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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 E₂ (iso[4]LGE₂), 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 crosslinking. We found elevated levels of calpain-1 in glaucomatous TM. However, calpain activity in glaucomatous TM is only about 50% to 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]LGE₂ *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]LGE₂-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 suggests 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.

Keywords

Glaucoma; Intraocular pressure; isolevuglandin; Primary open angle glaucoma; Retinal ganglion cell; Reactive oxygen species; Trabecular meshwork

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. World wide about 70 million people suffer from glaucoma¹. Primary open angle glaucoma (POAG) 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 POAG². 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

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peroxidase that are elevated in the aqueous humour of glaucoma patients. However, a deletion of the gene encoding for glutathione-S-transferase M1 may predispose POAG patients to ROS-induced oxidative injury³. 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 controls³⁻⁵. 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 stress⁶. 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 80kDa and a small subunit of about 30kDa. 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 sclerosis⁸. Autocatalysis of calpain, activated by calcium, converts the 80kDa subunit to a 76kDa form⁹. Differences in calpain-1 processing have been found in some disease conditions. For example, in Alzheimer's disease, altered ratios of 80 kDa to 76 kDa bands were observed⁹.

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 crosslink 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 protease¹².

Our previous proteomic and Western analyses of optic nerve¹³ 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 analysis¹⁶ 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 Table ST1). Controls 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 50–90 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 dithiothreitol, 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 ($8000 \times g$ for 5 min), and soluble protein 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]LGE₂ moiety¹⁷. 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, anti-mouse and anti-rabbit secondary antibodies linked to IR-dyes (700 nm or 800 nm) were used on an Odyssey infrared imaging system according to the manufacturer (Li-Cor Biosciences, Lincoln, NB).

Synthesis of iso[4]LGE₂

Iso[4]LGE₂ (5-acetyl-6-formyl-9-hydroxy-7(E), 11(Z)-heptadecadienoic acid), a highly oxidized lipid, is generated during autooxidation of arachidonic acid or arachidonate esters. Synthesis of iso[4]LGE₂ 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 α,β -unsaturated ketone as the key carbon-carbon bond forming step following methodology described previously¹⁷

Assay of calpain-1 activity

Calpain-1 assay was performed with 10 μg tissue extracts or recombinant control calpain-1 using a calpain activity assay kit (Biovision Inc, Mountainview, CA) following recommended protocols. The fluorometric assay is based on detecting the cleavage of a calpain substrate, Ac-LLY-AFC that fluoresces at 505nm while the cleaved substrate fluoresces at 400nm. 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 5mM Calpeptin¹⁸ (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 (SanDiego, CA) and stored at -80°C . About 10 μl of 1mM CaCl_2 solution was added to purified calpain-1 (per 100 μg 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 100–150 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 Krpm for 10 min. Purified protein fragments were stored at 4 °C for future experiments.

In vitro protein modification by iso[4]LGE₂

Purified calpain-1 and its fragments were subjected to modification using iso[4]LGE₂ prepared by total synthesis as described above. The individual fragments were subjected to varying concentrations of iso[4]LGE₂ 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 Krpm for 5 min and dried using a speed vac for 10 min. The modified proteins were washed and finally re-suspended 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 pre-formed 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 pre-cast 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 volts (30 mA) for 1 min and subsequently alternating pulses of 200 and 80 volts for 1 min for 5 cycles followed by constant 80 volts for 90 min.

TM cell culture and inhibition of proteasomal activity

Primary TM cells were derived from donor human eyes following methods described previously¹⁶. About 3000 primary TM cells were subjected to proteasomal inhibition using Epoxomicin (Peptides international, Catalog number: 4381-v), an equal amount of cells were used as control grown under identical conditions. Briefly, the cells were grown for 24 hours at 37°C and subjected inhibition with 0.04 µmol (final concentration) Epoxomicin¹⁹ for 6 hours. 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 hour. The supernatant was discarded and plates were blocked with 1% Ovalbumin in 1xPBS, 300µl/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 hour. The plates were washed and added with 100µl/well of 1mg/ml phosphatase substrate in diethanolamine buffer pH 7.5. The absorbance was measured at 405nm on a Synerg HT plate reader (Bio-Tek Instruments, Inc, Winooski, VA).

Dynamic light scattering

Dynamic light scattering was performed using about 1µg of protein mixture in a 12 µl holding cuvette using a Proteinsolutions micro sampler (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 micro sampler.

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 protocols²⁰.

Probing translation with poly adenylated 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 six hours (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, six hours). 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 (RNasequre™; Ambion Inc., Austin, Texas). The tissues were homogenized 10–20 times with a hand held homogenizer using 0.5 ml 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 proteins 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 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 (50µl). 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) 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 trehalose (per 50 µg of protein) was added to each extract. ³⁵S labeled methionine (540 Ci/mmol; MP Biomedical Inc, CA) was used to detect the translated product. Two identical gels (SDS-PAGE), one with ³⁵S labeled and the other with cold methionine was subjected to simultaneous side-by-side electrophoresis with Seebblue 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 anti-calpain-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 (TRICARB, 1900CA).

