Calcium-Activated Proteolysis in the Lens Nucleus during Selenite Cataractogenesis

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A single injection of 20 μmol sodium selenite/kg body weight in 10-day-old rats caused severe nuclear cataract within 4 days. By 4 days postselenite injection, nuclear calcium levels increased from 0.4 to 6.8 mmol/kg lens dry weight. The purpose of these experiments was to determine if this calcium increase was associated with proteolysis specifically in the lens nuclear region. Sodium dodecyl sulfate polyacrylamide electrophoresis of lens nuclear proteins following selenite injection showed: loss of 30, 27, and 26 K molecular weight polypeptides in the soluble fraction, loss of 83, 52, 30, 27, and 26 K polypeptides in the insoluble fraction, and loss of the major 26 K membrane protein. Gel chromatography of nuclear soluble proteins indicated a decrease in βH and βL crystallins following selenite injection. Two-hour in vitro incubation of nuclear lens homogenates with calcium duplicated many of the proteolytic changes occurring in lenses in vivo following selenite injection. Calcium induced proteolysis in vitro was inhibited by EGTA, leupeptin, and iodoacetate but was not inhibited by phenylmethylsulfonyl fluoride. These properties are similar to calcium activated protease (CAP) from other tissues. Activation of CAP, and subsequent degradation of nuclear proteins, may be causes of selenite cataract. Invest Ophthalmol Vis Sci 25:1275-1283, 1984

Materials and Methods

Isolation of Lens Protein

Ten-day-old Sprague-Dawley suckling rat pups (Simonsen Laboratories; Gilroy, CA) were given a single 0.05-ml subcutaneous injection of a solution containing 20 μmol sodium selenite/kg body weight. Pups were killed by decapitation at various times after injection, and the lenses were removed from the enucleated eyes by a posterior approach. The capsules were removed and the lenses dissected with tweezers under a microscope into cortical and nuclear regions in buffer 1 containing 20 mM sodium phosphate (pH = 7.3), 20 mM EDTA, and 10 mM iodoacetate at approximately 20°C. Development of cold cataract facilitated this separation, since cold cataract was localized in the nuclear region. The dissection also was aided by a tendency of the cortex to separate from the nucleus along an interface, which appeared when the cortex was removed. The cortex and nuclear regions, each consisting of approximately ½ the total lens protein, were homogenized separately in buffer 1 at a ratio of approximately ½ the total lens protein, were homogenized separately in buffer 2, containing 20 mM sodium phosphate (pH = 7.3), 20 mM EDTA, and 10 mM iodoacetate at approximately 20°C. Development of cold cataract facilitated this separation, since cold cataract was localized in the nuclear region. The dissection also was aided by a tendency of the cortex to separate from the nucleus along an interface, which appeared when the cortex was removed. The cortex and nuclear regions, each consisting of approximately ½ the total lens protein, were homogenized separately in buffer 1 at a ratio of approximately ½ the total lens protein. The insoluble pellet was washed once and dissolved in buffer 2, containing 20 mM sodium phosphate (pH = 7.3), 20 mM EDTA, 2 mM iodoacetate, and 2% sodium dodecyl sulfate (SDS). During the isolation of the intrinsic membrane proteins, the insoluble pellet was not dissolved in buffer.
2, but instead resuspended in buffer 3, containing 50 mM Tris (pH = 7.4), 5 mM EDTA, and 10 mM 2-mercaptoethanol, and the method of Russell et al. was used to obtain the intrinsic membrane proteins.

**Determination of Lens Calcium**

Lens capsules were removed in 1 mM EGTA/0.9% saline solution and the dissection of cortex and nuclear regions carried out in 0.9% saline. The dissected regions from four lenses were placed in tared acid washed glass vials, dried at 60°C for 12 hr and weighed to ±0.01 mg. The lens regions were digested as previously described, dissolved in 1.0 ml 0.2% lanthanum chloride/1% HCl solution, and analyzed for total calcium content by atomic absorption spectroscopy.

