Inhibition of Fiber Cell Globulization and Hyperglycemia-Induced Lens Opacification by Aminopeptidase Inhibitor Bestatin

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PURPOSE. To examine the role of calcium-dependent and independent proteolytic activity in the globulization of isolated fiber cells and glucose-induced lens opacification.

METHODS. Fiber cells from rat lens cortex were isolated, and the [Ca2+]i, and protease activity in the isolated fibers were determined by using a calcium binding dye and the protease substrate N-butoxy-carbonyl-Leu-Met-7-amino-4-chloromethylcoumarin (BOC-Leu-Met-CMAC). The activity of calpain in the lens cortex homogenate was determined with fluorescein-casein in the presence of Ca2+ and that of fiber cell globulizing aminopeptidase (FCGAP) with BOC-Leu-Met-CMAC and reduced glutathione (GSH) in the absence of Ca2+. The lens protease-calpain and the novel aminopeptidase FCGAP were partially purified by diethylaminoethyl (DEAE) gel column chromatography. Single fiber cells were isolated from rat lens, plated on coverslips, and placed in a temperature-controlled chamber. Their globulization time was determined by the appearance of light-scattering globules in the absence and the presence of protease inhibitors including the aminopeptidase inhibitor bestatin. To investigate the effect of the protease inhibitors E-64 and bestatin on the prevention of hyperglycemic cataract, the rat lenses were cultured in medium 199 in the presence of normal Ringer’s solution led to their globulization in 30 ± 3 minutes. Addition of 0.5 mM of the protease inhibitors E-64 and leupeptin increased the globulization time to 60 and 100 minutes, respectively, whereas no globulization of the fiber cells was observed for 4 hours in the presence of 0.05 mM bestatin. In rat lens cultured in medium containing 50 mM glucose, both E-64 and bestatin (0.05 mM each) significantly reduced the extent of opacification, indicating that an aminopeptidase, downstream to a Ca2+-dependent protease, may be involved in mediating cataractogenic changes.

RESULTS. Normal levels of lens fiber cell [Ca2+]i, determined by using a cell-permeable dye were approximately 100 nM, and the protease activity determined with BOC-Leu-Met-CMAC was maximum at [Ca2+]i of approximately 500 nM. A large fraction of the FCGAP that cleaves BOC-Leu-Met-CMAC was separated from calpain, which cleaves fluorescein-casein, by diethylaminoethyl (DEAE) gel column chromatography. The FCGAP did not bind to the column, whereas calpain bound to the column and was eluted by approximately 180 mM NaCl. Unlike calpain, the FCGAP did not require calcium for activation and did not cleave fluorescein-casein. However, the Ca2+-dependent calpain activated FCGAP, indicating that the latter may exist in pro-protease form. The FCGAP was selectively inhibited by the specific aminopeptidase inhibitor bestatin, indicating that FCGAP could be an aminopeptidase. However, the FCGAP was found to be immunologically distinct from leucine aminopeptidase and calpain. Perfusion of the isolated rat lens fiber cells with Ringer’s solution led to their globulization in 30 ± 3 minutes. Addition of 0.5 mM of the protease inhibitors E-64 and leupeptin increased the globulization time to 60 and 100 minutes, respectively, whereas no globulization of the fiber cells was observed for 4 hours in the presence of 0.05 mM bestatin. In rat lens cultured in medium containing 50 mM glucose, both E-64 and bestatin (0.05 mM each) significantly reduced the extent of opacification, indicating that an aminopeptidase, downstream to a Ca2+-dependent protease, may be involved in mediating cataractogenic changes.

CONCLUSIONS. In addition to calpain, a Ca2+-independent novel protease, FCGAP, a novel aminopeptidase, represents a significant fraction of the total proteolytic activity in the lens. Inhibition of FCGAP by bestatin attenuates Ca2+-induced globulization of the isolated fiber cells in vitro and hyperglycemia-induced opacification of cultured rat lens. (Invest Ophthalmol Vis Sci. 2002;43:2285–2292)

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ased on the presence of light-scattering centers in supranuclear cataract in humans and rats, it was proposed that fiber cell globulization is a major contributor to such cataracts.1,2 However, the hypothesis remained buried for more than two decades, until we isolated single fiber cells from rat lens cortex and demonstrated that the fiber cells disintegrate into light-scattering globules.3–8 The isolated fiber cells in non-ionic medium have been found to be stable for an extended period, whereas in ionic medium such as Ringer’s solution containing 1 to 2 × 10−3 M Ca2+, the fiber cells globulize in approximately 30 minutes.3,4 The globulization of fiber cells in Ringer’s solution is delayed significantly, however, by lanthanum, which blocks the uptake of calcium; by bis-(o-aminophenoxy)-N,N'-N''-tetraacetic acid-acectoyxymethyl ester (BAPTA-AM),4 which buffers intracellular calcium; by leupeptin and E-64,4 which inhibit calcium activated proteases; or by hypotonic solution, which prevents Donnan swelling.5 The globulization of isolated fiber cells is also delayed by removing Na+, or K+ from the Ringer’s solution.5 We also found that the globulization of fiber cells is associated with an increase in intracellular calcium ([Ca2+]i).5 The increase in [Ca2+]i, in the presence of the calcium ionophore A23197 has been shown to cause lens opacification,5 indicating that a change in [Ca2+]i may be a critical trigger of cataractogenesis.

