Changes in the Distribution of Lens Calcium during Development of X-ray Cataract

Kenneth R. Hightower, Frank J. Giblin, and V. N. Reddy

The present study was designed to examine the possible role of calcium in the opacification of x-ray-induced cataract in rabbit. The results demonstrate that the concentration of calcium in x-rayed lenses, just prior to lens hydration (7.5 weeks postirradiation), was twice that present in contralateral control lenses. At this stage of immature cataract, the lens nucleus remained transparent and maintained a normal level of calcium, but the lens cortex, containing regions of subcapsular opacification, accumulated a level of calcium that was twice that of the control. In the completely opaque mature cataract, (8–9 weeks post-x-ray), both the cortex and nucleus had gained significant amounts of calcium. As the concentration of total calcium increased in the immature x-ray cataract, the amount of the cation bound to membranes and insoluble proteins of the cytosol also increased comparably. However, the relative proportion of calcium in the various fractions remained unaltered in the immature cataract; in both control lenses and immature cataracts, 20% of the total calcium remained in the membrane pellet and 70% was located in the soluble protein fraction. Only in the mature stage of cataract was a shift in the distribution of calcium apparent, as the proportion of calcium in the soluble protein fraction increased to 90%. Although only 7% of the total calcium in a mature cataract was bound to membrane, the amount represented a fivefold increase over the control. The results of this study demonstrate that an elevation in lens calcium accompanies the opacification process in x-ray cataract.

The work also suggests that changes in calcium levels are not likely to result from inactivation of Ca-ATPase. Invest Opthalmol Vis Sci 24:1188–1193, 1983
tured stages of development. In addition, the potentially cytotoxic effects of x-irradiation on Ca-ATPase have been examined.

Materials and Methods

X-ray cataracts were produced in one eye of New Zealand White rabbits by irradiating the eye with a single 2000 rad (85 kVp, 5 mA) dose when the animals were 5 to 6 weeks of age. Details of the procedure, including a description of the shielding placed around the x-irradiated eye and the method employed for estimating the amount of radiation reaching the contralateral control eye (less than 50 rads) have been described earlier. Information pertaining to wet and dry weights in x-irradiated lenses and the state of hydration in x-ray cataracts has also been provided in a previous publication.

Various protein fractions were separated from control and x-irradiated lenses by differential centrifugation: 1000 g pellet (membrane), 10,000 g supernatant (soluble), 10,000 g pellet (insoluble). Lenses were blotted on filter paper moistened with 20 mM EGTA and homogenized in 150 mM histidine buffer, pH 7.4, as described in detail elsewhere. A small aliquot (0.1 ml) was removed for protein determination (Biorad assay) and calcium analysis. The remaining homogenate was centrifuged at 4°C for 20 min at 1000 g. The 1000-g pellet has been referred to as the membrane fraction in this investigation, since previous results have indicated that it contains approximately 80% of the total Na/K-ATPase activity present in the lens.

Following centrifugation, the 1000 g supernatant (Sn) was decanted and 0.1 ml was assayed for protein and calcium. The remaining Sn was centrifuged immediately at 10,000 g for 20 min. The 10,000-g Sn was decanted, analyzed for protein and calcium, and 0.5 ml dialyzed against calcium-free histidine buffer (50 ml) for 56 hrs with two replacements of buffer at 24 and 48 hrs. The concentration of calcium in the final dialysis buffer was less than 10^-6 M. The 10,000-g pellet was referred to as the water insoluble protein fraction in this study.

Analyses of calcium were carried out in solutions of 10% trichloroacetic acid containing 0.2% lanthanum chloride to minimize phosphate interference, using a Model 272 Perkin-Elmer atomic absorption spectrophotometer.

Calcium-stimulated, magnesium-dependent ATPase (Ca-ATPase) activity was measured in x-irradiated rabbit lenses according to techniques employed in earlier studies. A typical incubation medium consisted of the homogenate, histidine-EGTA buffer, and various electrolytes. The concentration of calcium used in this study was 10^-3 M, which we found to produce the maximum level of enzyme activity. Since very low levels of calcium being measured, all precautions were taken to ensure that contamination was minimized, including the routine washing of glassware with EGTA.