**In Vitro Incubation of Homogenates of Normal Lenses with Calcium**

Lens dissection and homogenations were carried out in buffer 4, containing 20 mM Tris (pH = 7.4), 1.0 mM EGTA, 2.0 mM 2-mercaptoethanol, and 0.01% sodium azide. The protein concentrations were adjusted to 15 mg/ml in the homogenates and the incubation was initiated by addition of calcium chloride in 2.0 or 3.0 mM excess over EGTA. In those samples incubated with protease inhibitors, the inhibitor was added 30 min prior to incubation. After 1 or 2 hr at 37°C, the incubation was terminated by the addition of EGTA at a final 5.0 mM concentration. The incubated homogenates then were centrifuged at 10,000 g for 15 min at 4°C to separate the soluble and insoluble fractions and the insoluble pellet washed once and dissolved in buffer 2, or resuspended in buffer 3 to isolate the intrinsic membrane protein as before. Synthetic leupeptin and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma Chemical Company (St. Louis, MO).

**Column Chromatography and Electrophoresis**

Lens soluble, insoluble, and intrinsic membrane proteins were separated on Laemmli 12% SDS-polyacrylamide gels following reduction using 2-mercaptoethanol, and stained with 0.1% Coomassie Blue. Apparent molecular weights were determined by comparison with a mixture of 7 proteins of known molecular weights (Dalton Mark VII-L, Sigma Chemical Co.; St. Louis, MO). Soluble lens crystallins were isolated using a 2.5 cm × 96 cm column of Sephadex G-200 (Pharmacia Fine Chemicals; Piscataway, NJ). After chromatography, the various crystallins were concentrated by ultrafiltration using a YM5 filter (Amicon Corporation; Lexington, MA). The α-cristallin fraction appearing at the void volume of Sephadex G-200 was applied to a 2.5 cm × 96 cm column of agarose A5M (Bio-Rad Laboratories; Richmond, CA) to separate high molecular weight protein from α-cristallin. The elution buffer of both columns used a flow rate of 15 ml/hr and contained 50 mM Tris (pH = 7.4), 100 mM sodium chloride, and 0.02% sodium azide. Protein was assayed by the method of

![Graph](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933343/ on 04/29/2017)
Lowry et al., using bovine serum albumin as a standard. Rats in this study were treated in accordance with the ARVO Resolution on the Use of Animals in Research.

Results

Calcium Concentrations in Lens Cortex and Nucleus

Nuclear cataract appeared in the selenite treated rats on approximately 4 days following injection. In the lens cortex, selenite injection caused calcium to increase from 0.34 to 1.71 mmol calcium/kg lens dry weight by 4 days postinjection (Fig. 1A). The lens nucleus (Fig. 1B) was, however, the major site of calcium increase since by 4 days post-selenite injection, the total calcium concentration increased from 0.40 to 6.81 mmol/kg lens dry weight. Following formation of the nuclear cataract, the lens calcium levels began to decrease.

Lens Protein Time Study

Figure 2A shows the results of SDS polyacrylamide electrophoresis (PAGE) of the total soluble nuclear protein from lenses of control and selenite treated pups. From 10 to 21 days of age, nuclear proteins from control animals (lanes 1, 2, and 3) showed few changes, except for a decrease in the apparent molecular weight $M_r = 30$ K band. However, by 2 days postinjection in the selenite treated animals (lane 5), a major decrease in $30$ K and $26$ K bands was noted. An additional loss of the $27$ K band occurred by 4 days postinjection (lane 7). The intensity of bands in the $25$ K region also increased following selenite injection, and then decreased with time, and were

![Fig. 2. SDS-PAGE of rat lens nuclear soluble (A) and insoluble (B) proteins. Lanes 1, 2, and 3 were from control rats 10, 17, and 21 days of age, respectively. Lanes 4, 5, 6, 7, 8, and 9 were from selenite-injected rats injected on day 10 of age and killed on 1, 2, 3, 4, 7, and 11 days postinjection, respectively. Lane 10 contains 7 molecular weight markers with corresponding molecular weights indicated to the right. Numbers to the left show positions and apparent molecular weights ($M_r$) of polypeptides referred to in text.](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933343/ on 04/29/2017)
Table 1. Localization of lens insoluble protein %

