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Neuroprotection in glaucoma using calpain-1 inhibitors: regional differences in calpain-1 activity in the trabecular meshwork, optic nerve and implications for therapeutics

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Abstract

Glaucoma is a group of irreversible blinding eye diseases affecting over 70 million people worldwide. Systemic delivery of calpain-1 inhibitors was proposed as a neuroprotection strategy for the prevention of progressive optic nerve damage in glaucoma. We present a general review of calpain-1 and an account of vast differences in processing of calpain-1 in the trabecular meshwork (TM) and the optic nerve. Calpain-1 accumulates in the glaucomatous TM tissues *in vivo*. However, calpain-1 activity is substantially lower in the glaucomatous TM compared to controls, apparently owing to partial degradation, and modification by lipid oxidation products such as iso[4]levuglandin E2 (iso[4]LGE₂). Treatment of calpain-1 with iso[4]LGE₂ *in vitro* results in covalent modification, inactivation, and resistance to protease digestion. iso[4]LGE₂-modified calpain-1 appeared to undergo ubiquitination in the TM by cellular degradation machinery mediated by ubch1–2, ubch5,6 and E6-AP, E2 and E3 enzymes respectively. In the TM, iso[4]LGE₂-modified calpain-1 loading impairs the cellular proteasome activity consistent with competitive inhibition and formation of suicidal high molecular weight aggregates. In contrast, higher calpain-1 activity, that appears to be under translational control, was observed in glaucomatous optic nerve compared to control. Therapeutic neuroprotection strategies using calpain-1 inhibitors will require consideration of such anatomic differences in its activity and biosynthesis.

Keywords

Glaucoma; Calpain; Trabecular meshwork; Isolevuglandin; Lipid oxidation; Posttranslational modifications; Neuroprotection

Introduction

Calpains belong to a family of cysteine proteases containing at least 14 different members. Among the different forms of calpain two of the best-studied forms are calpain-1 or e-calpain and calpain-2 or m-calpain. Calpains are typically dependent on calcium for activation and human calpain-1 and calpain-2 are activated by different levels of Ca²⁺, namely micromolar

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range for calpain-1 and millimolar range for calpain-2. The calpains 1 and 2 are ubiquitous in expression and have a catalytic subunit of molecular weights ranging from 78–80kDa and a common regulatory subunit of 26–30kDa range. Calpain-1 and 2 have four domains (domain I-IV) and at least 2 regions in the regulatory subunit, of which the domain II has been identified as a cysteine protease. Domain II of calpain is further divided into subdomain IIa and subdomain IIb that have a substrate binding cleft between them. Upon binding, calcium brings the subdomains IIa and IIb together and also helps cleave domain I thus activating calpain 1 or 2 [1]. The domain III is speculated to act as a linker region while domain IV contains several EF-hand structures that can bind calcium [2]. Calpains are activated by calcium and are part of a number of important cellular processes that require calcium for activation. Calpain-1 could be involved in many processes involving proteolysis although its broad functions seem to be skeletal remodeling and signal transduction, cell proliferation, differentiation, apoptosis, platelet activation and membrane fusion [3–6].

There are several other members of the calpain family, which are involved in more specific functions. Calpain-3 for example is specific to the skeletal muscle system, lens and retina, and has an important role to play in maintaining myofibril homeostasis[7]. Calpain-6 is highly expressed in the placenta[8], calpain- 9 is specific to the digestive tract and has a role in tumor suppression[9] and calpain-11 is specific to the testis[10]. The calpain family of proteases therefore plays important roles in different regions of the human body. Here, we will focus our attention on calpain-1 regulation in the eye and regional differences in calpain-1 in the anterior and posterior chambers of the eye.