We have shown that, in isolated fiber cells, the [Ca2+]i is approximately 100 nM. This is in contrast to earlier reports9,10 that indicate that [Ca2+]i in fiber cells may be as high as 1 to 2 mM, when measured by atomic absorption techniques and 1 to 2 μM when measured by Ca2+-selective electrodes. However, we observed that exposure to normal Ringer’s solution increases [Ca2+]i, which is accompanied by an increase in the protease activity in isolated fiber cells, as determined by using a fluorescent substrate, N-butoxy-carbonyl-Leu-Met-7-amino-4-chloromethylcoumarin (BOC-Leu-Met-CMAC).11 The maximum increase in protease activity is observed when the [Ca2+]i,
of the fiber cells is less than 0.5 μM, and most of the fiber cells globulize before the [Ca⁡²⁺]i reaches the level of 1.0 to 1.5 μM.⁸

The proteases calpains I and II have been implicated in cataractogenesis by a number of investigators.²⁻⁴ Based on in vitro studies with purified enzymes, it has been shown that calpain I requires at least 10 μM [Ca⁡²⁺]i for maximal activation, whereas calpain II is maximally activated by 1 to 2 mM calcium. Because lens fiber cells start globulizing when [Ca⁡²⁺]i, is less than 0.5 μM,⁸ it is likely that protease(s) other than calpain may also be involved in fiber cell globulization and possibly in supranuclear cataractogenesis. Because our initial studies indicated that the protease activity of the isolated lens fiber cells, determined with the fluorescent substrate BOC-Leu-Met-CMAC, is maximally activated in the presence of low (<0.5 μM) [Ca⁡²⁺]i, we partially purified the proteases from the rat lens cortex, using the method of David and Shearer⁴ for the isolation of calpain. Our results indicate that calpain and the novel protease, which we refer to as FCGAP, completely separate from each other in diethylaminoethyl (DEAE) gel column chromatography. The FCGAP did not bind to the column, whereas calpain was retained in the column and was eluted by approximately 180 mM NaCl, as described by David and Shearer. The FCGAP was inhibited by bestatin, an aminopeptidase inhibitor,⁵,⁶,¹⁰ whereas this inhibitor had no significant effect on calpain activity. In the current study, bestatin significantly delayed the globulization of isolated rat fiber cells, superfused with Ringer’s solution containing 2 × 10⁻⁵ M Ca⁡²⁺, and also prevented hyperglycemia-induced opacification of cultured rat lenses.

**Materials and Methods**

**Materials**

Leupeptin, chymostatin, bestatin, E-64, EDTA, EGTA, leucine-β-nitroanilide, glutathione (reduced form, GSH), β-mercaptoethanol (βME), antipain, and porcine kidney leucine aminopeptidase (LAP) were procured from Sigma (St. Louis, MO); and casein fluorescein conjugate, BOC-Leu-Met-CMAC, and a calcium-binding dye (Fluo-3 AM) were procured from Molecular Probes (Eugene, OR). DEAE gel (BioGel A) and horseradish peroxidase-labeled anti-mouse and anti-rabbit antibodies were procured from Bio-Rad Laboratories ( Hercules, CA), and mouse anti-μ and anti-λ-calpain antibodies were purchased from Chemicon International, Inc. (Temecula, CA). n-Calpain (human erythrocytes) and calcium ionophore A23187 were obtained from Calbiochem (La Jolla, CA). Derivitizing solvent (Deriva-sil) was purchased from Regis Technologies Inc. (Morton Grove, IL). Bovine lens anti-LAP antibodies were a gift from Allen Taylor (Tufts University, Boston, MA).

**Isolation of Fiber Cells and Determination of Globulization Time, Protease Activity, and [Ca⁡²⁺]i**

Sprague-Dawley rats (each weighing 200–250 g) were housed in accordance with the institutional guidelines and were killed by a single intraperitoneal injection of pentobarbital sodium. All animals were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The globs were removed and immersed in Ringer’s solution. The lenses were dissected with the cupula intact. Single fiber cells were isolated with trypsin and a temperature gradient, as described elsewhere.³⁻⁵ freshly isolated fiber cells, (2.0–2.5 mm in length) were allowed to attach to a coverslip and incubated with 0.5 mL of the indicated solutions. To determine the effect of bestatin, the fiber cells were incubated with Ringer’s solution containing 2.0 × 10⁻⁵ M Ca⁡²⁺, with or without 0.05 mM bestatin. Changes in fiber cell morphology were observed with an inverted microscope (Diaphot 300; Nikon, Tokyo, Japan). Tg refers to the time required for complete fiber cell globulization.

