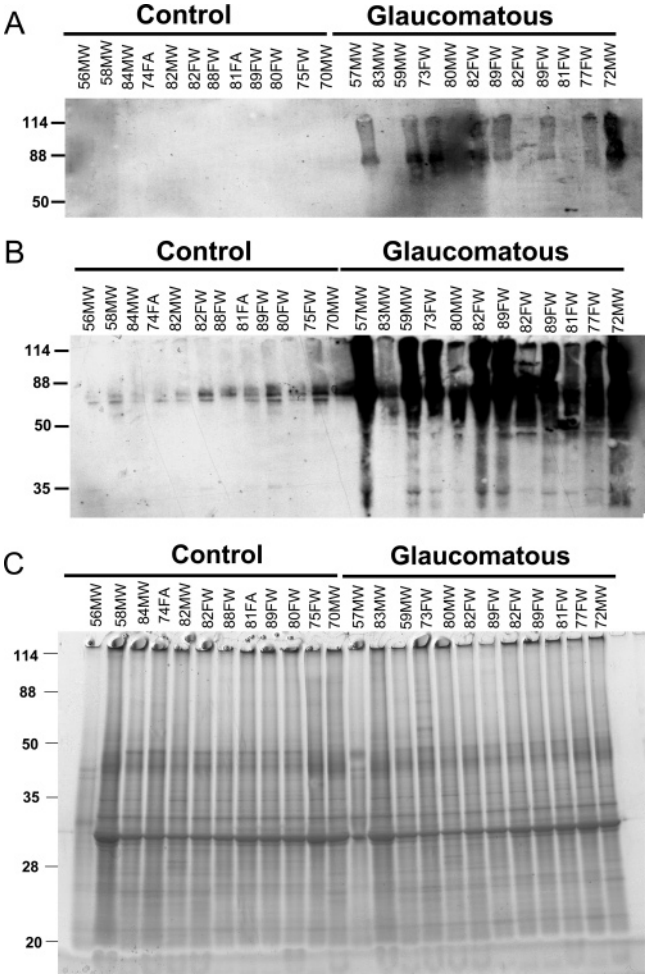

FIGURE 1: Western analysis of trabecular meshwork (TM) proteins. TM tissue extract (10 μg) was loaded on a 4−20% gradient gel (Criterion, Bio-RAD, CA) after transfer on a PVDF membrane, the blot was probed with antibodies. (A) rabbit polyclonal antibody against iso[4]LGE_2; (B) monoclonal antibody against calpain-1 large subunit; (C) coomassie-stained SDS−PAGE of proteins after partial transfer of proteins by electroblotting.

used antibodies to iso[4]LGE_2–protein adducts to immunoprobe proteins extracted from TM. Immunodetection with antibodies specific for these lipid-derived oxidative protein modifications demonstrated the presence of iso[4]LGE_2-modified proteins in glaucomatous but not in control TM (Figure 1A).

Inactive Calpain-1 Accumulates in Glaucomatous TM in vivo. If proteins in the TM are extensively modified by products of lipid oxidation, then their detection by proteomic analysis of tryptic peptides may be hidden by resistance to proteolysis. Therefore, we used anticalpain-1 antibodies to probe for calpain-1 epitopes in proteins that might be lipid-modified. As shown in Figure 1B, in contrast to controls, the glaucomatous TM revealed the presence of aggregated (high molecular weight) bands immunoreactive to anti-calpain-1 antibodies as well as degraded (low molecular weight) calpain-1. Antibody against large subunit of calpain-2 and small subunit of calpain-1 shows immunoreactivity for an ~80 kDa protein (Supporting Information Figure S1A) and 28−16 kDa protein (Supporting Information Figure S1B), respectively, in the normal but not in the glaucomatous TM protein extracts with equal amount of protein load consistent with formation of calpain aggregates as observed