<table>
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<th>Region</th>
<th>Control</th>
<th>Cataractous*</th>
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<tbody>
<tr>
<td>Cortex</td>
<td>1.8 ± 1.1†</td>
<td>3.6 ± 1.3</td>
</tr>
<tr>
<td>Nucleus</td>
<td>2.8 ± 0.4</td>
<td>17.8 ± 12.8‡</td>
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* Four days after selenite injection on day 10.
† Mean ± standard deviation (n = 5).
‡ Mean significantly different from controls at P < 0.05.

nearly absent by 11 days postinjection (lane 9). Over this time period, new low molecular weight bands also increased in the 18 K region. In contrast, the soluble protein from cortex showed no changes during selenite cataractogenesis when analyzed by SDS-PAGE (data not shown).

The nuclear insoluble protein from control lenses (Fig. 2B, lanes 1, 2, and 3) underwent age related changes from 10 to 21 days. These changes included a decrease in polypeptides above 25 K and an increase in polypeptides in the 21–25 K range. The band at 20 K also decreased in controls during aging. The nuclear insoluble protein from selenite-injected rats underwent similar changes as the control lenses, except the changes were more pronounced and occurred more rapidly. By 2 days postinjection (lane 5), a loss of 83, 52, 30, and 26 K polypeptides occurred. By 3 days postselenite injection (lane 6), the 27 and 20 K polypeptides were also lost. The age related increase in the 21–25 K bands occurring in the control lenses, proceeded more rapidly and to a greater extent in the selenite treated rats. By 4 days postinjection, insoluble protein was limited almost entirely to the 21–25 K bands. These changes in insoluble protein were localized in the nucleus since selenite injection did not cause significant changes in the polypeptides in insoluble protein from cortex (data not shown). Furthermore, a fivefold increase in the proportion of insoluble protein was found to be localized in the nucleus and not the cortex (Table 1).

Isolation of Lens Crystallins

Major loss of the β1, and a partial loss of the βL-crystallin fraction, occurred in the nucleus from cataractous selenite lenses 4 days postinjection (Fig. 3A). No changes were observed in the relative proportions of the crystallins from the cortex of cataractous selenite lenses. Thus, the loss of β-crystallins was localized in the lens nucleus, which contained the opacity.

The α-crystallin fractions were concentrated and applied to an agarose A5-M column (Fig. 3B) to isolate any high molecular weight species, which eluted together with α-crystallin on the Sephadex G-200 column. The amount of protein eluting at the void volume was very minimal in both the control

![Fig. 3. Gel filtration profiles of lens nuclear soluble protein from 14-day-old control rats (—), and 14-day-old, 4-day postselenite injection rats (— — ). A, Sephadex G-200 profile of 60 mg of control and selenite-treated rat soluble nuclear protein. The % of the total area under the peaks of control nuclear soluble protein was: α-15.2, βH-13.2, βL-17.0, and γ-54.7. The % total area under the peaks of selenite cataractous nuclear soluble protein was: α-18.9, βH-4.7, βL-11.7, and γ-64.9. B, A5M profile of 3.5 mg of control and selenite-treated rat alpha crystallin fraction pooled from part A, V0 indicates position of void volume. Vertical bars in A and B indicate which fractions were pooled for subsequent SDS-PAGE and integration of peak areas.](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933343/ on 04/29/2017)
and cataractous selenite lens nucleus, indicating that high molecular weight aggregates were not a significant proportion of the nuclear soluble protein in selenite cataract. The α-crystallin isolated from cortical regions also contained no significant amount of high molecular weight protein (data not shown). In addition to the α-crystallin peak, another peak eluted at approximately fraction number 85 in equal amounts in both control and cataractous lenses. This protein coeluted with α-crystallin during the G-200 separation, and its identity was unknown.