**Determination of [Ca⁡²⁺]i**

The [Ca⁡²⁺]i measurements in isolated fiber cells were performed using a calcium-binding dye (Fluo-3 AM; Molecular Probes). The calibration procedure was essentially the same as described previously.⁹ Briefly, the fibers were incubated with 10 μM of the dye for 3 hours at 37°C in HEPES-EDTA-sucrose (HES) solution (composition in millimolar: sucrose 280, Na-EDTA 10, HEPES 10 [pH 7.4], 300–310 mM). After incubation, the fibers were layered on the coverslip at the bottom of the tissue chamber. Fluorescence (F) of the fiber cell was measured at excitation wavelength 490 nm, and emission wavelength 520 nm. Maximum fluorescence (Fmax) was determined by the addition of ionophore A23187 (10 μM), and the minimum (Fmin) was determined by measuring the fluorescence after quenching by the addition of MnCl₂ (2.0 mM). The [Ca⁡²⁺]i, was calculated by the following equation

\[
[\text{Ca}^{2+}]_i = K_d \times [(F - F_{\text{min}})/(F_{\text{max}} - F)]
\]

A K_d of 400 nM, representing the dissociation constant of Ca²⁺ bound to the dye, was used for the calculations.

**Determination of Intracellular Protease Activity in a Single Fiber Cell**

Protease activity of individual fiber cells was determined by incubating the cells with the protease substrate BOC-Leu-Met-CMAC. This substrate readily permeates biological membranes and, once inside the cell, conjugates with GSH to form BOC-Leu-Met-CMAC-SG.¹⁷ Proteases cleave the conjugate between methionine and CMAC-SG, resulting in the fluorescent product CMAC-SG, which has an emission maxima at 410 nm when excited at 360 nm. BOC-Leu-Met-CMAC was dissolved in dimethyl sulfoxide (DMSO) to make a stock solution of 4.5 mM. The final working concentration used was 10 μM. The fibers were preincubated for 5 minutes in 0.5 mL of HES solution containing 10 μM substrate and then transferred to a circular tissue chamber, which had 3 mL of solution containing 10 μM substrate. The fluorescence of a single fiber was measured with a microfluorimeter, built around an inverted microscope (Diaphot 300; Nikon) that was equipped with an epifluorescence attachment and two photomultiplier tubes (PMTs).³ The fiber cell was placed on a coverslip attached to the bottom of the circular tissue chamber. The fibers were illuminated with a 150-W xenon lamp, powered by a constant current power supply (Universal Power Supply, model 68805; Oriel, Stratford, CT).

The light from the lamp was collimated through a beam probe and delivered to the filter assembly through a dichroic mirror installed in the microscope. The fiber cell was illuminated with excitation light at 560 nm and the emission fluorescence was measured at 410 nm using a long pass filter. The fluorescence from the fiber cell was collected through a 20× objective lens (CF Fluor; Nikon), and conducted through the side port of the microscope. To minimize collection of stray light, a rectangular shutter (model 85291; Nikon) was used to mask the portion of the image not occupied by the fiber cell. The masking cube was connected to a viewer (PFX; Nikon) for alignment and optical viewing of the fiber cell. The PMTs (HC 124-03; Hamamatsu, Hamamatsu City, Japan) were connected to the microscope through a beam splitter holder. The PMTs were energized using a 12-V power supply (LPS 11; Leader Electronic Corp., Cypress, CA). The gain of the PMT was set by adjusting the voltage on the analog-to-digital conversion board to be between 500 and 900 V. The setup was used in single-photon-counting mode, and the data were acquired by a set of concatenated counters (TIP-10), using a computer program (LabView; National Instruments, Austin, TX). The microfluorimeter was placed on a vibration-free table and covered with a dark cage to minimize interference from stray light. The protease activity was expressed as a change of fluorescence per minute. In each experiment, change in fluorescence of a single fiber cell was recorded for 30 minutes, but in all calculations of the enzyme activity, only the slope of the linear increase in the enzyme activity was used.

**Statistical Analysis**

All results are expressed as the mean ± SEM. Significance of difference was evaluated using Student’s t-test. Difference is significant at P < 0.05.
Determination of Enzyme Activity

Calpain. Calpain activity was determined according to the method of David and Shearer. Briefly, the reaction mixture (0.1 mL) contained 60 μL protein fraction, 20 μL fluorescein-casein (5 mg/mL) and 20 μL 20 mM Tris-HCl (pH 7.5), containing 15 mM CaCl₂ and 10 mM β-ME. The reaction mixture was incubated at 25°C for 30 minutes, the reaction was stopped by placing the sample on ice, and 50 μL (12 mg/mL) bovine serum albumin was added, followed by 0.2 mL 10% trichloroacetic acid. After centrifugation at 10,000 rpm for 5 minutes, 100 μL supernatant was removed and added to 100 μL 1.5 M Tris-HCl (pH 8.6). The fluorescence of the samples was determined at 525 nm after excitation at 455 nm, using a plate reader (FluoroCount; Packard Instruments, Meriden, CT). The amount of fluorescein-labeled, acid-soluble fragments was determined by comparison with standards of 0 to 5 μg undegraded fluorescein casein dissolved in 0.5 M Tris-HCl (pH 7.5; assuming that the casein was uniformly labeled by the fluorescein dye). One unit of calpain activity was defined as 1 μg of acid-soluble fragment released from casein per minute. Whenever described, calpain activity was also determined by using leupeptin as a substrate.