Calpain-1 is ubiquitous and has been well studied in terms of structure-function. It is known to be present in the spinal cord and also in several other parts of the nervous system. Small periods of ischemia can cause severe damage to the brain when prevalent over an extended time period. Calpain activation has been studied in the pathophysiology of cerebral ischemia [11]. Activation of calpain has been observed during ischemic episodes of the brain. Over activation of calpains can lead to excessive loss of cytoskeletal proteins, which may result in neuronal cell death. A number of studies have utilized calpain inhibitors on gerbils that serve as a model for ischemia[12,13]. The role of calpain in ischemia has been confirmed by treatment of gerbil brain with calpain inhibitor MDL 28170 which can reduce neuronal damage [12]. MDL 28170 is a potent inhibitor of calpains that is capable of passing through the blood-brain barrier. MDL 28170 administration even after three hours of induction of ischemia can still protect against cerebral cortical damage [12]. The mechanism of action of MDL 28170 is still under investigation. These experiments in gerbil provide support that calpain proteolysis play an important role in cerebral ischemia.

Central nervous system injuries also result in loss of neurofilament proteins, which are typically observed in advanced cases of neurodegeneration. Utilization of calpain inhibitors has been shown to reduce the loss of neurofilament 200 and neurofilament 68 in rat traumatic brain injury models [14]. Increase in calpain activity has also been observed in spinal cord injuries. Increased calpain expression has been observed in astrocytes within the injured spinal cord. Production of calpain may lead to breakdown of myelin or axonal degradation following an injury to the spinal cord [15].

Calpain-1 activation is thought to play an important role in cell death in several neurodegenerative diseases of the eye including retinitis pigmentosa, glaucoma and other retinal degenerative diseases [16]. Retinitis pigmentosa is a heterogeneous group of inherited degenerative disorders where progressive loss of vision occurs due to slow death of the rods and cones in the retina. The calpain levels are higher in a mouse model of inherited retinal degeneration (rd1 mice) than in normal mice. The retinal photoreceptor cell death and calpain overexpression in this model coincides with up-regulation of calpain-1 and down-regulation

of the calpain inhibitor calpastatin and cyclic AMP response element binding protein (CREB) [16]. Transcription of the calpastatin gene takes place through activated CREB and therefore down regulation of CREB is associated with calpastatin down regulation [17].

Calpains have been implicated in retinal degeneration in diabetic retinopathy and in the optic nerve and retinal degeneration in experimental allergic encephalomyelitis (EAE). The loss of pericytes in diabetic retinopathy is well documented[18]. Protection of pericytes from stress induced apoptosis has been reported in presence but not in the absence of calpain inhibitors and has been concluded as suggestive of the beneficial role of calpain inhibitors in preventing pericyte loss in diabetic retinopathy[18]. Increased expression of calpains has been found in the optic nerves of Lewis rats with EAE (an animal model for optic neuritis). A decrease in levels of myelin associated glycoprotein (MAG) and an increase in the translational expression of calpains were observed in the rats with optic neuritis compared to controls. Thus calpains are therefore implicated to play a role in degradation of MAG in optic neuritis. Calpain activity is also higher in microglia, reactive astrocytes and macrophages in optic neuritis [19,20].

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 glaucoma [21]. Glaucoma is a neurodegenerative disorder that is characterized by permanent and irreversible loss of vision. The hallmark of glaucoma is glaucomatous optic neuropathy (GON), characterized by retinal ganglion cell (RGC) death, physical changes and loss of axons at the optic nerve head. Increased intraocular pressure (IOP) is a risk factor for glaucoma [22]. The anterior segment of the eye consists of the cornea, the trabecular meshwork (TM), the iris, the ciliary body, and the lens. The posterior chamber consists of the retina, the optic nerve and the choroids. Elevated IOP occurs due to increased resistance to aqueous outflow at the TM tissue in the anterior eye chamber[23–25]. Lowering IOP often confers protection against glaucomatous damage [26]. Neuroprotection strategies require a better understanding of the etiology and molecular mechanisms underlying increased IOP and GON. At present the precise mechanisms of glaucomatous optic nerve damage are only poorly understood [26].