The various crystallins from control and cataractous lenses were further analyzed by SDS-PAGE (Fig. 4). In selenite lenses, dramatic changes took place in the α (lane 5), βH (lane 6), and βL (lane 7) fractions, while the γ (lane 8) fraction appeared to be unaffected. The α-crystallin fraction from the cataractous selenite nucleus contained three new polypeptides below 20 K. New 25 and 23 K bands were also present in α-crystallin, which corresponded to the size of several βH and βL polypeptides. The appearance of these putative β-polypeptides in α-crystallin was not due to poor resolution during column chromatography steps, since the α-crystallin was passed through 2 columns with different exclusion limits (Fig. 3) before SDS-PAGE. It is worth noting that these changes in α-crystallin polypeptides occurred without a major change in the concentration or molecular weight of α-crystallin (Fig. 3).

Figure 4, lanes 6 and 7 also show that changes in the 30, 27, 26, and 25 K polypeptides seen in cataractous total soluble proteins (Fig. 2A) were caused by changes in βH and βL-crystallin aggregates. The reduction in 30, 27, and 26 K polypeptides during selenite cataractogenesis can be explained both by the relative decrease of these individual polypeptides, and by the overall decrease in the concentrations of βH and βL-aggregates (Fig. 3A). A major proportion of the βH and βL-aggregates from the selenite cataractous nucleus was composed of a new 25 K polypeptide.

Unlike nuclear crystallins, isolated cortical crystallins showed no significant changes in polypeptide composition following selenite cataract formation (data not shown).

Cortical and nuclear soluble crystallins and insoluble protein from control and selenite cataractous rat lenses, at 4 days postselenite injection, also were separated by SDS-PAGE without prior reduction with 2-mercaptoethanol (data not shown). Although there was an appearance of a putative 40 K dimer of α-crystallin in the cortex of cataractous lenses, these gels did not reveal any increase in disulfide-linked high molecular weight aggregates. This finding was also in agreement with the chromatography data shown in Figure 3, where no high molecular weight aggregates were found following selenite cataract formation.

**Intrinsic Membrane Proteins during Selenite Cataractogenesis**

Intrinsic membrane proteins from the lens nucleus were isolated and analyzed by SDS-PAGE (Fig. 5). In control rats, the major lens intrinsic membrane protein (MP 26) appeared at 26 K (lane 1). Heating at 100°C for 5 min caused the characteristic coagulation of MP 26, and resulted in the loss of MP 26, and the appearance of protein that would not enter the gel (lane 2). Two days postselenite injection, a decrease of MP 26 and an increase in a smaller polypeptide at approximately 25 K occurred (lane 3).
Fig. 5. SDS-PAGE of rat lens nuclear intrinsic membrane proteins. Lane 1, 14-day-old control rats; lane 2, same as 1, except heated at 100°C for 5 min; lane 3, 12-day-old, 2-day postselenite injection rats; lane 4, same as 3 except heated; lane 5, 14-day-old, 4-day postselenite injection rats; lane 6, same as 5 except heated. Lane 7 contains molecular weight markers.

Both the remaining MP 26 and the 25 K polypeptide were coagulated by heating at 100°C (lane 4). By 4 days postinjection, MP 26 was decreased dramatically and 24 and 22 K polypeptides appeared (lane 5), which also were coagulated by heating (lane 6). Similar analysis of intrinsic membrane proteins from lens cortex following selenite injection indicated no loss of cortex MP 26 (data not shown).

Incubation of Normal Rat Lens Homogenates with Calcium

Incubation of cortical and nuclear homogenates of normal lenses from 10-day-old rat pups with 3.0 mM calcium produced many of the polypeptide changes observed after selenite administration in vivo. That is, in the soluble proteins, calcium incubation caused a decrease in the 30 K polypeptide and a disappearance of the 26 K polypeptide (Fig. 6A, lane 2). New bands also appeared in the calcium incubated sample at 25, 19, and 18 K. EGTA prevented these changes (lane 1). Calcium also activated proteolysis during incubation at 4°C, except the new bands at 19 and 18 K did not appear (lane 3). Heating the nuclear homogenates to 70°C for 1 min prior to addition of calcium inhibited the calcium-induced changes (lane 4). Addition of PMSF (a serine protease inhibitor) at 1 mM concentration did not inhibit the calcium-induced changes (lane 5), while addition of 0.2 mM leupeptin (an inhibitor of calcium activated protease in other tissues) did (lane 6). Five mM iodoacetate was also effective in inhibiting the calcium-induced changes (lane 7). The activation was specific for calcium, since addition of 3.0 mM magnesium did not activate the proteolysis (lane 8).