The division of lenses into nucleus and cortex was accomplished in the following manner. Using corneal scissors, a shallow incision was made along the lens equator and was followed by cross-cross incisions across the posterior surface of the lens intersecting at the pole. Placing curved forceps at the point of intersection, slight outward and downward pressure was applied to separate cortical fibers from the denser nucleus. Equivalent weights of lens nuclei were obtained by weighing the nucleus and removing adhering cortical fibers until the desired weight of 20–30 mg was obtained. This procedure yielded approximately 90% of the entire cortex and nearly 100% of the nucleus of normal and immature cataractous lenses. The mature cataract was treated differently because of its severe intumescence and tendency to rupture. Lenses were handled on EGTA-rinsed parafilm (2 × 2 cm sections) in order to collect the partially liquified cortex. After rupturing the lens with a forceps, the well-defined nucleus was merely lifted out of the ruptured lens mass. The cortex and cortical fluid were then rinsed from the parafilm into a homogenizing tube with between 80% and 90% recovery of the total weight. Lens tissues were weighed on parafilm and dried in tubes at 100°C for 48 hrs and weighed again before extracting in 1–2 ml of 10% trichloroacetic acid for calcium analysis. Vortexing and homogenizing the mixture with a pestle facilitated complete breakdown of the dried lens material.

Results

Prior to mature cataract development, opacification is localized mainly in the posterior subcapsular region of the lens and becomes progressively more dense (Figs. 1A, B), as evident under microscopic observation. At 3 weeks after x-irradiation opacification was limited to the superficial cortical fibers of the posterior surface, which was characterized by small hazy areas involving only 5–20% of the surface area of the lens (Fig. 1A). A steady increase in posterior subcapsular opacities occurred up to 7 weeks after x-irradiation at which point 75–100% of the surface area was opaque (Fig. 1B). In the present study, the term "immature cataract" is used to describe x-irradiated lenses isolated between 6 and 7.5 weeks after irradiation, when lens hydration appears normal.

Concentrations of calcium were measured in x-irradiated and control lenses and values are expressed...
Fig. 1. A, Photograph of rabbit lenses 3 weeks after x-irradiation—control lens on the left, x-irradiated lens on the right; B, photograph of rabbit lenses 7.5 wks after x-irradiation—control lens on the left, x-irradiated lens on the right; photographed with Zeiss OPMI 1 operation microscope.

in terms of whole lens and dry wt. (Fig. 2). The calcium content of a 5-week-old control lens was observed to increase from 1500 ng to 2000 ng during

Table 1. Dry weights and calcium for lens cortex and nucleus in control and x-irradiated rabbit lenses.

<table>
<thead>
<tr>
<th>Lens region</th>
<th>Control</th>
<th>Immature*</th>
<th>Mature†</th>
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<tbody>
<tr>
<td>Cortex</td>
<td>80 ± 8</td>
<td>70 ± 6</td>
<td>20 ± 6</td>
</tr>
<tr>
<td>Nucleus</td>
<td>25 ± 4</td>
<td>20 ± 3</td>
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<td>Cortex</td>
<td>22.8 ± 1.5</td>
<td>41.5 ± 7</td>
<td>1391 ± 291</td>
</tr>
<tr>
<td>Nucleus</td>
<td>21.1 ± 1.1</td>
<td>21.6 ± 1.7</td>
<td>945 ± 185</td>
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Table 2. Dry weights and calcium for lens cortex and nucleus in control and x-irradiated rabbit lenses.

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an 8-week growth period. However, the concentration of calcium expressed on a dry wt. basis remained constant at 19 ± 2 ng/mg dry wt. during this period. The first evidence for a change in the level of calcium in the x-irradiated lenses was at 6 weeks postirradiation when a 50% increase in the concentration of the cation was observed. No significant change in calcium level was detected at 3 weeks after x-ray. At 7.5 wks postirradiation, 1 week prior to hydration of the lens and formation of mature cataract, calcium was elevated by 100% on a dry wt. basis and 65% on a whole lens basis. In the mature cataract (8–9 weeks postx-ray) levels of calcium were approximately 18- to 75-fold higher than control lenses (whole lens and dry wt. basis, respectively). In terms of lens water, which was also greater by 50–70 μl per lens at this stage, the calcium concentration was approximately 3 mM, 15-fold higher than the control lenses (data not shown).

The next part of the investigation concerned the comparison of calcium levels in transparent and opaque regions of the lens. Observations were limited to the nucleus and cortex of immature and mature cataracts since significant changes in the calcium concentration were observed in these regions at the two stages. In view of the observed differences in lens growth between control and x-irradiated lenses, dry weights of the nucleus and cortex were determined (Table 1). The dry weight of the immature cataract was approximately 90% of the control in both the cortex and nucleus. In the mature cataract the cortical mass declined to 25% of the control and the nuclear mass decreased to 40%. Table 1 also shows calcium levels present in the nucleus and cortex of the two
types of lenses. While calcium was distributed homogeneously in the control lens, the concentration of calcium in the cortex of the immature cataract, which contained posterior subcapsular opacities, was nearly 100% higher than that in either the control cortex or transparent nucleus of the cataract. The nucleus of the mature cataract, which was densely opaque at this stage, contained calcium levels that were nearly 50-fold higher compared to controls.