Recent evidence has shown that in glaucoma with elevated IOP and ischemia, calpain activation leads to retinal cell death[27]. Calpain activation and retinal cell death has also been observed in retinitis pigmentosa[17,28]. Retinas from monkeys cultured under hypoxic conditions show increased cell death and leakage of lactate dehydrogenase [29]. Increased calpain expression and accumulation of α -spectrin have also been observed in hypoxic retinas. The α -spectrin accumulation in hypoxic retinas is thought to be due to calpain induced proteolysis. In hypoxia there is an increase in calcium influx which may aid proteolysis of calpain specific substrates. Calpain inhibitor SJA6017 reduces the proteolysis of α -spectrin in hypoxic retinas [29]. Experiments performed on hypoxic rat retinas revealed elevated presence of calpain-1 and calpain-2 [30]. Proteolysis of Tau, another substrate of calpain, and conversion of p35 to p25 has been observed in hypoxic retinas as well as in the brain in Alzheimer's disease. The calpain inhibitor SJA6017 partially inhibits the conversion of p35 to p25, and completely inhibits the phosphorylation of Tau [31]. The proteolysis is necessary for subsequent phosphorylation of Tau [31]. Based on their role to prevent proteolysis of calpain substrates, the calpain inhibitors have been postulated to be neuroprotective.

In neurodegenerative diseases, for example, in spinal cord injury models calpain inhibitors have been shown to be neuroprotective [32]. An extension of this strategy has been proposed in glaucoma[26]. Since mechanisms of optic nerve damage in glaucoma are only poorly understood therefore the concepts of neuroprotection have been borrowed from other neurodegenerative diseases such as stroke and ischemia [26]. However, it is important to look

at the processing of this protein closely before attempting to use calpain-1 inhibitors for neuroprotection in glaucoma[33,34]. Our work indicates that the processing of calpain-1 in the anterior chamber of the eye is vastly different from its processing in the posterior chamber. Thus the use of inhibitors in the anterior chamber may lead to disastrous consequences in the posterior chamber and vice versa.

Oxidative stress and neurodegenerative disorders

Oxidative stress has been implicated as a possible factor in several neurodegenerative disorders including Alzheimer's disease, Parkinson's disease and glaucoma. Apart from a host of other factors that contribute to oxidative stress, reactive oxygen species that lead to oxidation of lipids and carbohydrates play an important role in the pathology of several diseases. Oxidized lipids are capable of modifying proteins and DNA leading to unnatural post translational modifications of proteins. The anterior chamber of the eye is more prone to oxidative stress because of the formation of reactive oxygen species (ROS) [35,36]. The anterior chamber is in proximity to air and prone to diffusive transfer of oxygen, the anterior chamber is also constantly bathed in aqueous humor which has high dissolved oxygen. These factors render the anterior chamber relatively more prone oxidative stress than the posterior chamber. The reactive oxygen species give rise to potentially harmful substances in the anterior chamber. The aqueous humor may be an important route through which the ROS reach several tissues of the anterior chamber [37]. The trabecular meshwork is also a tissue that shows a distinct decrease in antioxidant capacity with aging. Studies have shown lower levels of antioxidant enzyme superoxide dismutase (SOD) and loss of cellularity in aging TM cells. These factors could also lead to an increase in oxidative stress with age in glaucomatous eyes[36]. The differential presence of certain lipids in the anterior chamber also renders it vulnerable to reactive oxidation products. The lipid peroxidation products, isolevuglandins, that are the subject of the present study, are derived from arachidonyl phospholipids, which are ω -6 fatty acids, that are present exclusively in the anterior chamber of the eye[38], unlike the ω -3 fatty acid (docosahexaenoate phospholipids) oxidation products that have been implicated in other eye diseases, for example, age-related macular degeneration and are prevalent in high levels only in the posterior regions of the eye[39]