The lens nuclear insoluble protein also was isolated from the homogenates incubated with calcium (Fig. 6B). Calcium incubation caused the decrease or disappearance of bands at 83, 52, 30, and 26 K and the appearance of a new band at 19 K (lane 2). As was found in the soluble protein, 4°C caused little inhibition of the calcium affect (lane 3). However, unlike the results with the soluble protein, heating the homogenates to 70°C prior to incubation did not completely abolish the calcium induced loss of insoluble polypeptide bands (lane 4). Heating to 70°C also caused insolubilization of various crystallins in the homogenates, which likely accounts for the increase in the 20 K band in the insoluble fraction. As was found in the soluble lens fraction, PMSF did not inhibit the polypeptide loss (lane 5), while leupeptin and iodoacetate inhibited polypeptide loss (lanes 6 and 7). Also, substitution of magnesium for calcium did not induce polypeptide changes (lane 8).

When lens cortical homogenates were incubated with 3.0 mM calcium, similar results were obtained, indicating that the lens cortex as well as the nucleus exhibits calcium activated proteolysis (data not shown).

Intrinsic Membrane Proteins Following Incubation of Lens Homogenates with Calcium

Homogenates of normal 10-day-old rat lens nucleus and cortex were incubated with 2.0 mM calcium, and the intrinsic membrane proteins isolated (Fig. 7). Calcium incubation caused a new band to appear at approximately 25 K (lane 3), which like MP 26 was coagulated by heating (lane 4). Similar results were obtained in cortical homogenates (lanes 5–8). The appearance of the 25 K polypeptide in the intrinsic membrane fraction caused by calcium also could be inhibited by 0.2 mM leupeptin (data not shown).
Discussion

The major findings of this study were that the selenite cataract was associated with proteolysis occurring specifically in the lens nucleus, and that this proteolysis appeared to be activated by increased lenticular calcium.

Proteolysis during selenite cataractogenesis was indicated because a rapid loss of numerous polypeptides in the lens nucleus was observed. Polypeptides were lost in all three soluble, insoluble, and intrinsic membrane fractions. In the insoluble protein of cataractous lenses, a rapid loss of all polypeptides above 25 K occurred, so that by 3 days postinjection, only 25-21 K polypeptides remained. In the nuclear soluble proteins, 30, 27, and 26 K β-crystallin polypeptides decreased. The nuclear intrinsic membrane protein MP 26 also was reduced drastically by 4 days postinjection. It should be noted that losses of higher molecular weight polypeptides also occurred in the insoluble nuclear fraction of normal control lenses during maturation from 10 to 21 days of age. Proteolysis in the nuclear insoluble protein, which required 11 days in control rats took place in only 2 days following selenite injection. The polypeptide pattern in the nuclear insoluble protein from 21-day-old control rats, and 12-day-old, 2 days postinjection rats was remarkably similar. Thus, selenite appeared to accelerate maturational proteolysis of the insoluble protein in the rat lens. We propose that the protease responsible for the maturational changes was also responsible for the proteolysis occurring in the nuclear soluble and membrane protein fractions following selenite injection.
Several pieces of evidence suggested that calcium activated the proteolysis observed in the selenite cataract. First, increased lens calcium and proteolytic changes occurred simultaneously during selenite cataractogenesis. At 1 day postinjection, only minor elevation of lens nuclear calcium and no nuclear proteolysis were observed. By 2 days postinjection, both calcium elevation and proteolysis occurred in the nucleus. This proteolysis continued during the time of calcium elevation, and essentially ended when the lens calcium levels began to decrease.