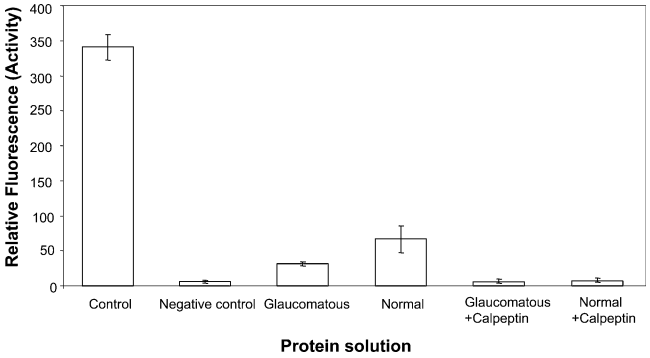

FIGURE 2: Determination of calpain enzymatic activity in TM tissue extracts. About 10 μg of TM tissue extract was used for calpain-1 activity assay using a kit from Biovision Inc. Recombinant calpain-1 (2 μg) served as control. The calpain activity in glaucomatous and normal TM extracts (10 μg) was determined in the presence of 5 mM cysteine protease inhibitor calpeptin as indicated.

in Figure 1B for calpain-1 antibody. As control, omission of the primary antibody for probing the immunoblot results in lack of a signal suggesting that observed calpain immunoreactivities are specific (Supporting Information Figure S1C). The weak signals around the 55 kDa region (Supporting Information Figure S1C) are likely to be cross-reactivity of low levels of endogenous IgG with secondary antibody; they remain very weak even with prolonged exposure. Calpain proteolytic activity was determined in glaucomatous and control TM tissue using equal amounts of protein extract and a chromogenic substrate for calpain (BioVision kit). Glaucomatous tissue showed about 50% less calpain activity than the same amount of protein from normal TM (Figure 2). The calpain activity in tissue extracts were found to be inhibited by calpeptin (Figure 2).

In Vitro Modification of Calpain-1 with Iso[4]LGE_2 Inactivates Its Protease Activity. Incubation of purified recombinant calpain-1 with iso[4]LGE_2 in vitro resulted in covalent modification of calpain-1. Substantial aggregation of purified calpain-1 occurs even in the presence of micromolar concentrations of iso[4]LGE_2 (less than micromolar protein to molar iso[4]LGE_2 ratio). At nonphysiological concentrations of iso[4]LGE_2, the protein becomes highly aggregated and cannot enter the gel. Such aggregates are formed in physiological concentrations of iso[4]LGE_2 as well. We found it difficult to capture all high-mass aggregates with regular electrophoresis and therefore embarked on using a modified electrophoresis, somewhat akin to pulse-field gel electrophoresis as described in the Materials and Methods, to trap and detect such high molecular weight aggregates of iso[4]LGE_2-modified calpain-1 using Western analysis (Supporting Information Figure S2, parts A and B). Sequential probing of the same blot with calpain-1 and iso[4]LGE_2 antibody showed that modification with iso[4]LGE_2 renders the protein less recognizable by calpain-1 antibody with increasing amounts of iso[4]LGE_2 (Supporting Information Figure S2A). In contrast, increasing amounts of iso[4]LGE_2-derived epitopes are immunodetected with increasing amounts of iso[4]LGE_2 (Supporting Information Figure S2B). In vitro iso[4]LGE_2 modification leads to a strong decrease in calpain-1 activity (Figure 3).

Iso[4]LGE_2-Modified Protein is Ubiquitinated and Aggregates on the Proteasome. Autolysis of calpain-1 generates protein fragments in addition to two intact subunits of about

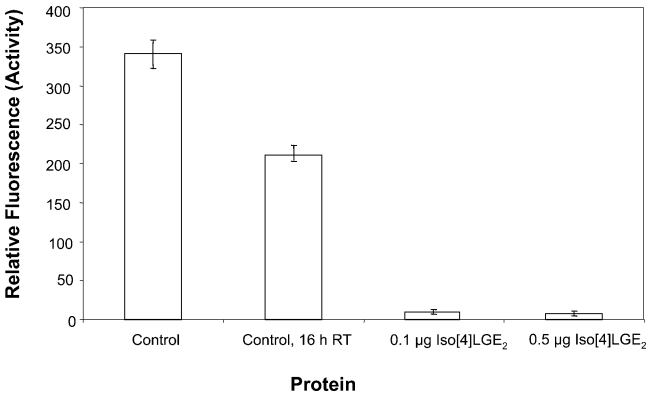

FIGURE 3: Formation of inactive covalent adducts of calpain-1 in vivo by iso[4]LGE_2. Purified calpain-1 (control) was incubated with an equal volume (15 μL) of PBS and precipitated with acetone. Activity assay of calpain-1 modified by iso[4]LGE_2. About 2 μg of iso[4]LGE_2-modified or purified calpain-1 was assayed using Biovision kit.