The distribution of calcium in control and x-rayed lenses was also investigated with respect to different protein fractions obtained from centrifugation of lens homogenates at 1,000 g and 10,000 g. Figure 3 shows clearly that the calcium content, on a ng calcium per mg dry wt. basis, in each fraction increased progressively during cataract development. The immature cataract was characterized by a small increase in "membrane" bound calcium (30%) and a fourfold increase in protein bound (10,000 g pellet) calcium relative to the control. Calcium levels measured in the 10,000 g Sn containing soluble proteins did not change appreciably. In the mature cataract a fivefold increase in calcium was observed in the "membrane" pellet (from 40 to 198 ng/mg dry wt.) and a 50-fold increase was found in the soluble protein fraction. Only in the mature stage did a portion of the calcium present in the soluble protein fraction remain undialyzable, although the amount remaining with the protein was only 1% (264 ± 42 ng calcium compared to 24,850 ng) of that initially present.

To determine whether a redistribution of calcium in addition to an increase in the level of the cation occurred during cataract development, the data were also expressed as total calcium per protein fraction (Table 2). In the control lens, the proportion of "bound" calcium (281 ng and 30 ng) is 30%, with the remaining 70% confined to the supernatant (1260 ng). This distribution remained essentially unchanged in the immature cataract in spite of the relative increase in calcium in each fraction. In the mature cataract, however, only 6% of the total calcium present was recovered in the "membrane" fraction, while nearly 90% was accounted for in the supernatant fraction of soluble proteins (24,850 ng).

The possible origin of the observed increase in the calcium content occurring in the x-rayed lenses was investigated by measuring the activity of the transport enzyme Ca-ATPase in immature x-ray cataracts at a stage where calcium levels are elevated (Table 3). The results of four paired experiments show that the average enzyme activity in the x-irradiated lens was 98 ± 7 nmol Pi/hr/lens compared with 119 ± 14 nmol P/hr/lens in the contralateral lens, representing a decrease of 15% but not statistically different (P = 0.1). Employing paired analysis, the mean difference in ATPase activity was 19 ± 16 with a "t" value of 1.19, again indicating no significant difference. To determine if x-irradiation might have otherwise altered the enzyme, the effects on the activity by the enzyme inhibitor propranolol were also studied. As seen in Table 3, propranolol had a nearly identical inhibitory effect on the Ca-ATPase activity in control and x-rayed lenses.

**Discussion**

The observed increase in the concentration of calcium in the lens during development of x-ray cataract supports the premise that the elevation of calcium in the tissue may be involved in the process of lens opacification. If calcium plays a role in lens opacification, a correlation would be expected between the increase in the concentration of lens calcium and the degree of opacity. However, the results (Fig. 2) indicated that an increase in the calcium content does not occur at 3 weeks post-x-irradiation. A possible explanation for this finding is that the degree of

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**Table 2. Total calcium (ng)** in pellets and supernatants obtained from centrifugation at 1000 g and 10,000 g in control and x-irradiated rabbit lenses.

<table>
<thead>
<tr>
<th>Protein fraction</th>
<th>Control lens</th>
<th>Immature</th>
<th>Mature</th>
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<tbody>
<tr>
<td>Membrane</td>
<td>281 ± 22</td>
<td>410 ± 85</td>
<td>1,555 ± 246</td>
</tr>
<tr>
<td>Insoluble</td>
<td>30 ± 5</td>
<td>155 ± 30</td>
<td>765 ± 120</td>
</tr>
<tr>
<td>Soluble</td>
<td>1,260 ± 90</td>
<td>1,560 ± 201</td>
<td>24,850 ± 3999</td>
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* n = 4-6 experiments
Ca++-ATPase activities measured in normal and x-irradiated rabbit lenses (nmol Pi/hr/lens)

<table>
<thead>
<tr>
<th>Experiment*</th>
<th>Normal</th>
<th>Immature Cataract†</th>
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<tbody>
<tr>
<td>Control</td>
<td>119 ± 14</td>
<td>98 ± 7</td>
</tr>
<tr>
<td>Propranolol‡</td>
<td>76 ± 8</td>
<td>63 ± 2</td>
</tr>
<tr>
<td>% Inhibition</td>
<td>36 ± 7</td>
<td>35 ± 3</td>
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* Ca++-ATPase measured in four paired lenses
† 6 weeks postirradiation
‡ 10⁻⁴ M

Opacification at 3 weeks is extremely slight, both in terms of depth and extent of involvement (Fig. 1B) and any increase in calcium restricted to such a small fraction of the lens would not be readily detectable. After 6 and 7.5 weeks of cataract development, both the degree of opacification and the level of lens calcium were found to increase.