Our work has focused on lipid peroxidation products derived from ω -6 fatty acids esters known as arachidonyl phospholipids, that act as precursors to a number of biologically active products either by enzyme oxidation or by free-radical induced oxidation[40] [41]. We are particularly interested in a molecule known as iso[4]LGE₂ which is formed exclusively by the free radical oxidation of arachidonic acid. Iso[4]LGE₂ is one type of isolevuglandin, a family of a γ -ketoaldehydes that has been known to form protein adducts and crosslink proteins orders of magnitude faster than 4-hydroxynonenal (HNE) or malondialdehyde (MDA), the other well known and more widely characterized lipid peroxidation products[33,42–45]. Due to the presence of the highly reactive γ -ketoaldehyde functionality iso[4]LGE₂ crosslinking with proteins may lead to the formation of aminal and pyrrole crosslinks “Fig. (1)”. Proteins modified by iso[4]LGE₂ have also been shown to inhibit proteasome activity [46,47].

Glaucoma and oxidative stress

Oxidative stress has been implicated as one of the factors in glaucoma[48,49]. Several studies have shown loss of antioxidant activity during aging, such as lower superoxide dismutase (SOD) activity in TM cells. Studies have also demonstrated that H₂O₂ treated TM cell cultures show reduced amounts of glutathione. The plasma of patients with primary open angle glaucoma (POAG) has shown lower levels of glutathione and higher levels of the lipid peroxidation product malondialdehyde [50,51].

Elevated IOP in glaucoma has always been closely associated with blockage of the drainage machinery in the anterior chamber of the eye at the level of trabecular meshwork. A proteomic analysis of normal and glaucomatous human TM captured calpain-1 only in the normal TM (Table 1)[52]. Western analysis of TM tissue extracts with monoclonal calpain-1 antibody showed the presence of both aggregated and degraded calpain-1[33,34] in the glaucomatous TM while enzymatic assay using a calpain activity assay kit (BioVision Inc., Mountain View, CA) clearly indicated that calpain-1 activity was higher in the normal TM tissue[33,34] “Fig. (2)”. The discrepancy between the immunoreactive detection of more calpain-1 (by Western and ELISA analyses) and less calpain activity (as well as no capture of calpain-1 peptides by proteomic analyses) led us to the hypothesis that calpain-1 is modified by the lipid oxidation products in the glaucomatous TM. Previous studies have indicated the presence of lipid peroxidation products such as 4-hydroxynonenal and isolevuglandins in the glaucomatous TM tissue [43]. Western analysis with rabbit polyclonal antibody specific to iso[4]LGE₂ showed increased immunoreactivity in the glaucomatous TM[34], which co-localized with calpain-1 immunoreactive bands. Since detection of proteins by proteomic analysis is carried out by tryptic digestion, we postulate that lack of proteomic detection of calpain-1 in glaucomatous TM could be due to the inability of trypsin to generate charged peptide species or due to technical limitations of mass spectrometry in analyzing calpain-1 peptides modified by lipid oxidation products. The products of oxidative stress, for example, iso[4]LGE₂ covalently modifies calpain. Modification of calpain-1 by iso[4]LGE₂ leads to aggregation and degradation of calpain-1 *in vivo*. *In vitro* studies were performed to capture and understand *in vivo* observations using iso[4]LGE₂ modified recombinant purified calpain-1 followed by ubiquitination and proteasome treatment [34].

Modification of calpain-1 by iso[4]LGE₂ *in vitro*

In order to study *in vivo* modification of calpain-1 by iso[4]LGE₂, modification was carried out *in vitro*[34]. Purified calpain-1 was procured commercially (EMD Chemicals Inc., San Diego, CA) and its fragments were further purified using gel purification/column chromatography[34]. Purified fragments of calpain-1 were subjected to modification using synthetic iso[4]LGE₂ and recovered[34] using acetone precipitation “Fig. (3A)”. Western analysis with iso[4]LGE₂ antibody of modified calpain-1 clearly indicated aggregation of calpain-1 upon modification by iso[4]LGE₂ “Fig. (3B)”. These aggregates were difficult to capture using regular Western analysis and we embarked on a modification of electrophoresis similar to pulse-field gel electrophoresis (PFGE) to trap and detect high molecular weight aggregates of iso[4]LGE₂-modified calpain-1 “Fig. (4B)”. When modified with increasing concentrations of iso[4]LGE₂, calpain-1 antibody failed to detect modified calpain-1 protein. These findings are supported by ELISA and activity analyses of the modified proteins which also shows that the activity of calpain-1 decreases with increasing concentrations of iso[4]LGE₂ (Table 2). Thus apparently as a result of modification by iso[4]LGE₂, the calpain-1 epitope is altered or lost and cannot be detected utilizing antibodies.