FCGAP. FCGAP activity was determined by using GSH and BOC-Leu-Met-CMAC. The 2.0 mL reaction mixture contained 10 mM Tris-HCl (pH 8.0), 20 μL 100 mM GSH, 20 μL 1 M CaCl₂, 50 to 200 μL protein sample, and 10 μL 2 mM BOC-Leu-Met-CMAC. FCGAP activity was determined at room temperature by noting the increase in fluorescence at an excitation wavelength of 360 nm and measuring emission at 410 nm with a spectrophotometer (F-4500; Hitachi, Ltd., Tokyo, Japan). One unit of enzyme activity was defined as 1 micromole substrate cleaved per minute.

Protease Purification

Eyeballs from 12 rats were removed in 10 mM Tris-HCl (pH 7.5) containing 1 mM EDTA, 1 mM EGTA, and 10 mM β-ME (buffer A). Lenses were dissected and the epithelium removed. The remaining lens was homogenized in buffer A with an Omnimixer (Sorvall, Newtown, CT) to make a 10% homogenate (wt/vol). The homogenate was centrifuged at 10,000 rpm for 30 minutes, and the supernatant was used to determine the protease activity with fluorescein-labeled casein (for calpain) and BOC-Leu-Met-CMAC (for FCGAP), as described earlier. The supernatant was applied to a DEAE gel column (21 × 100 mm) preequilibrated with buffer A at a flow rate of 30 mL/h. The column was washed with the same buffer for 2 hours and eluted by using a 200 mL linear gradient of 0 to 300 mM NaCl in Buffer A.

Immunoprecipitation

For the precipitation of protease activity, appropriate dilutions of antisera, raised against µ- and m-calpain or LAP were incubated with enzyme preparations overnight at 4°C. Subsequently, secondary antibodies raised against mouse or rabbit IgG were added, and the samples were allowed to stand for an additional 4 to 6 hours, followed by centrifugation at 10,000 rpm for 15 minutes. Supernatants were used to determine the calpain and FCGAP activity.

Effect of Protease Inhibitors on the Enzyme Activity

We investigated the effect of protease inhibitors by incubating 200 μL FCGAP fraction and 48 μL calpain fraction separately with 0.05 mM bestatin, and 0.5 mM each leupeptin, antipain, chymostatin, and E-64 for 30 minutes at room temperature (25°C). After incubation, the samples were assayed for FCGAP activity in a total volume of 2.0 mL, and for calpain activity in a reaction mixture of 0.1 mL, as described earlier.

Rat Lens Culture

The eyeballs were removed from the rats, and the lenses were dissected in phosphate-buffered saline under sterile conditions, with the aid of a dissecting microscope. Each of the dissected lenses was immersed in a separate well of a 24-well tissue culture plate containing medium 199 supplemented with 1% penicillin-streptomycin. Lenses were divided into four groups, one control and three experimental. Each group had three lenses. The control group lenses (group A) were incubated with medium 199 containing 5.5 mM glucose, and the experimental groups were incubated with medium 199 containing 50 mM glucose (group B), 50 mM glucose +0.05 mM bestatin (group C), and 50 mM glucose +0.05 mM E-64 (group D). The lenses were maintained in a humidified atmosphere of 5% CO₂ and 95% air at 37°C, as described previously. The incubation medium was changed every 24 hours. The incubations were staggered so that all the lenses, incubated for 0, 3, 6, and 8 days, were ready for biochemical measurements and digital image analysis at the same time under identical conditions.

The opacity of the lenses was examined by digital image analysis, as described elsewhere. Briefly, the imaging system consisted of a TV camera (Optronics Engineering, Goleta, GA) attached to the television port of an inverted microscope (Nikon). The condenser was adjusted for Köhler illumination. To view the entire lens, a ×2 objective was used. The first series of images were collected under a condition in which the illumination was increased so that the center of the control (untreated) lens (on day 0) saturated the acquisition system. No quantitative information was obtained from these images. For quantitative measurements, the illumination intensity was adjusted so that the maximum transmittance through the control untreated (on day 0) lens, measured by the camera, was just below the saturation threshold of the camera. The illumination remained unchanged for all subsequent measurements, and the images were acquired and analyzed using image-analysis software (Metamorph; Universal Imaging, West Chester, PA). Each lens was placed in a 2.5-cm Petri dish containing phosphate-buffered saline.