80 and 30 kDa. To determine whether iso[4]LGE_2-modified proteins are ubiquitinated by mammalian systems, we first performed in vitro modification of the intact calpain-1 (~80 and 30 kDa) and calpain-1 autolytic fragments with iso[4]-LGE_2. Calpain-1 fragments were purified either by column chromatography or gel electrophoresis with band excision and protein extraction. Subsequently, a HeLa cell-derived system, competent for ubiquitination of exogenous proteins, was used to ubiquitinate the unmodified and iso[4]LGE_2-modified calpain-1 (Supporting Information Figure S3). Purified calpain-1 (initial material) was rapidly heat inactivated (95 °C for 10 min). This control calpain-1, along with modified and unmodified calpain-1, was subjected to ubiquitination (Supporting Information Figure S3). The control calpain-1 and purified fragments show increased ubiquitination of iso[4]LGE_2-modified calpain-1 compared to the unmodified native calpain-1 or its fragments (Supporting Information Figure S3, parts B and C). In contrast to the unubiquinated calpain-1, the ubiquinated calpain-1 was efficiently recognized by HeLa cell-derived constituted proteasome 26S (Figure 4). The modified or unmodified calpain-1 when subjected to proteasome loading without subjecting to ubiquitination (Figure 4, parts A and C) does not show high molecular weight aggregate formation, in contrast, to calpain-1 which is ubiquitinated prior to proteasome treatment (Figure 4, parts B and D). The inhibition of proteasome activity using epoxomicin on cultured human primary TM cells also showed increased accumulation of calpain-1 immunoreactivity (Supporting Information Figure S4) supporting that proteasomal impairment likely leads to increased levels of calpain-1. Dynamic light scattering studies were performed to monitor the size of aggregates that were formed by feeding the ubiquitinated or unubiquitinated forms of iso[4]LGE_2-modified or unmodified calpain-1 to proteasome 26S. These studies confirmed the presence of large aggregates that correspond to ubiquitinated iso[4]LGE_2-modified calpain-1 (Table 1). The average size of the aggregated particle was found to be 4 times higher in the case of ubiquitinated iso[4]LGE_2-modified calpain-1 than for the ubiquitinated unmodified calpain-1 (Table 1).

These observations with the proteasome 26S subunit (Table 1 and Figure 4) suggest that iso[4]LGE_2-modified calpain-1 is ubiquitinated and loaded onto the proteasome 26S subunit but is refractory toward degradation. Human eyes apparently lack the capacity to degrade iso[4]LGE_2-modified proteins, resulting in their accumulation.

Elevated Calpain-1 Levels in Glaucomatous Optic Nerve Due to Enhanced Translation. Northern analysis of calpain-1 and GAPDH confirmed that there was no difference in the expression of calpain-1 either in the TM or in the ON (Figure 5). This suggested that levels of this protein may be under translational control. We recently developed a method to detect proteins when they are under translational modulation (*13*). This assay uses fresh human tissue depleted of all polyadenylated RNA and proteins of interest. Once the RNA, calpain-1, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein were depleted, equal amounts of the specific message for calpain-1 and GAPDH were introduced to depleted extracts containing equal amounts of total protein. Results reveal translational control of calpain-1 overexpression in the optic nerve (Figure 6, parts A and B). A similar assay, using TM extracts from cadaver donor eyes, showed a lack of such translational control in the TM (Figure 6, parts C and D).

