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Expression and proteolytic activity of calpain in lens epithelial cells of oxidative cataract*

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Abstract: Objective: To study the role of calpain in the mechanism of oxidative cataract through detecting the level of intracellular free Ca^{2+}, the expression and proteolytic activity of calpain in the lens epithelial cells (LECs) of H_2O_2-induced cataract. Methods: Rat lenses were cultured in vitro and cataract was induced by H_2O_2. The level of intracellular free Ca^{2+} was measured by fluorescence determination with fura-2/AM. The expression of m-calpain protein in LECs was detected with immunohistochemical method. The proteolytic activity in LECs was measured using a fluorogenic synthetic substrate. Results: There were significant differences of the level of intracellular free Ca^{2+} (P=0.001, 0.000, 0.000), the expression of m-calpain (P=0.001, 0.000, 0.000) and the proteolytic activity of calpain (P=0.001, 0.000, 0.000) between H_2O_2-induced and control group at 6, 12 and 24 h, respectively. Conclusions: H_2O_2 can increase intracellular free Ca^{2+}, then enhance the expression and proteolytic activity of calpain which may play a role in the mechanism of oxidative cataract of rat.

Key words: Cataract, Lens epithelial cell, Hydrogen peroxide, Calpain, Calcium
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INTRODUCTION

The association between oxidative stress and cataract formation is well known from both clinical and experimental data. The mechanism through which oxidative stress causes cataract has not yet been established. It is known that many kinds of cataract are related to increased levels of calcium. This has raised interest in the involvement of calcium-activated proteases.

Calpains are non-lysosomal, cysteine proteases activated by calcium, which are found in most mammalian cells. There are 2 types of calpains in the cells: μ-calpain and m-calpain, which require μ mmol/L and m mmol/L concentrations of Ca^{2+} respectively for initiation of activity. Several investigations showed that m-calpain is the predominant type in tissues including lens of animal eyes (Persson *et al.*, 1993). The purpose of this research was to investigate changes in the level of intracellular free Ca^{2+}, the expression of m-calpain protein and calcium-dependent proteolytic activity of calpain in lens epithelial cells (LECs) from whole rat lenses exposed to oxidative stress, and then study the role of calpain in oxidative cataract.

METHODS

Lens culture

The laboratory animals and controls were 5-week-old Sprague Dawley rats from Zhejiang

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Medical Scientific Academy without restriction of sex. The number of animals was 87 totally and the body weight was 180−200 g. Lenses of rats were obtained by a posterior approach, and then were cultured in basic medium including minimum essential medium (MEM) (Gibco BRL Inc., USA), 10% fetal bovine serum (Gibco BRL Inc., USA), 50000 U/L penicillin and 50000 U/L streptomycin for 8 h at 37 °C under 5% CO_2. The opaque lenses were discarded. The transparent lenses were divided into 2 groups randomly: the trial group was cultured in basic medium supplemented with 2 mmol/L H_2O_2, and the control group were cultured in basic medium. The medium was exchanged every 6 h.

Determination of the level of intracellular free Ca^{2+} (Tesfamariam *et al*., 1999)

