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HUMAN AND NON-HUMAN PRIMATE LENSES CULTURED WITH IONOPHORE FORM α B-CRYSTALLIN LACKING THE C-TERMINAL LYS, A PROMINENT FEATURE OF SOME HUMAN CATARACTS

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Abstract

Purpose—Elevation of lens calcium occurs in both human and experimental animal cataracts, and opacification may result from calcium-activated proteolysis. The purpose of the present study was to determine if calcium accumulation in cultured human and *Macaca mulatta* lenses results in proteolysis of crystallins, the major lens proteins.

Methods—Two-dimensional electrophoresis and mass spectrometry were used to construct detailed maps of human and monkey lens crystallins so that proteolysis following calcium accumulation could be monitored and the altered crystallins identified. Human and macaque lenses cultured in A23187 showed elevated lenticular calcium and superficial cortical opacities. The carboxypeptidase E (CPE) gene is expressed in human lens, and its presence in lens fibers was demonstrated by western blot. To investigate if CPE could cause similar truncation, purified α B-crystallin and CPE were incubated *in vitro*.

Results—The major change observed in the crystallins of these cultured lenses was the accumulation of α B₁₋₁₇₄-crystallin resulting from the loss of a C-terminal lysine. This result was significant, because similar appearance of α B₁₋₁₇₄ is a prominent change in some human cataracts. α B-crystallin and CPE incubation result in the formation α B₁₋₁₇₄-crystallin. This truncation was specific to α B₁₋₁₇₄-crystallin since other crystallins were not proteolyzed. While a weaker activator than zinc, calcium was capable of activating CPE *in vitro*.

Conclusions—Since zinc concentrations did not increase during culture in A23187, calcium uptake in lens may be responsible for CPE activation and α B₁₋₁₇₄ formation during cataract.

Keywords

monkey lens; human lens; calcium ionophore; α B-crystallin; cortical cataract; proteolysis; carboxypeptidase

Introduction

Cataracts are opacities in the lens of the eye. This disease is the leading cause of preventable blindness in the world ¹, yet an effective therapeutic strategy to slow the rate of cataract progression is unavailable. The human lens faces a number of challenges in attempting to remain transparent during its long life span. It is exposed to several possible oxidants ^{2, 3}, undergoes a large number of post-translational modifications ⁴, and is unable to replace the damaged proteins contained in its oldest central region due a programmed loss of organelles ⁵. Opacification results from a loss of the short-range order of crystallins, the major proteins of the lens, due to their aggregation upon unfolding or separation into protein-rich and protein-poor phases ⁶. Three factors which may normally prevent opacification with increasing age are: a healthy epithelium on the anterior surface of the lens where the bulk of metabolic activity resides ⁷, a complex network of pumps and channels allowing movement of ions and small molecules to and from the lens interior ⁸, and the maintenance of a reducing environment, especially in the center of the lens ⁹. While oxidation of proteins in the lens interior may directly precede opacification, alterations in lens epithelium may initiate the process.

Global genomic ¹⁰ and proteomic ¹¹ analysis have examined the differences between gene expression and protein composition in normal and cataractous human lenses. A major finding of the proteomic analysis, performed by two-dimensional gel electrophoresis (2-DE), was a 10–90% loss of the C-terminal Lys₁₇₅ from a major lens protein known as α B-crystallin ¹¹. Additional changes in this normally 20 kDa protein also included an N-terminal cleavage yielding a 16.4 kDa fragment, and overall elevation in the concentration of α B-crystallin.

α B-crystallin, and its binding partner α A-crystallin belong to the small heat shock protein family and form a polydisperse aggregate of 300–1200 kDa. These proteins are capable of binding to partially unfolded intermediates of the β/γ -crystallin families and prevent their irreversible aggregation and precipitation during aging ⁶. Thus, α -crystallins may function in the human lens, where they contribute 28% of the total protein ¹², by preventing opacity, until they are “titrated out” by other unfolded lens proteins ⁶. The study of lens epithelial cells from knockout mice missing α -crystallins suggests these proteins may also influence cell proliferation. Lens epithelial cells from α A^{-/-} mice exhibited reduced survival and growth ¹³, while epithelial cells from α B^{-/-} mice exhibited genomic instability and hyperproliferation ¹⁴. The reported cataract specific changes in α B may alter one or more α B functions and contribute to cataract formation.

