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# Involvement of calpain in diamide-induced cataract in cultured lenses

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Lenses cultured in diamide first developed outer cortical opacities followed by nuclear cataract. Lens hydration and total calcium were markedly increased by diamide. Proteolysis of crystallins were observed in nuclear cataract lenses. Calpain in the soluble fraction of lenses cultured with diamide was decreased, while calpain in the insoluble fraction was increased. Co-culture with E64d, an inhibitor of cysteine protease such as calpain, especially prevented nuclear opacities and proteolysis of crystallins, indicating that calpain was involved in cataract formation by diamide.

Calpain; E64d; Cataract; Diamide; Calcium; Lens culture

## 1. INTRODUCTION

Cataract is a phenomenon where the transparent lens in the eye becomes opaque. Several types of cataracts show increased calcium and proteolysis [1,2]. Calpains (EC 3.4.22.17) are calcium-activated, non-lysosomal cysteine proteases, and calpains are widely distributed in animal tissues [3]. Calpain II is a major protease in rat lens [4]. Calpain is believed to be involved in nuclear cataract formation in rat lens by causing limited proteolysis of the structural lens proteins (crystallins) [5,6]. Recent protein sequencing data indicate that almost all of the cleavage sites on partially proteolyzed $\beta$-crystallins from an in vivo model of cataract were calpain cleavage sites (L.L. David, unpublished). Furthermore, incubation of rat lens homogenates with activated calpain causes proteolysis, insolubilization of $\beta$-crystallins, and light scatter [7]. These effects can be prevented with E64, an inhibitor of cysteine proteases such as calpain, indicating an important role for calpain in nuclear cataract formation in rat models of cataract.

Oxidative stress is believed to be an early event in the development of some cataracts; and senile cataract, the most common type of cataract in humans shows oxidative damage [8]. Diamide (azodicarboxylic acid bis[dimethylamide]), a cataract inducer, specifically oxidizes sulfhydryl groups. Diamide caused changes in the $Na^+/K^+$ ratio, GSH levels, Na,K-ATPase activity, GSH reductase activity and the sulfhydryl level of the membranous protein in cataract [9]. However, the involvement of calpain in oxidation cataract has not been studied. Thus, the specific aim of the present experiment was to determine if calpain-induced proteolysis is part of the mechanism for diamide-induced cataracts.

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## 2. MATERIALS AND METHODS

Lenses from 4-week-old Sprague–Dawley rats were cultured at 37°C under 5% $CO_2$ in 4 ml of Eagle's minimum essential medium (MEM, Gibco) with 10% fetal calf serum (Gibco) (normal group). One mM diamide was present on day 1 only (diamide group), and 100 $\mu$M ethyl(+)-(2*S*,3*S*)-3-[(*S*)-3-methyl-1-(3-methylbutyl-carbamoyl) butylcarbamoyl]-2-oxiranecarboxylate (E64d, supplied by Taisho Pharmaceutical Co., Ltd., Japan), a membrane permeable inhibitor of cysteine proteases such as calpain [10], was present continuously (diamide + E64d group). Lenses were photographed under a dissecting microscope at several time points. After 4 days of culture, densities of cortical and nuclear opacities were quantitated using computerized image analysis (Image 1.31 software, Twilight Clone BBC, Silver Springs, MD). Lens hydration and calcium content in the lenses were measured as before [11]. Three to four lenses were pooled to obtain sufficient protein, and soluble and insoluble fraction were obtained by centrifugation [6]. Protein was determined by the dye binding reagent (Bio-Rad) using bovine serum albumin as standard. SDS-PAGE of the soluble and insoluble fractions were performed on discontinuous, 12% gels [12]. Immunoblots for calpain were performed on proteins electrotransferred to PVDF membrane (Millipore) using the method of Towbin et al. [13]. An affinity-purified polyclonal antibody against rat muscle calpain II [14] was used at 1:250 dilution, and immunoreactivity was visualized with alkaline phosphatase conjugated to anti-rabbit IgG secondary antibody and BCIP/NBT (Bio-Rad). The staining intensity of the immunoblots of calpain antigen were determined by densitometric image analysis. Statistical analysis of data was performed by Mann–Whitney U test.