Ubiquitination and aggregation of iso[4]LGE₂-modified calpain-1

Purified and iso[4]LGE₂-modified fragments of calpain-1 were subjected to ubiquitination using S100 HeLa conjugation kit (BIOMOL International LP., Plymouth Meeting, PA), and then subjected to further treatment with 26S proteasome fraction which is capable of degrading ubiquitinated proteins. A purified calpain-1 was used as control in these experiments, which was heat inactivated (95 °C for 10 min) prior to subjecting to ubiquitination and subsequent proteasome treatment. This control calpain-1, along with modified and unmodified calpain-1, was subjected to ubiquitination. 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[34]. In contrast to the un-ubiquitinated calpain-1, the ubiquitinated calpain-1 was efficiently recognized by HeLa cell derived constituted proteasome[34].

Iso[4]LGE₂ modified calpain-1 is ubiquitin conjugated by E2 enzymes ubch1, ubch 5–7 and E3 enzymes, E6-AP and ubch2 in TM tissue

E2 and E3 conjugation enzymes are tissue specific [53,54]. Lack of information about the presence of conjugation enzymes in TM and optic nerve motivated us to probe glaucomatous and control TM and optic nerve tissues using a battery of available antibodies to E2 and E3 enzymes (BIOMOL International LP., Plymouth Meeting, PA). Western analyses revealed the presence of ubch1, 2, ubch5,6 and E6-AP respectively in the TM tissue “Fig. (5A)”. Although very small amounts of Parkin and CHIP were also immunodetected in the optic nerve extracts “Fig. (5B)”, they were neither found co-immunoreactive with iso[4]LGE₂ modified calpain-1, nor observed in the TM extracts co-migrating with iso[4]LGE₂ immunoreactive bands. Thus in addition to ubch1, 2, 5 and E6-AP, the optic nerve has Parkin, CHIP and ubch7 but much lower levels of ubch5 compared to TM. The ubch7 is absent in the TM and ubch6 is present in relatively high abundance in the TM compared to optic nerve “Fig. (5B)”. Iso[4]LGE₂-modified calpain-1 presented to fresh TM extract results in their ubiquitination that appears to be mediated by ubch1, 5 and 6 (E2) and E6-AP (E3) enzymatic proteins “Fig. (6)”. Iso[4]LGE₂-modified calpain-1 is immunodetected with antibodies to iso[4]LGE₂ “Fig. (6A)”. A number of protein bands in the glaucomatous TM extract are immunoreactive to anti-iso[4]LGE₂-protein, Lane 1, “Fig. (6A)”. Several of the iso[4]LGE₂ immunoreactive bands in glaucomatous TM appear at identical positions to iso[4]LGE₂ immunoreactive bands that appear when normal TM extract, depleted of calpain-1 using an anti-calpain immunocolumn, is fed with iso[4]LGE₂ modified calpain-1, Lane 3–6, “Fig. (6A)”, suggesting that in glaucomatous TM, protein bands that are immunoreactive to iso[4]LGE₂ are modified calpain-1. The ubiquitination step appears to be relatively fast and the conjugation enzymes appear not to be affected by the iso[4]LGE₂-modification[34]. In our preliminary experiments, the conjugation enzymes do not appear to be covalently linked to iso[4]LGE₂-modified calpain-1 or to undergo suicidal complex formation during conjugation steps. Using calpain-1-depleted normal TM extract, we performed UV exposure (254 nm) for 10 min immediately after incubation as well as after 1 h “Figure (6A–E)”, this results in capture of the conjugation enzymes co-migrating with iso[4]LGE₂-modified proteins [55,56]. Immunodetection of the calpain-1 large subunit almost always revealed the formation of large aggregate in these experiments. We have captured aggregates after UV exposures that are immunoreactive to E6-AP, ubch1, 5 and 6 (data not shown). However, we have captured more E6-AP immunoreactivity after 1h incubation upon UV exposure that corresponds to a modified small subunit of calpain-1 “Fig. (6B)”. A clear shift of the immunoreactive ubch5 band is seen when iso[4]LGE₂-modified calpain-1 is fed to normal TM extract, and the ubch5 immunoreactive bands correspond to the position in glaucomatous TM extract (Lane 1, 5, 6 “Fig. (6C)”. Upon UV exposure the ubch5 immunoreactivity co-migrates with calpain-1 small subunit (indicated by the arrow) and a commensurate shift in unbound ubch5 immunoreactivity intensity (0h immunoreactivity between 14–17 kDa region), suggesting direct interaction of ubch5 with calpain-1 small subunit “Fig. (6C)”. Similarly upon UV exposure, an aggregate corresponding to iso[4]LGE₂-modified small subunit of calpain-1 is found with ubch1 and ubch6 “Fig. (6D, E)”. Observations with antibodies to proteasome 26S subunit suggest that iso[4]LGE₂-modified calpain-1 is ubiquitinated and loaded on to the proteasome 26S subunit, but fails to undergo degradation[34].