The protease producing truncated α B-Lys₁₋₁₇₄ is unknown, but this cleavage was most likely due to the activation of a carboxypeptidase. While aminopeptidases have been thoroughly examined in lens ¹⁵, carboxypeptidases have not been characterized. However, several metallo-carboxypeptidases have been described in other tissues that are capable of removing basic residues from both peptides and proteins. Transcripts for carboxypeptidases E/H (NEI Bank, NbLi0012), D and X ¹⁶ have been detected in human lens. Many human cataracts, especially those in the cortex contain increased levels of calcium ¹⁷, and calcium is an activator of some proteases. Therefore, the purpose of the present study was to determine if elevation of lens calcium in cultured primate lenses was capable of reproducing the previously reported alterations in lens α B-crystallin. We demonstrate that calcium loading in both human and monkey lenses caused the loss of the C-terminal Lys₁₇₅ from α B-crystallin, but was incapable of producing the 16.4 kDa form of the protein. These results were unlike similar experiments with rodent lenses, where calcium loading caused activation of the calcium-dependant endopeptidase calpain.

Materials and Methods

Source of lenses

Human eyes from 3-, 71-, 76-, and 81-year-old donors were obtained from Lions Eye Bank (Portland, OR). The research followed the tenets of the Declaration of Helsinki, and informed consent was obtained after explanation of the nature and possible consequences of the study; and the research was approved by the institutional human experimentation committee or institutional review board (IRB). Monkey (*Macaca mulatta*) eyes ranging in age from 1 to 12 years were obtained from procedures unrelated to the present studies. Experimental animals were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the Guiding Principles in the Care and Use of Animals (DHEW Publication, NIH 80-23).

Protein preparation

Samples for two-dimensional gel electrophoresis (2-DE) maps for soluble proteins from human lens were prepared by homogenizing whole decapsulated lenses from a 3-year old human donor in 1 ml of 20 mM sodium phosphate, 1.0 mM EDTA (pH 7.0) buffer, and centrifugation at 20,000 *g* for 30 min. Soluble proteins from 4-year-old monkey lenses were similarly prepared, except the cortical and nuclear region of the lenses were first isolated by dissection so that each contained approximately 50% of the lens fiber mass. For the analysis of the protein alterations caused by the accumulation of calcium, lenses were washed with PBS three times, after incubation. To obtain the epithelial samples, the lens capsule with attached epithelial cells was carefully dissected from the lens and homogenized in buffer A containing 20 mM Tris (pH 7.5), 1 mM EGTA, 1 mM EDTA and 2 mM dithioerythritol (DTE). Soluble protein was obtained by centrifugation at 13,000 *g* for 15 min at 4°C. To obtain concentric samples from the outer cortex of cultured lenses, each decapsulated lens was gently agitated for up to 2 hours in 1 mL buffer A in a 15 mL centrifuge tube. One mL buffer A was collected every 5 min and replaced with 1 mL of fresh buffer A. Protein concentrations were measured in each fraction, and samples were pooled to obtain 5 mg of protein designated as the superficial region of the lens cortex. Protein concentrations were measured by the BCA Protein Assay (Pierce Chemical Co. Rockford, IL) using BSA as a standard, and 400 µg portions of protein were dried by vacuum centrifugation in preparation for electrophoresis.

Lens culture

Lenses from donor human and monkeys at several ages were obtained by a posterior approach. For human lenses, culture was performed within 48 hours after death and within 2 hours for monkeys. Lenses were dissected in Eagle's minimum essential medium (EMEM; ATCC, Manassas, VA) containing 200 U/mL penicillin and 200 µg/mL streptomycin. For a 30 min pre-incubation, dissected lenses were placed in 2 mL EMEM containing 100 U/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL amphotericin, 50 µg/mL gentamicin, and 10% fetal bovine serum (Invitrogen Inc., Carlsbad, CA) using 6-well cell culture plates. Lenses were then transferred to 2 ml EMEM with 50 µg/ml gentamicin and 10% fetal bovine serum and cultured at 37°C under 5% CO₂ for up to 7 days. Ten µmoles of A23187 (EMD Biosciences, Inc., San Diego, CA) were added on day 1 only (A23187 group). Paired lenses were used for control and experimental protocols.