Calpain-1 Levels in Glaucomatous TM. Although both mass spectrometric proteomic analysis and immunodetection indicated elevated levels of calpain-1 in optic nerve, these analyses gave conflicting results for TM protein extracts. We postulated that the apparent discrepancy is the result of covalent modification of TM proteins by lipid oxidation products such as iso[4]LGE_2. Immunodetection did not show iso[4]LGE_2 modification of calpain-1 in optic nerve extracts (data not shown). In contrast, proteins in glaucomatous but not in control TM show extensive modification by iso[4]-LGE_2 (Figure 1). As noted above, owing to modification by lipid peroxidation products and concomitant aggregation that can impair tryptic digestion, it is not possible to detect calpain-1 in glaucomatous TM by mass spectrometry of tryptic peptides, and such modification may also complicate immunodetection of calpain-1. Levels of calpain-1 are apparently greatly elevated in glaucomatous TM versus control (Figure 1B). However, accurate quantification is obfuscated owing to modification by oxidized lipids, such as iso[4]LGE_2. As modification by iso[4]LGE_2 complicates immunodetection of calpain-1, it is not possible to determine absolute levels of calpain-1 in the TM.

DISCUSSION

Owing to their extraordinary reactivity, isoLGs are likely to modify proteins in the vicinity of their generation. It is tempting to speculate that proteins in the TM are more accessible to isoLGs than those in optic nerve tissue. Evidence presented here suggests that calpain-1 accumulates in the glaucomatous TM and optic nerve for vastly different reasons, both of which may contribute to the pathogenesis of POAG. Our observations suggest different fates, and presumably different roles, for calpain-1 in contributing to decreased permeability that impedes aqueous outflow through glaucomatous TM and in contributing to optic nerve damage. In both tissues, levels of calpain-1 are apparently elevated in glaucoma patients versus controls. However, in TM, calpain-1 is modified by products of lipid oxidation such as iso[4]LGE_2, resulting in inactivation and the accumulation of inactive proteins (Figure 2; Figure 1B) that may block