Treatment of iso[4]LGE₂-modified calpain-1 with *Phaenicia sericata* larvae

Human eyes apparently lack the capacity to degrade iso[4]LGE₂-modified proteins, resulting in their accumulation. In contrast, we found that the larvae of *Phaenicia sericata* (blow fly maggots) possess the ability to degrade the iso[4]LGE₂-modified proteins. The larvae of *P. sericata* secretions are known for their ability to degrade tissues that harbor secondary amines and lipids [57,58] and are likely to be cross-linked. *P. sericata* larvae extracts, when added to iso[4]LGE₂-modified calpain-1, showed the beginning of degradation products within 5

minutes of incubation and complete degradation of modified proteins within 3 hours of incubation “Fig. (7)”. We have used a gel purified ~250kDa aggregate of iso[4]LGE₂ modified calpain-1 for these experiments. At 5 minutes (we have depicted this as 0 h, the degradation of modified protein is already underway. The handling of this assay presents a lag period of about 5 minutes prior to gel loading for analyses and this period is depicted as 0 h, nevertheless the 0 h represents a 5 minute incubation period due to this lag period “Fig. (7)”. The presence of serine and metalloproteinases in maggot extracts may help in the degradation of lipid oxidation product modified proteins, which is not possible in human eyes. Further experiments have to be performed to determine which enzymes are present in maggot extracts and whether their orthologs are absent in human eyes.

Calpain-1 expression in the optic nerve

Primary proteomic analysis of normal and glaucomatous optic nerve (ON) indicated increased expression of calpain-1 in glaucomatous ON compared to normal ON. Western analysis with calpain-1 antibody also showed higher immunoreactivity in glaucomatous ON in comparison to the normal ON. Levels of modification with lipid peroxidation products such as iso[4]LGE₂ and HNE have been found to be negligible in the glaucomatous optic nerve in contrast to the TM[34].

RT-PCR analysis of calpain-1 in the optic nerve and the TM

RT-PCR analysis clearly indicated that there were no differences in the expression of mRNA in control compared to glaucomatous tissue in both the TM and ON “Fig. (8A, B)”. The levels of calpain-1 protein in the ON were examined using Western analyses “Fig. (8C)”, which clearly indicated higher levels of calpain-1 in the glaucomatous ON when compared to the normal ON. The calpain-1 mRNA level analysis shows similar mRNA levels in control and glaucomatous ON tissue “Fig. (8B)” suggesting a lack of transcriptional regulation that could have accounted for increased protein levels of calpain-1. However, translational control of calpain-1 expression leading to higher protein levels in glaucomatous ON “Fig. (8C)” cannot be eliminated. Recently we developed a method to detect translational level control of proteins [59]. This assay clearly indicated that in the ON calpain-1 expression is under the influence of some translational control[34], but in the TM no such translational control appears to exist. From these experiments we can ascertain that calpain-1 levels are regulated in different ways in the TM and ON[34].