Two-dimensional gel electrophoresis

First-dimension isoelectric focusing was performed using custom, 18-cm, linear pH 5–10 immobilized pH gradient (IPG) strips produced as previously described¹⁸, and reswelled in 340 µl of solution containing 400µg protein, 8M deionized urea, 2% CHAPS, 50 mM DTT,

2% glycerol, 2% pH 6–11 IPG buffer (GE Healthcare, Piscataway, NJ), and a trace of bromophenol blue. Isoelectric focusing was performed using a Protean IEF Cell (Bio-Rad, Hercules, CA) using a program with the voltage ramp set at rapid, a final 3,500V setting, 50,000 total v_h, 50 μ A limit per gel, and 20°C temperature. Gel strips were then reduced/alkylated, and the second dimension separation was performed on 24 $\times$ 18.5 cm 12% SDS PAGE gels as previously described¹⁸. Gels were then either stained with Coomassie blue G-250¹⁹ in preparation for in-gel digestion and identification of proteins, or negative stained with zinc-imidazole²⁰ in preparation for protein elution and mass measurement.

Protein analysis by mass spectrometry

In-gel trypsinization was performed as previously described²¹, and digests were analyzed by either LC-MS²¹ or atmospheric pressure MALDI-MS²² to collect MS/MS spectra using an LCQ Classic ion trap mass spectrometer (ThermoFinnigan, San Jose, CA). Proteins were identified using SEQUEST software (ThermoFinnigan) to correlate experimental MS/MS spectra with theoretical MS/MS spectra calculated from peptide sequences in a human subset of the Swiss-Prot protein database (Swiss-Prot, Swiss Institute of Bioinformatics, Geneva, Switzerland). SEQUEST results were filtered using DTASelect²³. DeltaCN values greater than 0.08 were required, and identified peptides had to exceed XCorr values of 2.0, 2.5, and 3.8 for +1, +2, and +3 ions, respectively, for the LC experiments using electrospray ionization; while +1 peptides derived by MALDI had to exceed an Xcorr value of 1.5. All identified proteins also required 2 or more unique peptide matches per entry for a positive identification.

Mass measurement of proteins eluted from 2-DE gels was performed as previously described²⁴, with the following modifications. Following negative staining using zinc-imidazole, spots from single gels were excised, shaken twice for 15 min in 192 mM glycine, 25 mM Tris base, 50 mM DTT, 0.1% SDS, and crushed by passing through a 20 μ m stainless steel frit using a 0.5 ml gas tight syringe. One-hundred and fifty μ l of 96 mM glycine, 12.5 mM Tris base, 50 mM DTT was then added to the syringe to transfer the remaining gel particles into a centrifuge tube, and the resulting slurry was shaken for 30 min. The slurry was then transferred to an Ultrafree-MC microcentrifuge filter (UFC30HV00, Millipore, Bedford, MA), centrifuged for 15 min at 13,000 g, an additional 50 μ l of the above solution added, and the device centrifuged again. The collected liquid was then dried by vacuum centrifugation, redissolved in 50 μ l of 5% formic acid, and the masses of the eluted proteins determined by injecting the sample onto a 1.0 $\times$ 250 mm C4 column. The same trap cartridge, column, and electrospray ionization technique was used as before²⁴, except a 20 μ l/min flow rate and 2–60% acetonitrile gradient over 50 min was used, and 0.05% TFA was added to the mobile phase to prevent formation of SDS-protein adducts during mass analysis.