## 3. RESULTS

By observation under the dissecting microscope, lenses cultured in MEM remained clear (Fig. 1). In contrast, culture in 1 mM diamide, present during the first day, caused opacities to appear in the peripheral and central regions (Fig. 1). The sequence of events in the development of the cataract was: equatorial subcap-

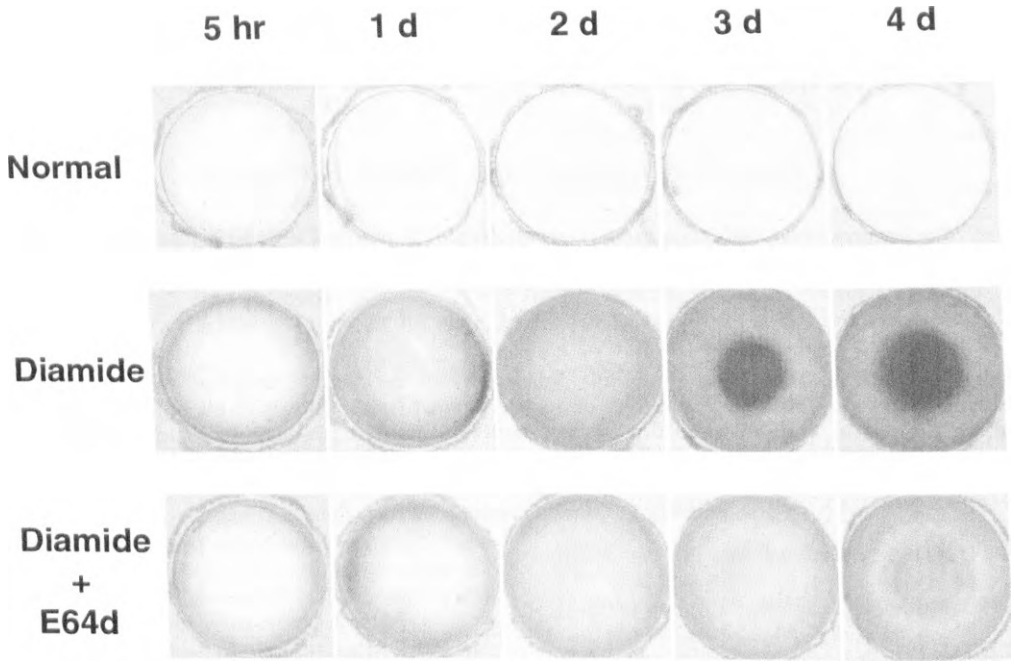


Fig. 1. Photomicroscopy of the development of diamide-induced cataracts and prevention by E64d. Darker areas are cataracts in these backlit lenses.

sular opacity by 5 h, generalized subcapsular opacity on day 1, cortical opacity on day 2, nuclear opacity on day 3; and dense nuclear opacity on day 4. These data suggested that the initiating cataractogenic event probably occurred in the epithelium or cortex, and this was followed by secondary cataract in the nucleus. E64d, present continuously during culture, reduced opacities caused by diamide in both the peripheral and central region of the lens (Fig. 1). Measurement of the mean density of both cortical and nuclear opacities in the lenses by image analysis confirmed that E64d was significantly effective in reducing both the cortical and nuclear opacities, although the effect of E64d was stronger against nuclear opacity (Fig. 2).

Diamide caused a decrease in the total protein and an increase in the insoluble protein in whole lens (Fig. 3). Analysis of the constituent polypeptides in the soluble fraction of normal cultured lenses by SDS-PAGE revealed a characteristic predominance of polypeptides belonging to lens crystallins in the molecular weight range of 19–31 kDa (lane N, Fig. 4). Previous studies showed that many of the soluble polypeptides in the approximate molecular weight ranges of 23–31 kDa are from $\beta$ crystallins, 19–20 kDa are from $\alpha$ crystallin, and the smear of polypeptides from 21–23 kDa are $\gamma$ crystallins. Diamide-induced cataract exhibited proteolysis of crystallins (lane D, Fig. 4). Determination of the molecular weight of each band by densitometric scanning of the gels confirmed proteolysis. The 31 kDa $\beta$