A second piece of evidence indicated that calcium-activated proteolysis may be involved in selenite cataractogenesis. This was the finding that in vitro incubation of normal lens homogenates with calcium reproduced some of the proteolytic changes caused by selenite in vivo. Changes that could be duplicated were: (1) degradation of soluble 30 and 26 K polypeptides and increase in 25 K polypeptide; (2) loss of insoluble 83, 52, 30, and 26 K polypeptides; and (3) partial degradation of MP 26. Calcium incubation did not cause as extensive degradation of lens polypeptides as observed following selenite injection. However, those polypeptides first decreased after selenite injection were also the polypeptides decreased by calcium incubation. Russell recently described remarkably similar soluble protein and membrane changes when rat lens homogenates were incubated with calcium. Our findings also are supported by Hess et al. Using the whole lens, these workers found a loss of insoluble polypeptides following selenite injection, which could be duplicated by incubation of lens homogenates with calcium. Several workers using bovine lenses also have demonstrated that calcium-activated proteolysis caused the degradation of the 57–58 K protein vimentin. In the present study we found a 57 K polypeptide in the cortex insoluble protein (data not shown) that was absent from the nucleus of both control and cataractous lenses. However, insoluble polypeptides with molecular weights both above and below vimentin were lost in the nucleus following selenite injection. Paradoxically, it also should be noted that incubation of cortex homogenates with 3.0 mM calcium activated proteolysis in vitro, but proteolysis was not observed in the cortex in vivo after selenite injection. We postulate that the calcium-activated proteolysis observed in cortical homogenates did not occur in vivo because calcium levels in the cortex of the selenite injected animals were not elevated sufficiently.

The third piece of evidence that supported the involvement of calcium-activated proteolysis in selenite cataract was the finding that the protease(s) responsible for the proteolysis in lens homogenates incubated with calcium exhibited properties similar to those of calcium activated protease. This enzyme has been extensively characterized in other tissues and recently purified in bovine and rabbit lenses. Some of the similar properties were: activation by mM calcium; inhibition by EGTA, leupeptin, and iodoacetate; lack of inhibition by PMSF; and little or no activation by magnesium.

One of the consequences of the extensive proteolysis observed in selenite-treated lenses may be the insolubilization of lens proteins. The nuclear region of the selenite cataractous lens showed major increases in insoluble protein. High molecular weight aggregates, that scatter light are thought to be a cause of lens opacity. Since no high molecular weight aggregates were found in the soluble nuclear proteins 4 days following selenite injection, the increased nuclear insoluble protein fraction may be responsible for the opacity. Nonreducing SDS-PAGE indicated that this insoluble protein did not contain intermolecular disulfide linkages. Thus, if the insoluble protein in

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selenium cataract does exist in light-scattering aggregates, its aggregation may be hydrophobic in nature instead of covalent.

Insoluble protein in selenium cataractous lenses reacted with antibody to \( \alpha \)-crystallin.\(^*\) However, our data suggested that the majority of the insoluble protein in the selenium cataract nucleus may be derived from \( \beta_H \) and \( \beta_L \)-crystallin fractions. During selenium cataractogenesis, there were major losses of \( \beta_H \) and \( \beta_L \)-crystallins. Loss of \( \beta_H \)-crystallin has been reported following posttranslational modifications of proteins in the cataractous nucleus supports this conclusion, however this cannot be proven until the insoluble protein and \( \beta \)-crystallin polypeptides are compared by peptide mapping or immunologic tests.

An important consequence of proteolysis in selenium cataract was the loss of membrane protein. By 2 days postselenite injection, the lens nuclear MP 26 protein began to decrease and was replaced by new 24 and 22 K polypeptides at day 4 postinjection. The loss of MP 26 is significant because it is the major protein associated with lens gap junctions.\(^2\)\(^6\) Its degradation in the selenite cataractous lens may cause isolation of the nuclear region and contribute to the opacity.

Thus, the present investigation has led to the hypothesis that elevated calcium may initiate a series of extensive proteolytic changes following selenite treatment. Proteolysis of nuclear \( \beta \)-crystallins may lead to production of insoluble aggregates, which scatter light. Proteolysis of membranes also may contribute to cataract formation. We suggest that calcium-activated protease also may play a role in other cataracts containing elevated calcium.

Key words: selenium cataract, rat lens, calcium-activated protease, proteolysis, lens proteins

References

\(^*\) J. L. Hess, Department of Biochemistry and Nutrition, Virginia Tech., Blacksburg, Virginia, personal communication.

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