SDS-PAGE and immunoblotting

SDS-PAGE of lens proteins was performed on 10% NuPAGE bis-Tris gels with the MES buffer system (Invitrogen Inc.) for carboxypeptidase E (CPE) detection. Immunoblots were performed by electrotransferring proteins from NuPAGE gels onto polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA) at 100 V (constant) for 60 min at ice-cold temperature using Tris Glycine buffer. Antibody source and dilution were goat polyclonal antibodies against CPE (Santa Cruz Biotechnology Inc., Santa Cruz, CA) used at 1:100. Immunoreactivity was visualized with alkaline phosphatase conjugated to anti-rabbit IgG secondary antibody and BCIP/NBT (Bio-Rad).

CPE assay

To inactivate CPE, human recombinant CPE (rhCPE) (more than 95% purity, R&D Systems, Inc.) was incubated with 2 mM EGTA (Sigma-Aldrich, Saint Louis, MO) for 10 min at room temperature. After chelating ions, 50 µg/mL of CPE and 2.0 µg of crystallins: α B (Abcam, Inc., Cambridge, MA), human recombinant β B1 (kindly provided by Dr. Kirsten Lampi, Oregon Health & Science University, Portland, OR), and recombinant human γ D/ γ S containing N-terminal His tags (kindly provided by Dr. Johnathan King, Massachusetts Institute of Technology, Cambridge, MA) were incubated with calcium or zinc for 1 h at room temperature. CPE inhibitor, 1,10-penanthroline (Sigma) was used to confirm CPE dependent α B degradation. Crystallin masses were measured following incubation by mass spectrometry as described above for proteins eluted from 2-DE gels.

Zinc measurement

Wet weight of lenses was determined by weighing lenses before and after heating at 80°C for 16 h. For zinc measurement, each dried lens was digested overnight with gentle agitation in 1.0 mL concentrated HCl. Water was added to dilute the samples at 1:100 dilution for lens, 1:2 dilution for EMEM medium and 1:5 dilution for CPE assay samples and zinc was determined at 206.20 nm by inductively coupled plasma emission spectrometry (ICP-ES), on an Optima 2000 DV (Perkin-Elmer Life and Analytical Sciences Inc., Boston, MA). Zinc content in the lenses was expressed as milliequivalents zinc per kg lens water.

Results

Two-dimensional electrophoresis maps of human and monkey lens proteins

Prior to analysis of the protein alterations caused by the accumulation of calcium in primate lenses, all major proteins resolved by 2-dimensional electrophoresis (2-DE) of soluble proteins from a non-cultured 3-year-old human whole lens (Figs. 1A and 1B), and the cortex of a 4-year-old monkey lens (Figs. 1C and 1D) were trypsinized, subjected to mass spectral analysis, and identified (Tables 1 and 2). The pattern found in 3-year-old whole human lens was similar to a previous IPG-based, 2-DE gel of a human adult lens cortex²⁵. Similarity was due to the young age of the crystallins in the fibers of both samples²⁵. Comparison of human and monkey lens crystallins by 2-DE suggested that young lens fibers of both species contain similar high abundances of β B2, α A, α B, β A3/A1, and β B1-crystallins. Furthermore, the majority of distinct crystallins in both species contained multiple forms due to similar partial truncation and heterogeneity of isoelectric points. This suggested that crystallins from monkey lenses were subjected to similar age-related truncation, deamidation and phosphorylation as are human lenses^{26,27}. Since calcium elevation in rodent lenses causes rapid truncation of crystallins due to activation of proteases^{24, 28, 29}, experiments were next performed with cultured human and monkey lenses to determine if calcium elevation could further enhance the observed truncation of crystallins.

Human lens culture in calcium ionophore

Culture of both human and monkey lenses in calcium ionophore A23187 induced superficial cortical opacities³⁰. The proteins from these effected regions comprised of the lens epithelium and superficial cortex were examined by 2-DE. In the absence of calcium ionophore, the crystallins from the epithelium (Fig. 2A, normal) and superficial cortex (Fig. 2B, normal) from the lenses of a cultured 81-year-old human donor were remarkably similar to the crystallins from whole lenses of the 3-year-old donor (Fig. 1A). Culture in the absence of calcium ionophore also did not introduce changes in lens crystallins, since a 2-DE gel of epithelial protein from an uncultured 71-year-old donor lens was identical to lenses cultured for 7 days without ionophore (data not shown).