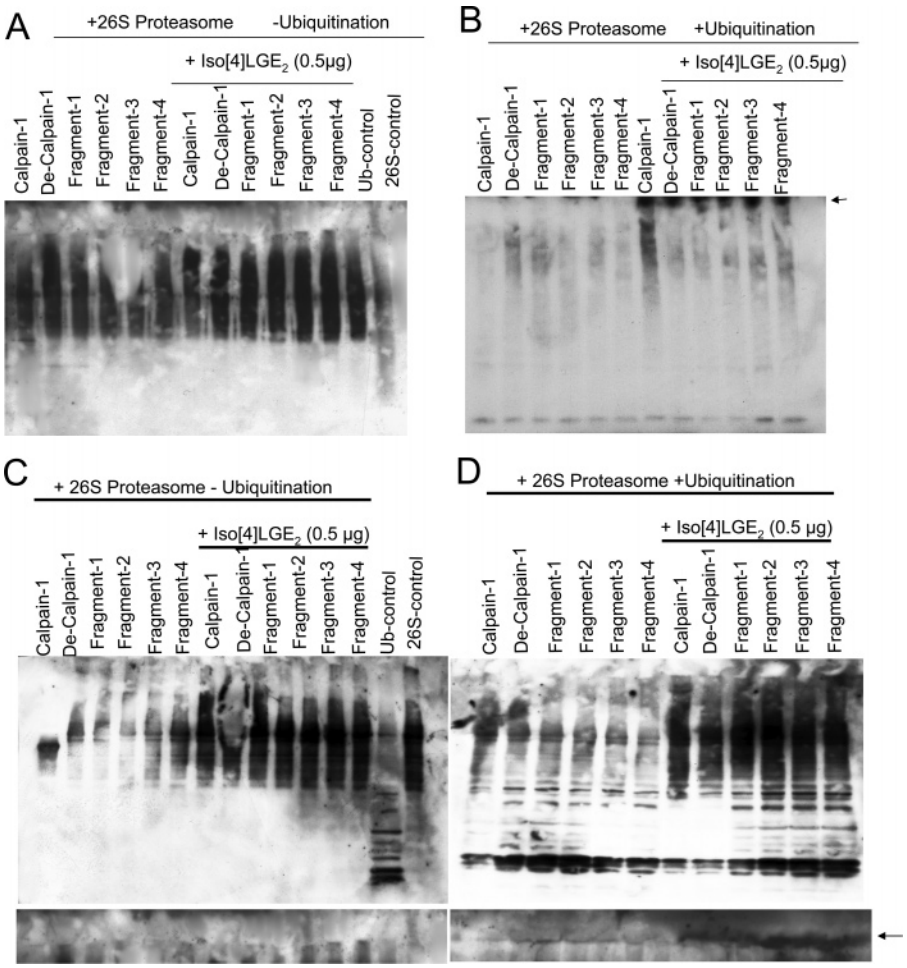

FIGURE 4: Aggregate formation by iso[4]LGE_2-modified but not unmodified proteins (ubiquitinated or unubiquitinated) upon in vitro proteasome 26S loading. Using plugged variable field gel electrophoresis we show the differences between unubiquinated and ubiquinated unmodified and iso[4]LGE_2-modified calpain-1 with or without in vitro proteasome 26S loading. Ubiquitinated or unubiquitinated calpain-1 species (purified calpain-1 or its fragments shown in Supporting Information Figure 3A) subjected to proteasome 26S recognition using a kit (Boston Biochemicals, MA) was loaded on 4% acrylamide and 0.5% agarose plugs and subjected to variable field electrophoresis on a 4−20% SDS−PAGE. Western blot probed first with antiubiquitin antibody (A and B) and subsequently with proteasome 20S antibody (C and D). Proteasome 26S loading of proteins without ubiquitination (A and C) and after ubiquitination (B and D). Lower parts of the panels (A and B) show the Western analysis of plugged field gel electrophoresis (top of the gel). Arrows indicate the position of the aggregates. Ub-control and 26S control are solutions from the respective kit used for protein ubiquitination and proteasome 26S subunit containing the fraction without any calpain addition.

Table 1: Cumulative Biomodal Dynamic Light Scattering Data

experiment[a]	Rh (nm)	Cp (nm)	MW (kDa)
unubiquitinated calpain-1 + proteasome	71.36	27.77	7.56×10^4
ubiquitinated calpain-1 + proteasome	87.43	30.71	1.18×10^5
unubiquitinated iso[4]LGE_2 calpain-1 + proteasome	80.21	31.93	1.32×10^5
ubiquitinated iso[4]LGE_2 calpain-1 + proteasome	145.1	50.5	4.33×10^5

[a] Dynamic light scattering shows enhanced aggregation on in vitro proteasome 26S loading of iso[4]LGE_2-modified calpain-1. The ubiquitinated iso[4]LGE_2-modified calpain-1 subjected to proteasome 26S recognition using a kit (Boston Biochemicals, MA) and dynamic light scattering on DynaPro. Rh is the hydrodynamic radius and Cp is cumulative average particle size. The results presented here were found to be nearly identical in three independent experiments.

aqueous outflow resulting in increased IOP. In contrast, the upregulation of calpain-1 expression that occurs in optic nerve tissue generates elevated levels of active protease that may contribute to degradation of proteins and concomitant damage of this tissue.

IsoLGs are extremely reactive γ-ketoaldehydes that damage proteins by covalent adduction and cross-linking. Previous studies show the presence of proteins modified by lipid peroxidation products in the glaucomatous TM (*21*). In order to understand the possible events that may occur in vivo we modified calpain-1 in vitro with iso[4]LGE_2. Several independent mechanisms can lead to both aggregated and degraded calpain-1; smaller calpain-1 bands are not immunoreactive for iso[4]LGE_2 (Figure 1, parts A and B) suggesting they are degradation products of the calpain-1. Smaller calpain-1 fragments (Figure 1B) are likely to have been generated by mechanisms unrelated to modification by products of lipid peroxidation. Iso[4]LGE_2 binds to proteins and modifies them by forming Schiff bases, protein-bound pyrroles, and various other covalent adducts. This product