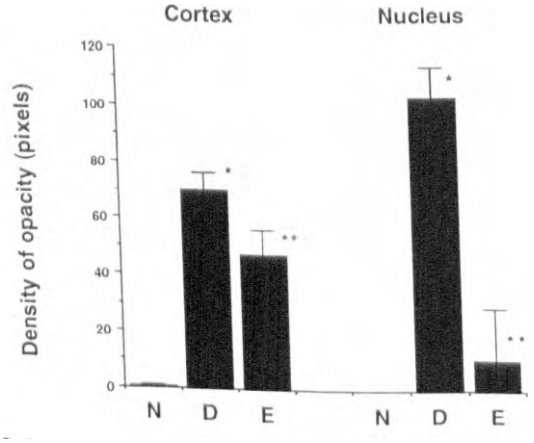


Fig. 2. Density of opacities in cortex and nucleus of lens after 4 days culture. Data are mean ± S.D. ($n$ = 9–10). * $P < 0.05$ relative to N and ** $P < 0.05$ relative to D. N = Normal; D = Diamide; E = Diamide + E64d.

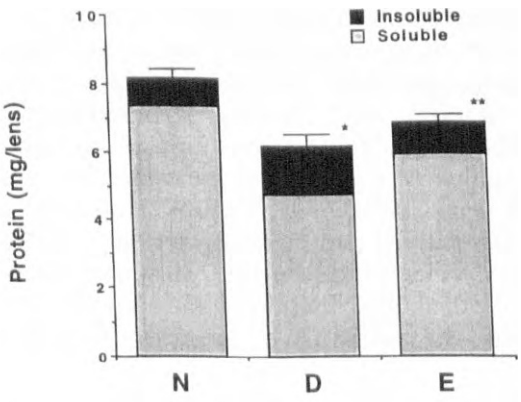


Fig. 3. Amounts of soluble and insoluble proteins in cultured lenses. Error bar shows standard deviation for total amount of protein ($n$ = 6). * $P < 0.05$ relative to N and ** $P < 0.05$ relative to D. N = Normal; D = Diamide; E = Diamide + E64d.

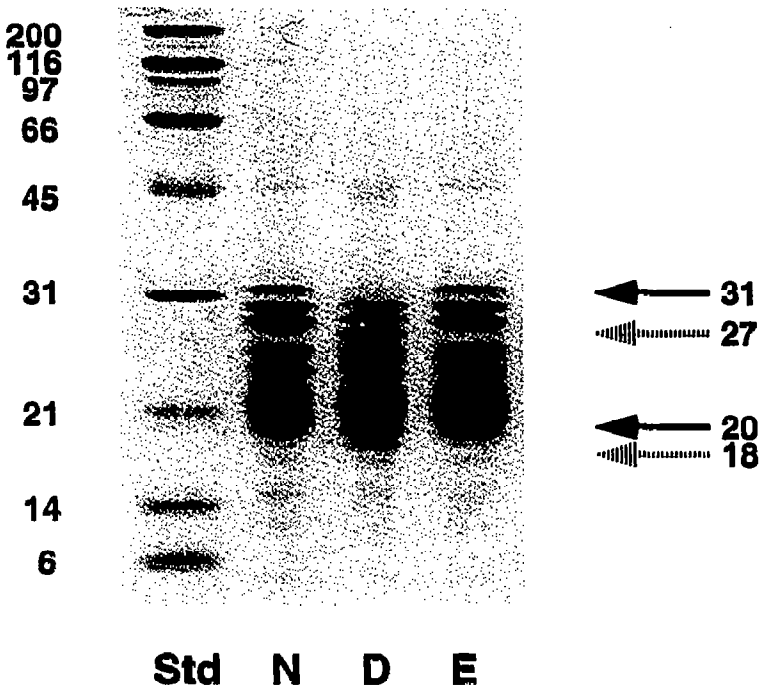


Fig. 4. SDS-PAGE of soluble proteins (5 μg/lane) from lenses. Lane marked N = Normal, lane D = Diamide, and lane E = Diamide + E64d. Molecular weight standards (Std) are indicated as kDa on left. In cataract lenses, note loss of bands at approximately 31 and 20 kDa (solid arrows), and new bands at 27 and 18 kDa (stippled arrows); these changes were prevented by E64d.