In contrast, elevation of calcium by incubation of human lenses in A23187 caused a major increase in a spot migrating at a slightly lower molecular weight and more acidic position next to intact αB_{1-175} in both epithelium and superficial cortex (Figs. 2A and 2B, “A23187,” arrows). This change was quite pronounced, in that few changes were observed in other crystallins. The novel protein was excised, digested, and peptides analyzed by tandem MS. These results indicated that the novel protein was derived by modification of intact αB_{1-175} . The modified αB was further analyzed by eluting it from a zinc-imidazole stained 2-DE gel and measurement of its whole mass by electrospray mass spectrometry. Following elution from a 2-DE gel, intact human αB had a measured mass of 20,201 (Fig. 3A) that was identical to the calculated mass. In contrast, the acidic and lower molecular weight form of αB produced by culture in A23187 had a mass that was 129 mass units lower (Fig. 3B), indicating this protein was αB_{1-174} missing a C-terminal lysine. The 2-DE migration and MS identification of truncated αB_{1-174} was the same as previously described in some cataractous human lenses (Fig. 2C). Thus, elevating calcium in cultured human lenses was able to reproduce a major protein change observed in vivo in human cataracts.

Monkey lens culture in calcium ionophore

In order to test if the formation of truncated αB_{1-174} could be reproduced in non-human primate lenses, similar experiments were conducted in monkey lenses. Similar to human lens, culturing monkey lenses from animals of 6-, 8-, and 12-years of age in A23187 caused a major increase in a spot migrating at a slightly lower molecular weight and more acidic position next to intact αB_{1-175} (Fig. 4, arrows). Intact *Macaca mulatta* αB_{1-175} was eluted from a 2-DE gel and its mass measured (Fig. 3C). Within experimental error, its 20,187 mass was identical to the calculated mass of *Macaca fascicularis* αB_{1-175} (Swiss Prot accession Q60HG8), suggesting the sequences of intact αB are identical in these two species. The new spot migrating at a lower molecular weight and more acidic position compared to αB_{1-175} had a mass of 20,059 (Fig. 3D). This 128 mass decrease was again due to the loss of the C-terminal Lys_{175} from αB . Thus, monkey lenses provided a useful model to study the response of human lenses to calcium loading due to culture in A23187.

CPE activity against αB_{1-175}

Three carboxypeptidases (E/H, D and X) are known to be expressed in human lens¹⁶. Commercially available human recombinant carboxypeptidase E (hrCPE) was used to digest recombinant human αB -crystallin (αB_{1-175}). Prior to the incubation study, CPE expression in monkey lens tissues was confirmed by immunoblotting (Fig. 5A). CPE was expressed only in the lens fiber region and not in lens epithelium. This band did not appear if the CPE antibody was first neutralized with the immunizing peptide. These data indicated that the strong 50 kDa band in lens fiber protein was indeed CPE. αB_{1-175} was truncated in human lens epithelium sample (Fig. 2A). This might be due to lens cortex contamination of lens epithelium during dissection. The A23187 treated lenses absorbed significant amounts of water and this caused difficulties in dissecting epithelium without fiber contamination.

CPE is known as a zinc metallopeptidase that removes basic amino acids from the C-terminus of peptides³¹. We tested if Ca^{2+} would also activate CPE and hydrolyze αB -crystallin. γD - and γS -crystallins were used as negative control substrates since the C-termini do not contain a basic amino acid. βB1 -crystallin was used as another negative control since it has a Pro-Lys C-terminus, and other basic carboxypeptidases did not hydrolyze substrates having a penultimate proline^{32–35}. When either calcium or zinc were incubated with CPE and αB -crystallin for one hour, an αB -crystallin fragment at 20,030 Da missing the C-terminal lysine was detected. 2.5 μM zinc caused 86% αB degradation (Fig. 5B), 4 mM calcium caused 33% degradation (Fig. 5C), and 2.5 μM zinc plus 4 mM calcium caused 93% degradation (Fig. 5D). Four mM zinc with CPE caused 100% degradation of αB .