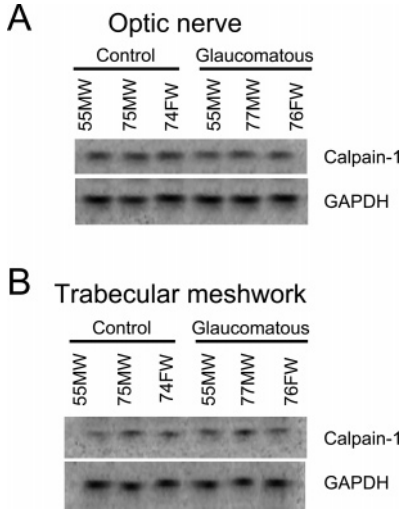

FIGURE 5: Representative Northern analyses of total RNA (5 μg) isolated from normal control and glaucomatous human optic nerve. Northern analysis of total RNA (~2 μg) from normal and glaucomatous trabecular meshwork (TM). Donor age and gender are indicated. Upper and lower panel shows the signal for calpain-1 and GAPDH, respectively.

of free radical-induced lipid oxidation also cross-links proteins to generate high molecular weight aggregates (*22*).

We now also find major differences in translational control of calpain-1 production and posttranslational modification of this protease by lipid oxidation products in TM and optic nerve, respectively. Previously, in spinal cord tissue calpain-1 was found to be under translational control; similar control appears to account for its increased presence in diseased optic nerve tissue (*8*).

There are 37 and 9 lysines and 57 and 13 arginines in the protein sequence of human calpain-1 large subunit (80 kDa; P07384) and in the small subunit (30 kDa; P04632), respectively. Treatment of calpain-1 in vitro with iso[4]LGE_2 leads to aggregate formation (Supporting Information Figure S2, parts A and B). Proteins observed on a coomassie-stained gel and determined using theoretical estimates of recovery after acetone precipitation and that immunodetected with anti-iso[4]LGE_2-protein adduct antibodies did not match. One possibility was that high molecular weight aggregates are formed due to modification and they are refractory to entry into the gel and are lost due to eddies or non laminar flow around wells during handling. We therefore used a plugged variable field electrophoresis. Several trial experiments suggested a polyacrylamide−agarose hybrid plug to be effective in efficient capture and Western transfer of such aggregates. For lower iso[4]LGE_2 to calpain-1 ratios, modification is such that calpain-1 epitopes remain available for detection. However, with increased modification, the calpain-1 epitope appears to be lost. Thus, although immunodetection with anti-iso[4]LGE_2 is possible, calpain-1 is not detected (Supporting Information Figure S2, parts A and B). Although plugged variable field electrophoresis helps capture the protein aggregates and allows Western transfer, the method is not perfect and is less efficient for small amounts of protein aggregates. In vitro modification of calpain-1 with iso[4]-LGE_2 abolishes its proteolytic activity (Figure 3). This is consistent with accumulation of lipid-modified calpain-1 but reduced protease activity observed in glaucomatous TM compared to normal controls (Figure 2).