Quantitative measurements were made from each lens for each condition measured in triplicate. Each lens was divided into four concentric circular regions by measuring the radius of each lens and dividing it into four equal segments. An annulus, the width of which was equal to one fourth the radius of the lens, was constructed and the average pixel intensity was measured in each region. The average pixel intensity, measured from homologous regions for each of the three lenses for each treatment, was averaged.

Biochemical Measurements

The lenses from all the groups, after the indicated days in culture, were homogenized in 0.5 mL 20 mM potassium phosphate (pH 7.0) by sonication for 30 seconds using a sonifier cell disruptor (model W185 Heat Systems; Ultrasonics Inc., Plainview, NY). For measuring GSH, 0.2 mL homogenate was mixed with 0.3 mL precipitating reagent (0.2 M glacial meta-phosphoric acid, 5.1 M NaCl and 5.9 mM EDTA). After centrifugation at 10,000g for 15 minutes, 0.2 mL supernatant was added to 0.8 mL 0.3 M NaH₂PO₄, followed by the addition of 0.1 mL 5.5’ dithiobis-(2-nitrobenzoic acid; DTNB; 0.04% in 1% sodium citrate). The change in optical density (OD) at 412 nm was recorded using a spectrophotometer (UV-Vis; Varian, Sunnyvale, CA), as described previously. Soluble and insoluble proteins were determined by the Bradford method, using 5 μL aliquots of homogenate before and after centrifugation at 10,000g for 15 minutes. For measuring sorbitol, the homogenate was ultrafiltrated (YM10 Centricon; Millipore, Bedford, MA). An aliquot of the filtrate was lyophilized in a Savant Asigo Automatic Speed Vac (Farmington, NY) until completely dry and stored overnight in a vacuum desiccator containing calcium chloride as the desiccant. The samples were derivatized by adding 0.1 mL of a solvent (Deriva-sil) under anhydrous conditions. The derivatized mixture (1 μL) was injected into a gas chromatograph (GC) system (model 3400; Varian). The temperature gradient was set to increase from 140°C to 170°C at 4°C/min and from 170°C to 250°C at 50°C/min. The amount of sorbitol present in a sample was calculated using reagent sorbitol measured by GC under similar conditions. Aldose reductase (AR) activity was determined by using an aliquot of 10,000g supernatant and glyceraldehyde, as described elsewhere.
RESULTS

As shown in Figure 1, the basal level of \([\text{Ca}^{2+}]_i\) in isolated fiber cells was approximately 100 nM, similar to that observed in various other cells, including cardiac myocytes. In the presence of Ringer’s solution containing \(2 \times 10^{-3}\) M \(\text{Ca}^{2+}\), the \([\text{Ca}^{2+}]_i\) levels increased to approximately 1.2 \(\mu\)M in 20 minutes. The protease activity in the fiber cells, as determined by increased fluorescence at 410 nm and excitation at 360 nm, was maximally increased in approximately 8 minutes when the \([\text{Ca}^{2+}]_i\) was less than 400 nM. Because this concentration of \([\text{Ca}^{2+}]_i\), at least in vitro, is significantly lower than that required for the maximum activation of calpain, we reasoned that all the protease activity in the lens fibers may not be due to calpain. Hence, to investigate further the source of the increase in the protease activity at low concentrations of \([\text{Ca}^{2+}]_i\), we partially purified the putative proteases.

Partial Purification and Characterization of FCGAP

For the partial purification of the proteases, the 10,000g supernatant of rat lens cortex was subjected to DEAE gel column chromatography. FCGAP did not bind to the column, whereas calpain bound to the column and was eluted by approximately 180 mM NaCl (Fig. 2). FCGAP activity was lost after heating at 60°C for 10 minutes, indicating that the activation may be due to a protein. It is interesting to note that the FCGAP did not hydrolyze casein, which is the substrate used for assaying calpain. Correspondingly, the calpain fraction which was eluted by approximately 180 mM NaCl did not cleave the BOC-Leu-Met-CMAC that was used for measuring the FCGAP activity in cells. The leucine-p-nitroanilide was cleaved by the commercial LAP, but not by the FCGAP or calpain fractions. The commercial LAP did not cleave BOC-Leu-Met-CMAC or fluorescein-labeled casein. Furthermore, unlike calpain, the FCGAP did not require \(\text{Ca}^{2+}\) for activation, although we observed a good correlation between low levels of \([\text{Ca}^{2+}]_i\) (<0.4 \(\mu\)M) and FCGAP activity in single fiber cell (Fig. 1). It is likely that a \(\text{Ca}^{2+}\)-dependent protease(s) is required to activate FCGAP.