and 20 kDa α crystallins were lost, along with the accumulation of new polypeptide bands at 27 kDa and 18 kDa. Addition of E64d prevented proteolysis of crystallins observed in cataract induced by diamide (lane E, Fig. 4).

Hydration was elevated in lenses cultured with diamide for 4 days to a hydration value of 78.0 ± 1.06%, compared to 58.6 ± 0.43% in normal control lenses (Fig. 5). Addition of E64d was slightly effective in reducing lens hydration, since the % lens hydration was 73.4 ± 0.77. Concentrations of total calcium in lenses after 4 days of culture in diamide were markedly elevated to 3.9 ± 0.21 meq $Ca^{2+}$/kg water compared to normal cultured lenses containing 0.4 ± 0.01 meq $Ca^{2+}$/kg water (Fig. 6). E64d did not inhibit this marked elevation in calcium.

Amounts of calpain 80 kDa subunit in lens soluble fractions were reduced in lenses cultured with diamide (Fig. 7; soluble, lane D), and E64d prevented this loss of calpain 80 kDa subunit (soluble, lane E). Amounts of calpain 80 kDa subunit in lens insoluble fractions were increased in lenses cultured with diamide (insoluble, lane D). E64d caused an accumulation of calpain 80 kDa subunit in insoluble fraction above diamide alone (insoluble, lane E).

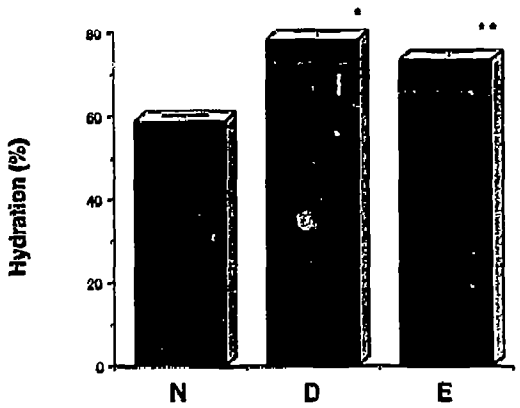


Fig. 5. Increase in the lens hydration in cataract lenses and prevention by E64d. Data are mean ± S.D. ($n$ = 6). * $P$ < 0.05 relative to N and ** $P$ < 0.05 relative to D. N = Normal; D = Diamide; E = Diamide + E64d.

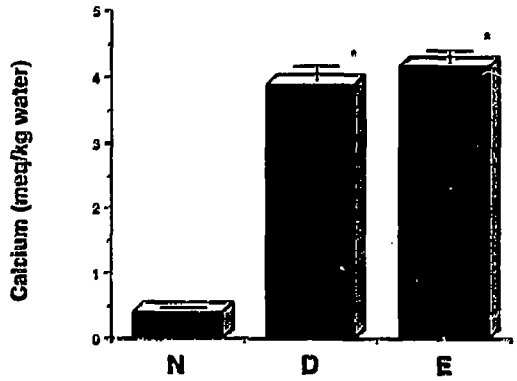


Fig. 6. Marked and equal elevation of calcium in lenses cultured with diamide or with diamide plus E64d. Data are mean ± S.D. ($n$ = 6). * $P$ < 0.05 relative to N. N = normal; D = Diamide; E = Diamide + E64d.