(data not shown). All α B degradations were inhibited 100% by 1 mM 1,10-penanthroline chelating reagent (data not shown). 1,10-penanthroline was a better inhibitor of CPE for chelating zinc ions than EGTA. As we expected, none of the negative controls, β B1-, γ D- and γ S-crystallins, were degraded by CPE even with 4 mM zinc (data not shown). These data confirmed that calcium, as well as zinc, is an activator of CPE.

Unexpectedly, α B was degraded 19% by CPE without external addition of calcium or zinc, even following pretreatment with 2 mM EGTA (Fig. 5E). The assay buffer did not contain zinc, however CPE and α B mixture was found to contain 0.89 ± 0.02 mEq zinc/kg water (Table 3). This suggests that CPE binds zinc strongly enough to prevent removal during pretreatment with EGTA and to allow partial activation without addition of ions.

The A23187 ionophore used in the present lens culture experiments is known to increase influx of calcium and zinc from the medium. Only 0.07 ± 0.02 mEq Zn/kg water was detected in our culture medium (Table 3). Further, the zinc concentration actually decreased in A23187-treated lenses, probably due to the massive water uptake (Table 3). In contrast, A23187 increased lens calcium concentrations to almost four times over normal cultured lenses. These results suggested that endogenous CPE in the present experiments was activated by A23187-induced calcium influx.

Discussion

Elevated amounts of truncated α B₁₋₁₇₄ have been observed in cataracts from some human patients¹¹, in Soemmerring's ring "after" cataract³⁶, and in an infant with persistent hyperplastic primary vitreous where cells invade the posterior lens¹¹. A major finding of the present investigation was that elevation of lenticular calcium by A23187 also produced α B₁₋₁₇₄ in cultured human lenses, and that the process could be reproduced in cultured monkey lenses. However, calcium-elevation was not able to reproduce the previously observed 16.4 kDa fragment of α B¹¹.

Our findings are important because the cause of elevated α B₁₋₁₇₄ was unknown, and the data now implicate elevated calcium in the mechanism for production of α B₁₋₁₇₄. Previous studies have ruled out premature termination of the α B-crystallin gene, splice variants, or other mutations as causes¹¹. We found that simply adding calcium to the soluble proteins from homogenates of human or monkey lenses did not produce α B₁₋₁₇₄ (data not shown), indicating that the intact structural organization of the lens may be important for the process.

The comparative 2-DE maps of protein identities in human and monkey lenses produced in this study indicated that the positions of the various crystallins were remarkably similar. The major difference was that monkey lenses contained a greater number of acidic forms of all the major crystallins, especially α B-crystallin. While a major portion of this acidification was likely due to accumulation of deamidation in these long-lived proteins⁴, crystallins in monkey lenses also appeared to undergo a more extensive phosphorylation than human crystallins. The train of α B spots with increasing acidity in Fig. 1C and D exhibited decreased migration in the second dimension compared to intact α B. This would be expected if they were phosphorylated. While 2-DE maps of human lens crystallins have been previously published^{12, 22, 25}, they were either constructed using 2-DE gels focused with free carrier ampholines, or were IPG based with less complete spot identifications. The comparative 2-DE maps between human and monkey lenses in this study should facilitate the use of monkey lenses to investigate the contribution of crystallin modifications in the formation of human cataract.

The further usefulness of cultured monkey lenses to study human lens biology was demonstrated by the similar manner in which both species lost the C-terminal Lys of α B-

crystallin. We hypothesize that this cleavage was likely due to carboxypeptidase activation by calcium, rather than zinc, influx into the lens. However, note that our data showed a much greater sensitivity of CPE to zinc ion, even with pre-treatment with EGTA. Normal lens contains a significant level of zinc (~ 1 mM), and zinc was not elevated in our A23187-treated lenses. Zinc binds to approximately 3,000 proteins, especially 200 enzymes and 200 transcription factors³⁷. Thus, zinc might have been bound to these other proteins in normal lens, leaving too little to activate carboxypeptidases. In calcium-elevated lenses, however, calcium may directly bind to carboxypeptidases for activation. The specific cleavage of α B may result from the nearly complete absence of basic residues at the C-terminal end of other crystallins. While β B1-crystallin also contains a C-terminal Lys residue, an adjacent Pro residue prevented cleavage. Similarly, Lys 174 of α B-crystallin may be resistant to further cleavage due to Pro at residue 173.