Activation of Partially Purified FCGAP by \(\text{Ca}^{2+}\)-Activated Protease(s). To understand further how \([\text{Ca}^{2+}]_i\) activates FCGAP in fiber cells, we incubated the FCGAP fraction (DEAE-gel unadsorbed) alone and a mixture of the FCGAP fraction and the calpain fraction (eluted from DEAE gel column by approximately 180 mM NaCl), for up to 60 minutes at ambient temperature, without or with \(2 \times 10^{-3}\) M \(\text{Ca}^{2+}\). The presence of \(\text{Ca}^{2+}\) had no effect on the FCGAP activity in the absence of the calpain fraction, whereas in the mixture (FCGAP + calpain fraction) incubated with \(\text{Ca}^{2+}\), the activity of the FCGAP increased approximately twofold compared with the mixture incubated without \(\text{Ca}^{2+}\) (Fig. 3). This indicates that calcium could activate calpain and/or some other protease, which in turn could lead to the activation of the FCGAP.

Effect of BOC-Leu-Met-CMAC on Calpain Activity. It is possible that the chloro group of BOC-Leu-Met-CMAC inactivates calpain by reacting with its sulfhydryl residue(s). Therefore, we preincubated the calpain fraction, obtained from the rat lens cortex, as well as the commercially produced \(\mu\)-calpain, with BOC-Leu-Met-CMAC for 30 minutes at room temperature, and then determined the activity of calpain with fluorescein-labeled casein. There was no inhibition of calpain activity by the FCGAP substrate.

Immunologic Nature of FCGAP, Calpain, and LAP. Our results show that FCGAP is immunologically distinct from cal-

![Figure 1](https://iovs.arvojournals.org/)

**Figure 1.** Correlation between \([\text{Ca}^{2+}]_i\) and protease activity. \([\text{Ca}^{2+}]_i\) (●) and protease activities (■) were determined in isolated rat lens fiber cells, superfused with \(2 \times 10^{-3}\) M \(\text{Ca}^{2+}\), at different time intervals. The results are expressed as the mean ± SD; \(n = 5\).

![Figure 2](https://iovs.arvojournals.org/)

**Figure 2.** DEAE-gel column chromatography of rat lens supernatant. Rat lenses were homogenized and centrifuged at 10,000g, and the supernatant was applied on a DEAE gel. The FCGAP did not bind to the column, whereas calpain, determined by using fluorescent casein as the substrate, bound to the column and was eluted by a gradient of NaCl (0–300 mM). Solid line: calpain activity; dashed line: protein absorbance at 280 nm; dotted line: NaCl gradient.

![Figure 3](https://iovs.arvojournals.org/)

**Figure 3.** Activation of FCGAP by calcium-activated calpain. A mixture of an equal amount of FCGAP (unadsorbed) and calpain (eluted at approximately 180 mM NaCl) fractions from a DEAE gel column were incubated without () and with (■) 2 mM \(\text{Ca}^{2+}\) at room temperature, and FCGAP activity was determined at different time intervals.
We used several protease inhibitors to inhibit FCGAP and calpain. Leupeptin, chymostatin, antipain, and E-64 significantly (60%–90%) inhibited the activity of the calpain fraction and the commercial calpain, but did not inhibit (<3%) FCGAP activity (Table 1). In contrast, 0.05 mM of the aminopeptidase inhibitor bestatin inhibited FCGAP by more than 80%, but had little or no effect on calpain activity. Thus, based on the effects of protease inhibitors, FCGAP appears to be distinct from calpain. These results indicate that even at low [Ca\textsuperscript{2+}], some Ca\textsuperscript{2+}-activated protease activity in the fiber cells increases, which activates the FCGAP that may be responsible for the globulization of fiber cells as well as cataractogenesis. Therefore, we examined the effect of bestatin, a FCGAP inhibitor, on the globulization of isolated fiber cells and also on glucose-induced opacification of rat lens in culture.

### Inhibition of Isolated Fiber Cell Globulization by Bestatin.

The freshly isolated lens cortex fiber cells incubated with Ringer's solution containing 2 × 10\textsuperscript{-3} M Ca\textsuperscript{2+} globulize in 50 ± 3 minutes. Addition of 0.5 mM E-64 increased the Tg to 70 ± 5 minutes as published earlier, whereas the addition of 0.05 mM bestatin increased Tg to more than 4 hours. Of the 40 fibers examined, more than 80% of the fiber cells did not show any changes for 4 hours, indicating that bestatin is the best protease inhibitor of fiber cell globulization identified to date.