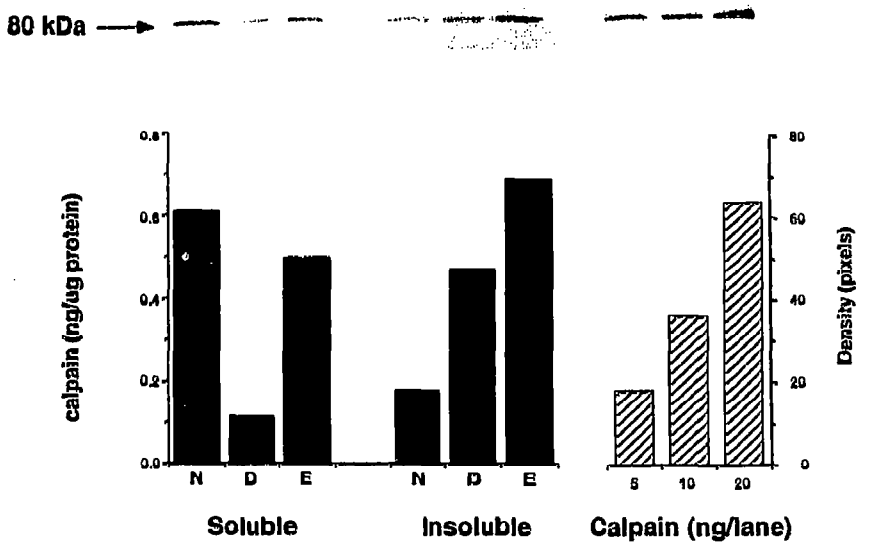


Fig. 7. Immunoblots for calpain of soluble and insoluble proteins (10 μg/lane) from cultured lenses (top of graph). Calpain in the soluble (left 3 lanes) and insoluble (center 3 lanes) fractions migrate to the same 80 kDa position as authentic purified pig heart calpain II (right 3 lanes). Each bar below is the densitometric image analysis of the immunoblot. The standard curves gave proportional increases in calpain staining as the amount of purified calpain II from pig heart (5, 10, 20 ng/lane) was increased (right graph). In the diamide cataract (middle lanes), note the decrease of calpain in soluble fraction (left graph), and the increase of calpain in insoluble fraction; calpain in both soluble and insoluble proteins from lenses cultured with diamide + E64d was higher than in lenses with diamide alone. Lane marked N = Normal, lane D = Diamide, and lane E = Diamide + E64d.

## 4. DISCUSSION

The major new finding of the present experiments was that calpain was involved in the mechanism of nuclear cataract formation in lenses cultured in the sulfhydryl oxidant diamide. The initial insult due to diamide in lens was probably oxidation of the sulfhydryl groups in the epithelium and outer cortex, allowing influx of sodium, water, and calcium [9]. Diamide cataracts developed rapidly, and within 4 days a dense nuclear opacity was present. Markedly elevated lens calcium, increasing calpain in insoluble fraction (a marker of calpain activation) and proteolysis of crystallins were observed in cataractous lenses cultured in diamide alone.

Another new finding was that E64d, a cell-permeable lipophilic inhibitor of cysteine proteases such as calpain, prevented nuclear cataract and associated proteolysis induced by diamide. Inhibition by E64d occurred even in the presence of increased lens calcium and hydration. This indicated that the cataract was due to proteolysis and was not caused by increased hydration, or direct precipitation of proteins with calcium. Similar biochemical events and cataract prevention by E64 were observed in lenses cultured in xylose, calcium ionophore, and selenite [6]. This indicates that calpain-induced proteolysis is a common mechanism of secondary nuclear cataract in rat lens initially insulted by a variety of cataractogenic agents.

Results with the diamide cataract in this report are also the first demonstration of cataract showing a spontaneous increase in the concentration of calpain in the insoluble fraction. At this time we do not know if this is non-specific insolubilization, or if the data indicate that the insoluble fraction is another locus for calpain activity in addition to calpain activity in the cytosol. Association of calpain with the phospholipids of membranes of the insoluble fraction may also reduce the concentration of calcium required for activation of calpain [15–17]. Calpain concentrations in both soluble and insoluble fraction from lenses cultured with diamide plus E64d was larger than diamide alone, indicating that E64d prevented further autolytic degradation of calpain [18] as well as inhibiting the proteolysis of crystallins. This suggests that calpain inhibitors should be further tested against other oxidation-induced cataracts.

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