The functional significance of truncated α B₁₋₁₇₄ in lens remains unknown. While intact α B-crystallin plays an important role as a chaperone in maintaining the solubility of other proteins in aged lens⁶, removal of the C-terminal lysine does not appear to alter chaperone activity¹¹. However, it is possible that other functions, such as those leading to genomic instability and hyperproliferation in α B^{-/-} mice¹⁴ may be affected by this truncation. Alternatively, the α B truncation may have no functional significance, but could be an indicator that unregulated carboxypeptidase activity in calcium loaded or stressed lens cells alters the function of other proteins that then could contribute to cataract.

Human lens is known to contain calpains³⁸, a class of proteases activated by calcium. In an earlier study, these enzymes were implicated in the proteolysis of the cytoskeletal protein vimentin in human lenses cultured with ionomycin¹⁷. However, the present experiments, and our earlier study³⁰ suggested that the activity of calpains in human and monkey lenses were not significantly directed towards crystallins. Calpains are known to extensively truncate the C-terminus of both α A- and α B-crystallin³⁹. The lack of any observable truncation of α A-, or more extensive truncation of α B-crystallin, suggested that elevation of calcium by A23187 in human and monkey lenses does not lead to widespread activation of calpains. This apparent lack of calpain activity against the crystallins in human and monkey lenses is due to the low activity of calpains, and the relatively high abundance of calpastatin, the endogenous inhibitor of calpains, in these lenses^{30, 38}. The lack of calpain activation was unlike rodent lenses, which undergo extensive opacification and calpain-induced truncation of crystallins during culture in A23187⁴⁰. The present study also suggested that calcium elevation by A23187 did not cause extensive transglutaminase mediated cross-linking of crystallins, since no cross-linking of β A3-crystallin, a known substrate for lens transglutaminase⁴¹, was observed. These results were unlike a previous study of human lenses cultured in ionomycin that observed possible transglutaminase cross-linking of vimentin¹⁷. Thus, unlike vimentin, crystallins in primate lenses appear to be resistant to modification by both calpains and transglutaminase.

Further studies are necessary to investigate why calcium elevation leads to carboxypeptidase activity. These studies are important because inappropriate carboxypeptidase activity could contribute to the mechanism of human cataract, and potentially be a target for therapeutic intervention.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Two-dimensional electrophoresis gels of soluble proteins from 3-year-old whole human lens (A), and 4-year old monkey lens cortex (C). Each gel image is duplicated in (B) and (D) in order to show regions numbered, excised, digested with trypsin, and proteins identified by mass spectral analysis (Tables 1 and 2, Supplementary Figs 1 and 2.)

Fig. 2. Two-dimensional electrophoresis gels of the soluble proteins from the epithelium (A) and superficial cortex (B) of human lenses cultured without (“Normal”) or with A23187. Only the area containing the majority of crystallins is shown. Landmark spots for intact αA , intact αB_{1-175} , and $\beta B2$ -crystallins are indicated in relation to the increased amounts of truncated αB_{1-174} (arrow) observed only in A23187-treated lenses. (C) Similarity of the above data to abundant truncated αB_{1-174} found in cataractous human lenses ¹¹.

Fig. 3. Deconvoluted spectra showing the masses of intact αB_{1-175} (A) and truncated αB_{1-174} (B) from human lens; and intact αB_{1-175} (C) and truncated αB_{1-174} (D) from monkey lens, corresponding to spots with arrows in Figs. 2 and 4.

Fig. 4. Two-dimensional electrophoresis gels of the soluble proteins from the superficial of monkey lenses of ages 6, 8 and 12 years cultured without ("Normal") or with A23187. Arrows indicate increased amounts of truncated increased αB_{1-174} observed only in A23187-treated lenses.

Fig. 5.