### Prevention of Glucose-Induced Lens Opacification by Bestatin and E-64.

As shown in Figure 4, digital image analysis of control and treated rat lenses clearly illustrates the increase in lens opacity in the presence of high (50 mM) glucose and the protective effect of the protease inhibitors bestatin and E-64. Figure 4 (left) shows images of the lenses with the light adjusted so that the central portion of the day 0 control lens saturated the camera. Under these light conditions, the differences in the effect of the treatment, although visually apparent, do not allow accurate quantification. Therefore, for quantification (Fig. 4, right), the light intensity was adjusted so that the camera was just below saturation for the day 0 control lens. Each row illustrates a lens representative of each treatment as indicated in the figure legend. Both bestatin (row C) and E-64 (row D) were effective in preventing the lens opacification compared with glucose alone (row B).
Figure 5 shows the quantification of the changes in lens opacity as a function of treatment, days in culture, and the region of the lens in which opacity was measured. These measurements were made under the conditions shown in Figure 4 (right). The mean intensity in each region of the lens was analyzed using analysis of variance for a two-factorial experiment. The two factors were treatment (glucose 5.5 mM, glucose 50 mM + bestatin, and glucose 50 mM + E-64) and days in culture (3, 6, and 8 days). Fisher's least-significant difference procedure with Bonferroni adjustment for number of comparisons was used for multiple comparisons, including comparison with the means of the control group (glucose, 5.5 mM) with the mean of 5.5 mM glucose at day 0. All tests were assessed at the 0.05 level of significance. The results showed that, even in the control lens, there was a decrease in the transmittance from the entire lens as a function of number of days in culture (Fig. 5). However, regardless of the region, the mean of the control group at 3 days in culture is not statistically significant from the mean at day 0. The means of the control group at 6 and 8 days in culture are significantly lower than the mean at day 0.

The differences between the different regions of the treatment groups (three lenses in each group) were the same, regardless of the days in culture. In region A, there was no significant difference between the control group and the bestatin-treated group, but both high glucose and E-64 significantly decreased transmittance through the lens. In regions B and C, all groups were significantly different from the control group and from each other. The treatment with bestatin resulted in a significantly higher transmittance through the lens than either E-64 or high glucose. Similarly, treatment with E-64 resulted in a significantly higher transmittance through the lens than high glucose. In region D, there was no significant difference between bestatin-treated lens and the control. The treatment with E-64 resulted in a significantly lower transmittance through the lens than the control. However, there was no significant difference between lenses treated with bestatin or E-64. The transmittance through the lenses treated with bestatin or E-64 were all significantly better than the 50 mM glucose-treated lenses treated with protease inhibitors.

Biochemical Changes in Lenses Cultured with High Glucose and Protease Inhibitors

Biochemical changes on various days of lens culture are given in Table 2. In the lenses of all the groups, there was no significant change in the ratio of soluble to insoluble proteins and GSH on day 3, but AR activity dropped by more than 50% in the lenses cultured in 50 mM glucose compared with the
TABLE 2. Determination of Biochemical Parameters in Rat Lenses Cultured in Medium 199 Supplemented with 50-mM Glucose, in the Presence or Absence of 0.05 mM Bestatin and E-64

<table>
<thead>
<tr>
<th>Group</th>
<th>Protein (Sol./Insol.)</th>
<th>GSH (nmol/mg protein)</th>
<th>AR (mU/mg protein)</th>
<th>Sorbitol (nmol/mg protein)</th>
</tr>
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<tbody>
<tr>
<td>Day 0</td>
<td>2.16 ± 0.10</td>
<td>9.39 ± 0.25</td>
<td>6.09 ± 0.37</td>
<td>ND</td>
</tr>
<tr>
<td>Day 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.10 ± 0.16</td>
<td>9.06 ± 0.31</td>
<td>7.60 ± 0.63</td>
<td>1.26 ± 0.25</td>
</tr>
<tr>
<td>Glucose 50 mM</td>
<td>1.99 ± 0.10</td>
<td>8.32 ± 0.23</td>
<td>2.99 ± 0.53**</td>
<td>2.38 ± 0.10*</td>
</tr>
<tr>
<td>Glucose 50 mM+Bestatin</td>
<td>2.16 ± 0.10</td>
<td>8.73 ± 0.20</td>
<td>6.17 ± 0.51***</td>
<td>63.91 ± 8.75***###</td>
</tr>
<tr>
<td>Glucose 50 mM+E-64</td>
<td>1.96 ± 0.08</td>
<td>8.65 ± 0.11</td>
<td>5.45 ± 0.53***</td>
<td>47.18 ± 4.05***###</td>
</tr>
<tr>
<td>Day 6</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>Control</td>
<td>2.41 ± 0.08</td>
<td>7.99 ± 0.11</td>
<td>5.84 ± 1.60</td>
<td>1.27 ± 0.08</td>
</tr>
<tr>
<td>Glucose 50 mM</td>
<td>0.79 ± 0.05**</td>
<td>4.11 ± 0.15**</td>
<td>2.29 ± 0.20**</td>
<td>5.36 ± 1.16*</td>
</tr>
<tr>
<td>Glucose 50 mM+Bestatin</td>
<td>2.23 ± 0.28**</td>
<td>8.14 ± 0.21**</td>
<td>5.73 ± 0.21**</td>
<td>55.6 ± 6.56***###</td>
</tr>
<tr>
<td>Glucose 50 mM+E-64</td>
<td>2.29 ± 0.41**</td>
<td>7.71 ± 0.17**</td>
<td>4.82 ± 0.53**</td>
<td>22.61 ± 4.36***###</td>
</tr>
<tr>
<td>Day 8</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.18 ± 0.16</td>
<td>7.95 ± 0.25</td>
<td>5.71 ± 0.44</td>
<td>3.00 ± 0.07</td>
</tr>
<tr>
<td>Glucose 50 mM</td>
<td>1.02 ± 0.03**</td>
<td>4.60 ± 0.16*</td>
<td>1.68 ± 1.01**</td>
<td>2.12 ± 0.53</td>
</tr>
<tr>
<td>Glucose 50 mM+Bestatin</td>
<td>2.15 ± 0.13**</td>
<td>6.22 ± 0.16**</td>
<td>4.27 ± 0.17**</td>
<td>19.81 ± 0.52**##</td>
</tr>
<tr>
<td>Glucose 50 mM+E-64</td>
<td>2.14 ± 0.12**</td>
<td>6.38 ± 0.55**</td>
<td>3.68 ± 0.12**</td>
<td>13.40 ± 0.60***###</td>
</tr>
</tbody>
</table>