To ascertain whether the modified calpain-1 is ubiquitinated, we utilized a commercially available ubiquitination competent system derived from HeLa cells. Calpain-1 modified by iso[4]LGE_2 is ubiquitinated (Figure 4). Results show that without ubiquitination (Figure 4 and Table 1), there is less aggregate formation, which corroborates with a previous report (*12*) that protein/peptides modified by iso-[4]LGE_2 or its isomers can significantly reduce the activity of the proteasome 20S, a subunit of the 26S form, leading to the inference that, perhaps, unubiquitinated-modified protein is not efficiently processed by the proteasome 26S subunit. We used a commercially available 26S proteasome system to perform these experiments. Neither calpain-1 nor its denatured fragments appear to be recognized by the cellular proteasome system in the absence of ubiquitination. Ubiquitinated-modified calpain-1 forms large aggregates that could be captured and transferred onto a blot using plugged variable field electrophoresis (Figure 4A−D indicated by the arrows). We also utilized another method, dynamic light scattering (DLS), to verify whether there is a significant difference in aggregation with ubiquitinated versus unubiquitinated iso[4]LGE_2-modified calpain-1 when fed to the proteasome 26S system (Table 1). DLS uses scattered light to measure the rate of diffusion of the protein particles in the solution. This motion data is processed to derive a size distribution for the sample, where the size is given by the hydrodynamic or Stokes radius (Rh) of the protein particle. DLS results corroborated the presence of large aggregates when ubiquitinated iso[4]LGE_2-modified calpain-1 was fed to the proteasome 26S (Rh 145) compared to unubiquitinated iso[4]LGE_2-modified calpain-1 (Rh 80.21). Unubiquitinated calpain-1 always had a lower Rh value compared to the ubiquitinated counterpart when fed to proteasome 26S. Neurodegenerative diseases are often characterized by oxidative stress and malfunction of the proteasome. Previous studies have shown that reactive aldehydes formed due to free radical-induced lipid peroxidation, e.g., isoLGs, inhibit proteasome function. 4-Hydroxynonenal (HNE), the most studied of lipid peroxidation products, has been shown to be capable of modest inhibition of proteasome activity in millimolar concentrations. Also, it was observed that proteins modified by HNE are highly ubiquitinated, forming high molecular weight aggregates (*23*). Immunoprecipitation has shown the formation of HNE−proteasome conjugates and the attachment of HNE to proteins. Accumulation of oxidized and modified proteins may occur due to impairment of the ubiquitin−proteasome pathway (*23*). Isolevuglandins (but not their modified products) also were shown to inhibit the ubiquitin−proteasome pathway and, at very high concentrations, induce cell death (*12*). Inhibition of proteasome activity due to post translational modifications may lead to neurodegeneration (*24*, *25*); a loss of proteasome activity has also been observed in patients with Alzheimer's disease, where the expression of the proteasome remains unaffected.

TM cells are in an environment of high oxidative stress owing to the presence of ROS in the aqueous humor. Proteasome malfunction can occur by excess accumulation of protein components that lead to direct oxidation and inactivation of the proteasome system affecting cell viability. In vitro oxidative stress causes accumulation of oxidized and cross-linked proteins in cells and inhibits proteasome activity.

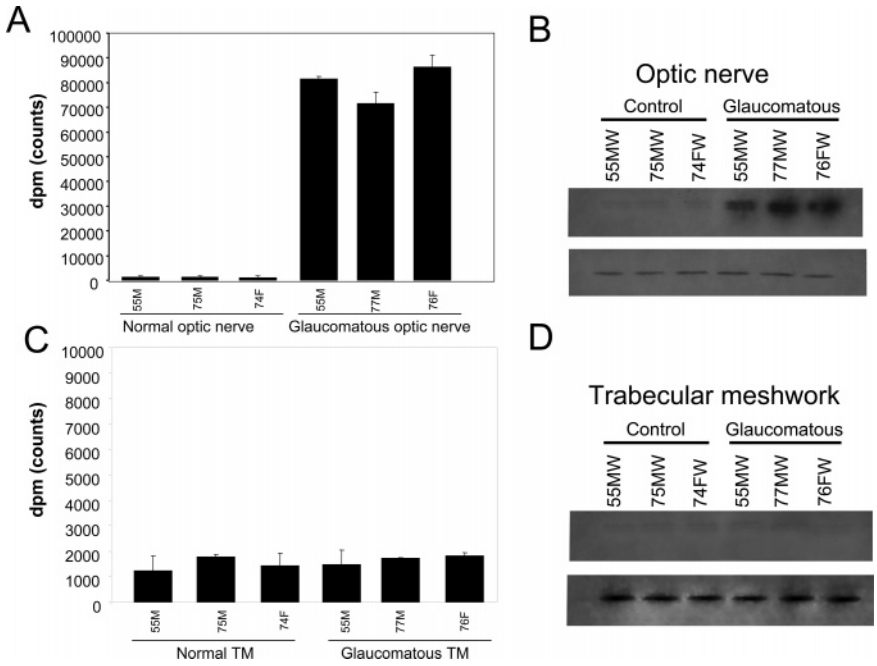

FIGURE 6: Modulation of calpain-1 in optic nerve at the translation level. (A) In vitro translation of calpain-1 (measured as dpm) was monitored in poly A RNA, calpain-1- and GAPDH-depleted control, and glaucomatous optic nerve extracts. Radioactive calpain-I relative to GAPDH is shown. (B) Parallel Western analysis of in vitro translated products in (A) using with rabbit polyclonal calpain-1 antibody (top) and anti-GPDH (bottom). (C) In vitro translation of calpain-1 (measured as dpm) was monitored in poly A RNA, calpain-1- and GAPDH-depleted control, and glaucomatous TM extracts. Radioactive calpain-I relative to GAPDH is shown. (D) Parallel Western analysis of in vitro translated products in (C) using with rabbit polyclonal calpain-1 antibody (top) and anti-GAPDH (bottom). Donor age and gender are indicated.