Capsules were obtained from the cultured lenses at 6, 12 and 24 h, and washed twice with Ca^{2+} and Mg^{2+} free phosphate buffered saline (PBS), which contained 2 mmol/L EDTA and 2 mg/ml bovine serum albumin. Cells suspension of lens epithelia adhering to their capsules was made on 300-mesh copper grids and was centrifuged (500 r/min for 3 min) at room temperature. The pellet was resuspended in HEPES-buffer saline (HBS, pH 7.4 at 37 °C) with the following composition (mmol/L): NaCl, 130; KCl, 5.0; $MgSO_4$, 1.0; $CaCl_2$, 1.6; glucose, 10; HEPES, 20 and bovine serum albumin, 1 mg/ml. Cell concentration of the suspension was determined on a hemacytometer slide and adjusted to 1×10^6 cells/ml. Cells were loaded with Ca^{2+}-sensitive fluorophore fura-2 acetoxylmethyl ester (fura-2/AM) (Calbiochem Inc., USA) by incubating the suspension with 5 μmol/L fura-2/AM for 40 min at 37 °C. The fura-2/AM loaded lens epithelial cells (LECs) were washed twice with HBS by centrifugation and then incubated in HBS for additional 30 min for complete deesterification of the dye. The cell suspensions were then transferred to a cuvette and placed in a temperature-controlled chamber (37 °C) under constant stirring and allowed to equilibrate for 3 min. The dye was alternately excited at 340 and 380 nm, and the fluorescence was measured at 505 nm in a spectrofluorophotometer (Hitachi F-4000, Japan). Intracellular free Ca^{2+} ($[Ca^{2+}]_i$) was calculated from the fluorescence signals according to the formula described by Grynkiewicz *et al.*(1985): $[Ca^{2+}]_i=\mathrm{Kd}\beta[(R-R_{\min})/(R_{\max}-R)]$. R was the ratio of the fluorescence at 340 nm to that at 380 nm. $R_{\max}$ were the ratio in the presence of 0.2% Triton X-100 and $R_{\min}$ were the ratio in Ca^{2+}-free media with 5 mmol/L EGTA; Kd was assumed to be 224 nmol/L; β was determined from the ratio of 380 nm fluorescence measured in a Ca^{2+}-free media to that measured in a Ca^{2+}-replete media.

Immunohistochemistry

The cultured lenses of the trial group and the control group were fixed at 6, 12 and 24 h in 10% formalin overnight and embedded in paraffin. Paraffin-embedded sections (5 μm) were subjected to immunostaining. Non-specific background staining was eliminated by incubating sections with rabbit non-immune serum. A purified goat polyclonal antibody against rat m-calpain (Santa Cruz Inc., USA) was used at 1:250 dilution and immunoreactivity was visualized with Strept Avidin-Biotin-enzyme Complex (SABC) method according to the protocol of SABC Kit (DAKO Inc., USA). Phosphate buffer saline (PBS) took the place of m-calpain antibody as the negative staining control while paraffin-embedded sections of human skeleton muscle took the place of lenses sections as the positive staining control. The result of immunostaining was assessed by 2 independent observers. The staining intensity was classified into 4 grades according to the color of granules in cytoplasm of LECs: "negative (–)" indicates no granules; "mild positive (+)" indicates weak-yellow granules; "moderate positive (++)" indicates dark-yellow granules; "severe positive (+++)" indicates brown granules. The numbers of LECs of every grade were counted in every visual field under microscope (100×) and 5 visual fields were chosen randomly in every sample.

Calpain activity assay (Potter *et al.*, 1998)

The method to make cell suspension was as described above. Calpain activity in intact cells was determined by measuring calcium-specific hydro-

lysis of the peptidyl 7-amino bond of the calpain substrate succinyl-leucyl-leucyl-valyl-tyrosyl-7-amino-4-methylcoumarin (suc-LLVY-AMC) (Calbiochem Inc., USA). To assay calpain activity, the cells suspension was prewarmed to 37 °C for 10 min with stirring in a spectrofluorophotometer (Hitachi F-4000, Japan). At t=−1 min, ionomycin (Calbiochem Inc., USA) in DMSO (2.5 μmol/L final concentration) or DMSO carrier was added to the cells suspension. At t=0 min, suc-LLVY-AMC was added to 50 μmol/L of concentration. At t=3 min, the result was obtained with spectrofluorophotometer. The excitation wavelength was 360 nm and the emission detection wavelength was 460 nm. The final result of this assay measuring the initial rate of calcium-dependent substrate cleavage was determined by subtracting the ionomycin-independent rate from the total rate.

Statistics

Statistical significance was tested using independent-sample t test and Mann-Whitney U test.

RESULTS

Determination of the level of intracellular free Ca^{2+} in rat LECs of H_2O_2-induced cataract: $[Ca^{2+}]_i$ was not increased in rat LECs of the control group after 24 h but was increased obviously in rat LECs of the trial group with continuing of the action of H_2O_2. There were significant differences of $[Ca^{2+}]_i$ between 2 groups at 6, 12 and 24 h (P=0.001, 0.000, 0.000) (Fig.1).