RESULTS

Iso[4]LGE₂-modified proteins accumulate in glaucomatous TM in vivo

Our previous proteomic analyses of the TM detected several proteases in normal but not in glaucomatous TM¹⁶. 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 used antibodies to iso[4]LGE₂-protein adducts to immuno probe proteins extracted from TM. Immunodetection with antibodies specific for these lipid-derived oxidative protein

modifications demonstrated the presence of iso[4]LGE₂-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 anti-calpain-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 a ~80kDa protein (Supporting information Figure S1A) and 28-16kDa (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 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 55kDa 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 control 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]LGE₂ inactivates its protease activity

Incubation of purified recombinant calpain-1 with iso[4]LGE₂ *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]LGE₂ (less than micromolar protein to molar iso[4]LGE₂ ratio). At non-physiological concentrations of iso[4]LGE₂, the protein becomes highly aggregated and cannot enter the gel. Such aggregates are formed in physiological concentrations of iso[4]LGE₂ 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 methods section, to trap and detect such high molecular weight aggregates of iso[4]LGE₂-modified calpain-1 using Western analysis (Supporting information Figure S2A,B). Sequential probing of the same blot with calpain-1 and iso[4]LGE₂ antibody showed that modification with iso[4]LGE₂ renders the protein less recognizable by calpain-1 antibody with increasing amounts of iso[4]LGE₂ (Supporting information Figure S2A). In contrast, increasing amounts of iso[4]LGE₂-derived epitopes are immunodetected with increasing amounts of iso[4]LGE₂ (Supporting information Figure S2B). *In vitro* iso[4]LGE₂ modification leads to a strong decrease in calpain-1 activity (Fig. 3).

Iso[4]LGE₂-modified protein is ubiquitinated and aggregates on the proteasome

Autolysis of calpain-1 generates protein fragments in addition to two intact subunits of about 80kDa and 30kDa. To determine whether iso[4]LGE₂-modified proteins are ubiquitinated by mammalian systems, we first performed *in vitro* modification of the intact calpain-1 (~80kDa and 30kDa) and calpain-1 autolytic fragments with iso[4]LGE₂. 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]LGE₂-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]LGE₂-modified calpain-1 compared to the unmodified native calpain-1 or its fragments (Supporting information Figure S3B,C). In contrast to the unubiquitinated calpain-1, the ubiquitinated 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 4A,C) does not show high molecular weight aggregate formation, in contrast, to calpain-1 which is ubiquitinated prior to proteasome treatment (Figure 4B,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]LGE₂-modified or unmodified calpain-1 to proteasome 26S. These studies confirmed the presence of large aggregates that correspond to ubiquitinated iso[4]LGE₂-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]LGE₂-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]LGE₂-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]LGE₂-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 results (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 modulation¹³. 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 over expression in the optic nerve (Figure 6A, B). A similar assay, using TM extracts from cadaver donor eyes, showed a lack of such translational control in the TM (Figure 6C, D).

Calpain-1 levels in glaucomatous TM

While 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]LGE₂. Immunodetection did not show iso[4]LGE₂ 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]LGE₂ (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]LGE₂. As modification by iso[4]LGE₂ 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 glaucomatous trabecular meshwork 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]LGE₂, resulting in inactivation and the accumulation of inactive proteins (Figure 2; Figure 1B) that may block aqueous outflow resulting in increased IOP. In contrast, the up regulation 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 crosslinking. Previous studies show the presence of proteins modified by lipid peroxidation products in the glaucomatous TM²¹. In order to understand the possible events that may occur *in vivo* we modified calpain-1 *in vitro* with iso[4]LGE₂. Several independent mechanisms can lead to both aggregated and degraded calpain-1, smaller calpain-1 bands are not immunoreactive for iso[4]LGE₂ (Figure 1A,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]LGE₂ binds to proteins and modifies them by forming Schiff bases, protein bound pyrroles, and various other covalent adducts. This product of free radical-induced lipid oxidation also cross links proteins to generate high molecular weight aggregates²².

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 tissue⁸.