Impairment of proteasome function has been shown, when TM cells are subjected to chronic oxidative stress (*6*, *26*). In addition to the proteasome, cellular lysosymes were also found unable to digest oxidatively modified proteins leading to impaired lysosomal activity and accumulation of modified proteins (*27*). Inhibition of proteasomal activity using epoxomicin resulted in greater calpain-1 immunoreactivity (Supporting Information Figure S4) indicating accumulation of calpain-1. This result suggests that isoLG modification of calpain and the interference with the proteasomal activity by the modified calpain is likely to result in accumulation of calpain in the TM.

The hallmark of glaucoma is glaucomatous optic neuropathy, characterized by retinal ganglion cell (RGC) death, physical changes, and loss of axons at the optic nerve head. At present the precise mechanisms of glaucomatous optic nerve damage are only poorly understood (*28*). Therefore, the concepts for neuroprotection in glaucoma have been designed by analogy with other neurological problems such as ischemic damage, stroke, spinal chord injury, and other neurodegenerative diseases (*28*). Effective neuroprotection in glaucoma is currently an unrealized therapeutic goal and far from being a clinical reality (*29*). Approaches to the development of neuroprotection fall under three broad strategies: prevent RGC damage and death, promote endogenous RGC survival mechanisms, and promote protection of optic nerve axons. Molecular mechanisms of axonal survival in a spinal chord injury model have led to several neuroprotection strategies (*30*, *31*). For spinal chord and ischemic neuronal injuries, inhibition of calpain-1, a cysteine protease, was proposed as a neuroprotectant strategy (*8*, *32*, *33*). In an ischemic brain injury model, a calpain inhibitor was shown to provide neuroprotection by reducing cortical neuronal damage after ischemia (*34*). Entry of calcium from the extracellular space in ischemia has been implicated in optic nerve damage. Increased intra-axonal calcium activates calpain, resulting in specific degradation of axolemmal and cytoskeletal proteins (*14*, *15*, *33*). Elevated intra-axonal calcium was noted in glaucomatous optic nerve, and calpain-1 was proposed as a therapeutic target for neuroprotection (*28*). Although the eyes are diminutive organs, unlike the brain or the spinal cord where a uniform unregulation of calpain-1 occurs throughout the tissue and can be subjected to inhibition, the TM and optic nerve vastly differ in terms of calpain-1 upregulation complicating utility of a straightforward calpain-1 inhibition strategy.

A potential therapeutic strategy may rely on sophisticated upregulation of degrading enzymes in the TM while reducing calcium concentration in the optic nerve. Both endogenous and exogenous inhibitors of calpain-1 exists which include substances like iodoacetamide and iodoacetate that inhibit cysteine proteases. In a spinal cord injury model, some consideration of the type and delivery of calpain-1 inhibitors was part of a therapeutic strategy that was proven useful. Also, leupeptin, a calpain-1 inhibitor, was shown to protect motor neurons from cell death and help in recovery after nerve injury (*35*). The present study suggests that while in the optic nerve overexpression of calpain-1 occurs in glaucoma, no such event occurs in control optic nerve or in the TM of either type of eyes. Overall, our observations show that in age-associated progressive diseases different parts of the same organ may have intricate and different processing behavior of the same protein, and adopting a therapeutic strategy formulated by analogy with other organ systems must be carefully evaluated in light of those differences.

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SUPPORTING INFORMATION AVAILABLE

Table ST1 and Figures S1−S4. This material is available free of charge via the Internet at http://pubs.acs.org.

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