Lenses, three in each group cultured for various periods, were individually homogenized in 20 mM phosphate (pH 7.0), and aliquots were taken for the determination of biochemical parameters. The results are given as mean ± SE. The significance values *P < 0.1, **P < 0.01, and ***P < 0.001 for 50 mM glucose, 50 mM glucose+bestatin and 50 mM glucose+E-64 groups were compared with 5.5 mM glucose, and #P < 0.01, ##P < 0.001. ###P < 0.001 for 50 mM glucose+bestatin and 50 mM glucose+E-64 groups were compared with 50 mM glucose. Sol./Insol., ratio of soluble to insoluble protein.
This possibility is supported by a twofold activation of FCGAP activity when the FCGAP and calpain fractions were incubated together in 2 × 10^{-3} M Ca^{2+}. Nonetheless, the possibility that calpain or any other protein in the calpain fraction behaves like calmodulins and delivers Ca^{2+} to FCGAP cannot be completely ruled out. Further investigation is needed to delineate this pathway(s), which is downstream to the Ca^{2+}-activated protease(s).

Cysteine protease inhibitors, such as leupeptin, pepstatin, and E-64 inhibited calpain activity,\textsuperscript{12,13} but did not significantly inhibit FCGAP activity. FCGAP was inhibited, however, by more than 80% by the aminopeptidase inhibitor bestatin, whereas this inhibitor did not inhibit calpain. We therefore, reasoned that bestatin should be able to prevent or significantly delay the globalization of isolated rat lens cortex fiber cells. Our results show that, indeed, bestatin prevented globalization of fiber cells when incubated in Ringer’s solution containing 2 × 10^{-3} M Ca^{2+} for more than 4 hours. This was the best protection that we have observed so far by any protease inhibitor or calcium chelator. These interesting observations prompted us to investigate whether bestatin could prevent or significantly delay cataract in rat lenses cultured in high glucose.

Digital image analyses of rat lenses, cultured in 50 mM glucose for up to 8 days, showed that the lenses had progressive development of more than 80% opacity in the nuclear region (Fig. 5A) in 8 days, as determined by transmittance measurements.\textsuperscript{19} In contrast, the group of lenses cultured in 50 mM glucose +0.05 mM bestatin, had approximately 50% opacity, which was only slightly greater than the control lenses cultured in 5.5 mM glucose, which had approximately 40% opacity, indicating excellent protection from hyperglycemic cataractogenesis by bestatin. The calpain inhibitor, E-64 also prevented the opacification of lenses cultured in 50 mM glucose, as described by Nakamura et al.\textsuperscript{12} and Shearer et al.\textsuperscript{13} except that bestatin provided significantly better protection against opacification in the nuclear region. The biochemical determinations such as ratio of soluble-insoluble proteins and GSH also substantiated the protective effect of bestatin. Sorbitol and aldose reductase levels were unexpectedly more than 80% by the aminopeptidase inhibitor bestatin, whereas this inhibitor did not inhibit calpain. We therefore, reasoned that bestatin should be able to prevent or significantly delay the globalization of isolated rat lens cortex fiber cells. Our results show that, indeed, bestatin prevented globalization of fiber cells when incubated in Ringer’s solution containing 2 × 10^{-3} M Ca^{2+} for more than 4 hours. This was the best protection that we have observed so far by any protease inhibitor or calcium chelator. These interesting observations prompted us to investigate whether bestatin could prevent or significantly delay cataract in rat lenses cultured in high glucose.

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Based on these observations, we propose the sequence of events that cause hyperglycemia-induced lens opacification to be enhancement of calcium influx, which in turn activates the FCGAP(s). The activated protease(s) cleaves cytoskeletal and membrane proteins that form the substratum for the elaboration of light-scattering centers and the development of cataract.

Acknowledgments

The authors thank Elias B. Jackson for technical assistance.

References