It is therefore evident that processing of calpain-1 is different in different parts of the eye. In glaucoma, whereas in the anterior chamber, calpain-1 undergoes modification by lipid peroxidation products leading to aggregation and degradation, no such modification was found in the optic nerve. *In vitro* studies have clearly indicated that modification of purified calpain-1 and their fragments by iso[4]LGE₂ leads to malfunction of the cellular proteosomal machinery. The TM proteasome machinery is incapable of degrading iso[4]LGE₂ modified forms of the protein. Oxidative stress has been implicated as an important factor in several neurodegenerative diseases. Lipid peroxidation product, HNE has been implicated in the inhibition of proteasome activity in millimolar concentrations. Highly ubiquitinated proteins also seem to undergo modification by HNE and form high molecular weight aggregates. Thus oxidation of proteins can lead to impairment in the function of the ubiquitin-proteasome pathway [60]. The reason for the modification of calpain-1 in glaucoma in the TM but not in the ON could be due to the presence of higher levels of fatty acids such as arachidonic acid (AA) and elevated levels of reactive oxygen species in the anterior chamber [38]. These lipids undergo oxidation when exposed to free radicals and light and modify proteins such as calpain-1.

Conclusions

The findings presented here suggest 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. The cellular degradation machinery for modified calpain-1 exists in trabecular meshwork but loading iso[4]LGE₂ modified proteins often impairs it. Such impairment of the degradation machinery was not observed for optic nerve in the posterior eye segment. 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 an organ must be carefully evaluated in light of those differences for application into another organ.

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Fig. (1).

A schematic diagram showing modification of proteins by lipid peroxidation product iso[4]LGE₂.

Fig. (2).

(A) Calpain-1 enzymatic activity as relative fluorescence units from individual donor TM tissue extracts. Assay was performed under identical conditions with TM tissue extracts (10 μ g) from normal (hollow squares) and glaucomatous (black solid squares) donors using calpain-1 activity assay kit (Biovision Inc., Mountain View, CA). (B) Average calpain-1 activity in relative fluorescence units as shown for individual donors in (A) for 10 normal and 10 glaucomatous donor TM (10 μ g protein extract) measured under identical conditions have been presented, error bars indicate standard deviation for all 10 donors. Recombinant purified calpain-1 (2 μ g; EMD Chemicals Inc., San Diego, CA) served as positive control. Negative control was normal TM extract (10 μ g) that was added with 10 mM of a general calpain/cysteine protease inhibitor calpeptin prior to measurement. For positive and negative control, average and standard deviation from three independent experiments have been presented.

Fig. (3). Formation of *in vitro* covalent adducts by calpain-1 and iso[4]LGE₂. (A) Coomassie stained SDS-PAGE. Purified calpain-1 (2 µg in 2 µl) was incubated with indicated amount of synthetic adduct (10-50 µl in volume) of iso[4]LGE₂ in a total volume of 70 µl for 16 hours at room temperature. Control purified calpain-1 was incubated with an equal volume (50 µl) of phosphate buffered saline and precipitated with acetone. (B) Western analyses for modification. The PVDF blot was probed with rabbit polyclonal antibody specific for iso[4]LGE₂.

Fig. (4).

Formation of inactive covalent adducts of calpain-1 *in vitro* by iso[4]LGE₂. (A) Silver stained SDS-PAGE. Purified calpain-1 (2 µg in 2 µl) was incubated with indicated amount of synthetic adduct (5–15 µl in volume) of iso[4]LGE₂ for 16 hours at room temperature. Purified calpain-1 (control) was incubated with an equal volume (15 µl) of phosphate buffered saline and precipitated with acetone. (B) Western analyses for iso[4]LGE₂ modification. The PVDF blot was probed with rabbit polyclonal antibody specific for iso[4]LGE₂.