A further correlation between the increase in calcium concentration and opacification follows from analysis of the nucleus and cortex during a late stage of cataract development just prior to lens hydration. At this stage the calcium level in the transparent nucleus remained normal, while the concentration of the cation in the cortex, containing the opaque subcapsular region, increased twofold (Table 1). A similar correlation of calcium accumulation with opacification has been demonstrated in the valinomycin-induced cataract which also develops subcapsular opacities in the initial stages of development. Thus, following exposure of young rabbit lenses to the ionophore valinomycin, superficial opacities develop when the calcium concentration in the whole lens approaches 0.6 mM. This level of calcium is approximately equal to that measured in x-rayed lenses 6 weeks after irradiation, a point at which posterior opacities in the lenses are prominent. Worgul has reported that rabbits exposed to doses similar to those employed in this study develop a 3+ cataract at this point, based on a scheme which assigns a 4+ to a completely opaque lens.

The elevation of calcium in the x-rayed lenses did not appear to result from impaired calcium transport since the activity of the transport enzyme Ca-ATPase was not reduced significantly (Table 3). Moreover, the fact that propranolol inhibited Ca-ATPase equally in control and x-irradiated lenses further suggested that the enzyme was not functionally altered by x-irradiation. Since lenses in the immature stage are not hydrated, osmotic entry of calcium from the ocular fluids can also be discounted, although microdomains of lens swelling cannot be eliminated. The observed accumulation of calcium in the cortex of the immature x-ray cataract was probably due to an increase in membrane permeability. This conclusion is supported by results of previous studies that indicate that an increase in membrane permeability to rubidium is first observed at 4 weeks following irradiation and continues to increase until the mature cataract develops. An explanation for the relatively low levels of calcium found in the nucleus of the immature x-ray cataract is not evident from the present data. It is possible that once calcium enters the peripheral lens fibers it becomes bound to cortical cell membranes and proteins, as has been reported in calcium loaded rabbit lenses.

In view of the increase in the calcium content during the development of x-ray-induced cataract, it is conceivable that the increase in water insoluble proteins associated with this type of cataract is derived from calcium induced aggregation of water soluble proteins. It is well established that calcium is able to induce aggregation of isolated lens proteins. In the present study, the insoluble protein fractions (1000 g and 10,000 g pellets) were found to contain fivefold more calcium than identical fractions in control lenses, indicating that the process of insolubilization or aggregation may involve calcium binding. Further evidence for the association of calcium with insoluble proteins in the lens comes from studies of human cataracts in which calcium remained firmly bound to proteins despite repeated washing with solutions of EGTA.

It is important to recognize that the increase in bound calcium that occurs in the developing x-ray cataract does not constitute a redistribution of total internal calcium. That is, the excess of bound calcium is not derived from the pool of free calcium present in the immature cataract, since the dialyzable calcium present in the 10,000 g supernatant fraction represented 70% of the total calcium present in both control lenses and immature cataracts. In this sense, x-ray cataract is similar to the immature cortical and brunescent human cataracts recently examined, where it was also demonstrated that the opacities were not accompanied by a redistribution of calcium. The mature x-ray cataract is also similar to the mature human cataract in that the proportion of calcium increased significantly in the soluble protein fraction.

It is interesting to note that the activity of Ca-ATPase was not adversely affected in the immature x-ray cataract at a time when Na/K-ATPase activity has been shown to be inactivated by 40%. It is possible to resolve this situation if it is suggested that inactivation of Na/K-ATPase is not directly the result of x-irradiation; rather, inactivation is the result of calcium accumulation. This possibility was substantiated by the finding that culturing rabbit lenses in media with added calcium leads to an increase in...
membrane calcium and subsequent inhibition of the cation transport system and inactivation of Na/K-ATPase by 50% (20). In the present study, a twofold increase in bound calcium was found in the 1000 g and 10,000 g pellets of the immature x-ray cataract. It is, therefore, reasonable to expect that this increase in membrane bound calcium might explain some of the inactivation of Na/K-ATPase observed in the initial stage of cataract development.

In summary, the present study demonstrates that an increase in the concentration of calcium accompanies lens opacification and may be associated with the formation of HMW proteins. The observation that “membrane-bound” calcium increases in x-ray cataract suggests that the decrease in Na/K-ATPase detected in x-irradiated lenses may be due, in part, to calcium inactivation of the enzyme and thus may indirectly contribute to Na/K imbalances and eventual lens hydration and lens opacification. It remains to be ascertained, however, whether local areas of cell disruption and hydration might accompany early cataract development and contribute to the light scattering processes.

Key words: cataract, x-irradiation, calcium, lens, Ca-ATPase

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References