Carboxypeptidase E immunoblot image of monkey lens epithelium (Epi) and fiber region (Fiber) lysate (A). Deconvoluted spectra showing the masses of recombinant αB_{1-175} incubated with CPE and 2.5 μM Zinc (B), αB_{1-175} incubated with CPE and 4 mM Calcium (C), and αB_{1-175} incubated with CPE and 2.5 μM Zinc and 4 mM Calcium (D) and αB_{1-175} incubated with CPE and no ions (E).

Table 1Identification of major proteins from a 3-year-old whole human lens.⁺

Spot Number	Protein	Accession#
43, 46, 48, 49, 50, 53, 54, 55	α A	P02489
29, 32 [*] , 36	α B	P02511
1–12, 16, 21, 24, 25	β B1	P53674
22, 23, 26, 27, 28, 34, 37	β B2	P43320
40 [*]	β B3	P26998
31, 35, 41, 44, 51	β A3/A1	P05813
39, 42, 45, 47, 52	β A4	P53673
14, 17, 18, 32 [*] , 33	γ C	P07315
19, 20, 30	γ D	P07320
38	γ S	P22914
40 [*]	HSP 27	P04792
13, 15, 56, 57	N.I.	-

⁺ From spots numbered, excised, digested, and identified from tandem MS spectra of proteins shown in Fig. 1B.

^{*} Denotes spots that contained more than one detected protein. NI: not identified. Peptides identified from tandem MS data are summarized in Supplementary Fig. 1.

Table 2Identification of major proteins from cortex of a 4-year-old monkey lens.⁺

Spot Number	Protein	Accession #
1, 2, 3 [*]	Aldehyde dehydrogenase	P00352
4	Alpha enolase	P06733
10	Glyceraldehyde-3-phosphate dehydrogenase	P04406
14	Phosphatidylethanolamine-binding-protein	P30086
58	Fatty acid-binding protein	Q01469
59 [*]	Actin	(P60709)
73 [*] , 75 [*]	HSP 27	(P04792)
3 [*] , 56, 59 [*] , 60, 61, 62, 64, 65, 68	α A	(P02489)
27, 45, 55, 69	α B	(P02511)
12, 13, 35, 36, 38, 39, 50	β B1	(P53674)
5, 20, 21, 32, 33, 34, 37, 40, 41, 47, 48, 49 [*] , 52 [*] , 53, 57, 72 [*]	β B2	(P43320)
49 [*] , 52 [*]	β B3	(P26998)
30, 31, 42, 43, 46, 54 [*] , 70, 72 [*] , 73 [*] , 74, 75 [*]	β A3/A1	(P05813)
71, 76, 77	β A4	(P53673)
17, 18, 19	γ C	(P07315)
N.D.	γ D	
23, 29, 54 [*]	γ S	(P22914)
6, 7, 8, 9, 11, 15, 16, 22, 24, 25, 26, 28, 44, 51, 63, 66, 67, 78, 79	N.I.	

⁺From spots numbered, excised, digested, and identified from tandem MS spectra of proteins shown in Fig. 1D.^{*}Denotes spots that contained more than one detected protein. ND: not detected, NI: not identified. Peptides identified from tandem MS data are summarized in Supplementary Fig. 2.

Table 3

Ion concentrations and water levels during monkey lens culture and enzyme assays

Sample	Zinc (mEq/kg water)	Calcium* (mEq/kg water)	Water (mg)
Lens (no culture)	0.48 ± 0.06	2.57 ± 0.14	101 ± 4
Lens (cultured)	0.42 ± 0.04	4.88 ± 1.69	113 ± 4
A23187 lens (cultured)	0.28 ± 0.03	9.91 ± 1.07	153 ± 8
CPE assay buffer	ND		
CPE + αB	0.89 ± 0.02		
EMEM medium	0.07 ± 0.02		

ND, not detectable. Data are means ± SEM (n=3 for ion concentrations, n=6 for lens water level). Three groups of 9 lenses each were analyzed for ions and water.

* Calcium data is referenced from previous publication (30), which should be corrected to mmol Ca/kg lens water instead of mEq/kg lens water.