There are 37 and 9 lysines, 57 and 13 arginines in the protein sequence of human calpain-1 large subunit (80 kDa; P07384) and in the small subunit (30kDa; P04632) respectively. Treatment of calpain-1 *in vitro* with iso[4]LGE₂ leads to aggregate formation (Supporting information Figure S2A, 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]LGE₂-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]LGE₂ 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, while immunodetection with anti-iso[4]LGE₂ is possible, calpain-1 is not detected (Supporting information Figure S2A,B). Although plugged variable field electrophoresis helps capture the protein aggregates and allows Western transfer, the method is not perfect and less efficient for small amounts of protein aggregates. *In vitro* modification of calpain-1 with iso[4]LGE₂ 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]LGE₂ is ubiquitinated (Figure 4). Results show that without ubiquitination (Figure 4 and Table 1), there is less aggregate formation, which corroborates with a previous report¹² that protein/peptides modified by iso[4]LGE₂ 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]LGE₂-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]LGE₂-modified calpain-1 was fed to the proteasome 26S (Rh 145) compared to unubiquitinated iso[4]LGE₂-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., isolevuglandins, 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 aggregates²³. 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 pathway²³. Isolevuglandins (but not their modified products) also were shown to inhibit the ubiquitin-proteasome pathway, and at very high concentrations, induce cell death¹². 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 reactive oxygen species 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. Impairment of proteasome function has been shown, when TM cells are subjected to chronic oxidative stress^{6, 26}. In addition to the proteasome, cellular lysosomes were also found unable to digest oxidatively modified proteins leading to impaired lysosomal activity and accumulation of modified proteins²⁷. Inhibition of proteasomal activity using epoximycin resulted in greater calpain-1 immunoreactivity (Supporting information Figure S4) indicating accumulation of calpain-1. This result suggests that isolevuglandin 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 understood²⁸.

Therefore, the concepts for neuroprotection in glaucoma have been designed by analogy with other neurological problems such as ischemic damage, stroke, spinal cord injury and other neurodegenerative diseases²⁸. Effective neuroprotection in glaucoma is currently an unrealized therapeutic goal and far from being a clinical reality²⁹. 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 cord injury model have led to several neuroprotection strategies^{30, 31}. For spinal cord 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 ischemia³⁴. 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 neuroprotection²⁸. 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 trabecular meshwork 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 trabecular meshwork 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 injury³⁵. The present study suggests that while in the optic nerve over expression of calpain-1 occurs in glaucoma, no such event occurs in control optic nerve or in trabecular meshwork 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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The abbreviations used are

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

Figure 1.

Western analysis of trabecular meshwork proteins. TM tissue extract (10 μ g) was loaded on a 4–20% gradient gel (Criterion, Bio-RAD, CA) after transfer on a PVDF, the blot was probed with antibodies. A) rabbit polyclonal antibody against iso[4]LGE₂, B) monoclonal antibody against calpain-1 large subunit, C) monoclonal antibody against calpain-1 small subunit, D) Comassie stained SDS-PAGE of proteins after partial transfer of proteins by electroblotting.

Figure 2.

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

Figure 3.

Formation of inactive covalent adducts of calpain-1 *in vivo* by iso[4]LGE₂. Purified calpain-1 (control) was incubated with an equal volume (15 µl) of phosphate buffered saline and precipitated with acetone. Activity assay of calpain-1 modified by iso[4]LGE₂. About 2 µg of iso[4]LGE₂ modified or purified calpain-1 was assayed using Biovision Kit.

Figure 4.

Aggregate formation by iso[4]LGE₂ 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 unubiquitinated and ubiquitinated unmodified

and iso[4]LGE₂ 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 Fig. 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 anti-ubiquitin antibody (A,B) and subsequently with proteasome 20S antibody (C,D). Proteasome 26S loading of proteins without ubiquitination (A,C) and after ubiquitination (B,D). Lower panels (A,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 fraction without any calpain addition.

Figure 5. Representative Northern analyses of total RNA (5 μ g) isolated from normal control and glaucomatous human optic nerve. Northern analysis of total RNA ($\sim$ 2 μ g) from normal and glaucomatous trabecular meshwork. Donor age and gender are indicated. Upper and lower panel shows signal for calpain-1 and GAPDH respectively.

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 panel) and anti-GAPDH (bottom panel). (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 panel) and anti-GAPDH (bottom panel). Donor age and gender are indicated.

Table 1

Cumulative biomodal dynamic light scattering data

Experiment [*]	Rh (nm)	Cp (nm)	MW (kDa)
Un-ubiquitinated Calpain-1 + Proteasome	71.36	27.77	7.56×10^4
Ubiquitinated Calpain-1 + Proteasome	87.43	30.71	1.18×10^5
Un-ubiquitinated Iso[4]LGE2 Calpain-1 + Proteasome	80.21	31.93	1.32×10^5
Ubiquitinated Iso[4]LGE2 Calpain-1 + Proteasome	145.1	50.5	4.33×10^5

^{*} Dynamic light scattering shows enhanced aggregation on in vitro proteasome 26S loading of Iso[4]LGE2 modified Calpain-1. The ubiquitinated Iso[4]LGE2 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 near identical in three independent experiments.