Fig. (5).

Western analysis for detection of ubiquitin conjugation enzymes (E2 and E3) in the trabecular meshwork and optic nerve tissue extracts. Proteins in the extracts (10 μ g) were fractionated on a 4–20% SDS-PAGE and subsequently transferred onto a PVDF membrane. (A) Western analysis was performed with TM tissue extracts (10 μ g protein) with a battery of antibodies to indicated E2 and E3 conjugation enzymes (Parkin, CHIP, E6-AP, ubch2, ubch6, ubch7 and ubch1) (B) Western analysis was performed on optic nerve extracts (10 μ g of protein) and probed with antibodies to E2 and E3 conjugation enzymes as indicated. Position of the corresponding protein molecular weight standards has been as indicated.

Fig. (6).

iso[4]LGE₂ modified purified calpain-1 was incubated with TM extract (normal TM extract depleted of calpain-1) for indicated length of time with or without subsequent crosslinking using UV light (254 nm for 10 minutes) and probed for conjugation enzymes. Control TM extract shown in first lane of each Western analysis prior to modified calpain was derived from a glaucomatous donor eye (66 year old Caucasian female). (A) Western analysis with iso[4]LGE₂ antibody, arrow indicates position of immunoreactivity upon addition of modified calpain-1 and corresponds to the immunoreactivity in the same position in control glaucomatous TM. (B) probed with E6-AP antibody (C) probed with ubch5 antibody (arrow indicates a shift in ubch5 immunoreactivity after addition of modified calpain-1 to the TM and UV cross linking co-migrating with calpain-1 small subunit), (D) probed with ubch1 antibody, and, (E) probed with ubch6 antibody. Upper row in this panel corresponds to small subunit of calpain-1, the lower row indicated with arrow corresponds to ubch6 immunoreactivity.

Fig. (7). iso[4]LGE₂ modified proteins are degraded by proteins derived from larvae of *Phaenicia sericata*. (A) Silver stained gel. The iso[4]LGE₂ modified calpain-1 (2 µg; a single modified species of molecular weight around 250kDa was gel purified and used for this experiment) was incubated with 10 µg of *P. sericata* extract for indicated time period. The molecular weight, control *P. sericata* extract without any addition and gel purified control iso[4]LGE₂ modified protein are shown in lane 1–3 respectively as indicated. The 0 h represent approximately 5 minutes of incubation. This is due to a processing lag period which was estimated to be about 5 minutes (B) Western analysis of an identical gel as in (A) was blotted onto a PVDF membrane and probed with rabbit polyclonal antibody against iso[4]LGE₂.

Fig. (8).

Analysis of calpain-1 mRNA and protein levels. (A) Products of Reverse Transcription and PCR (RT-PCR) in the TM. RT was performed using cloned AMV reverse transcriptase (Invitrogen Inc., Carlsbad, CA) and a 22 mer oligo dT primer. PCR was performed using following primer pairs and product of reverse transcription as template: Calpain-1 (5'-atgtcggaggagatcatcac-3' and 5'-caatgcaaacatggctcagct-3') and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (5'-cttcaccaccatggagaaggc-3' and 5'-ggcatggactgtggtcatgag-3'). RT-PCR utilized recommended and standardized protocols [61]. All Caucasian (indicated by W) donor eyes of indicated age and gender (M= male, F= female) on top of each lane was used for this analysis. (B) RT-PCR analysis for calpain-1 and GAPDH in optic nerve, rControl: PCR using a Plasmid as template that has calpain-1 and GAPDH genes. Upper and lower panel in RT-PCR analyses in (A) and (B) represent calpain-1 and GAPDH respectively as indicated. (C) Western analysis of optic nerve extracts with rabbit polyclonal antibody against calpain-1.