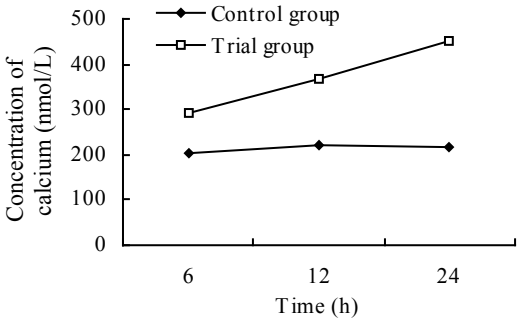

Fig.1 Concentration of intracellular free Ca^{2+} in rat LECs of 2 groups

Immunohistochemical detection of the expression of m-calpain protein in rat LECs of H_2O_2-induced cataract: negative staining was found in cytoplasm of most cells except for a few cells which showed light-yellow staining in the control group at 6, 12 and 24 h; in the trial group, the percentage and intensity of positive-staining LECs were increased with continuing of the action of H_2O_2 (Figs.2–3). There were significant differences of the expression of m-calpain protein between 2 groups at 6, 12 and 24 h (P=0.000, 0.000, 0.000) (Table 1).

Proteolytical assay of calpain in rat LECs of oxidative cataract: the proteolytical activity of calpain was not increased in rat LECs of the control group after 24 h but was increased obviously in rat LECs of the trial group with continuing of the action of H_2O_2. There were significant differences of calpain activity between 2 groups at 6, 12 and 24 h (P=0.001, 0.000, 0.000) (Fig.4).

DISCUSSION

Calpain is a soluble calcium-activated neutral protease which has been identified in a wide variety of tissues and species and has been implicated in

Table 1 Comparison of the ratio of m-calpain positive expression in rat LECs

	Positive ratio ($\bar{x} \pm s$, %)		
	6 h (n=5)	12 h (n=5)*	24 h (n=5)†
Trial group			
(−)	88.59±5.39	14.40±5.63	17.45±9.87
(+)	11.21±5.70	38.48±15.38	9.89±2.47
(++)	0	28.72±13.26	39.11±10.20
(+++)	0	7.37±5.13	32.08±10.79
Control group			
(−)	98.13±1.79	97.73±2.39	99.29±1.07
(+)	3.12±0.77	3.79±1.67	1.79±0.88
(++)	0	0	0
(+++)	0	0	0
U	112769.5	19644.5	22036.0
P	0.000	0.000	0.000

*Comparison of control group at 6 and 12 h: U=124500.0, P=0.667
†Comparison of control group at 12 and 24 h: U=123250.0, P=0.069

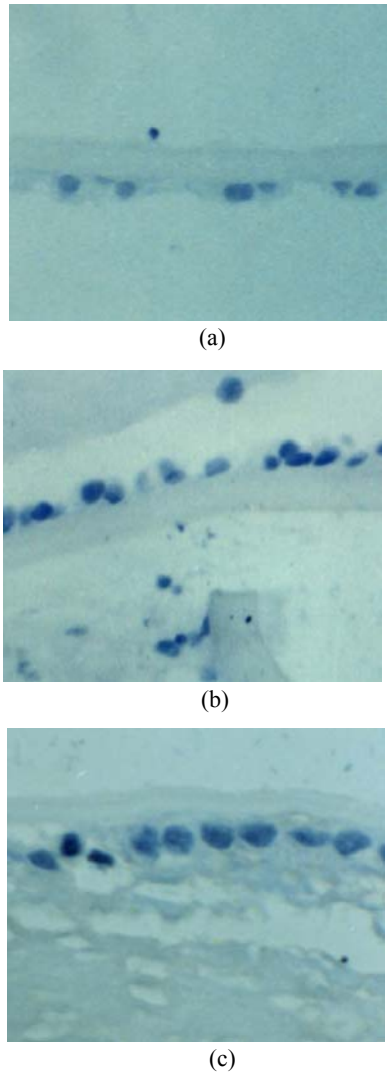

Fig.2 Expression of m-calpain in rat LECs of control group (a) at 6 h; (b) at 12 h; (c) at 24 h; (Immunohistochemistry ×400)

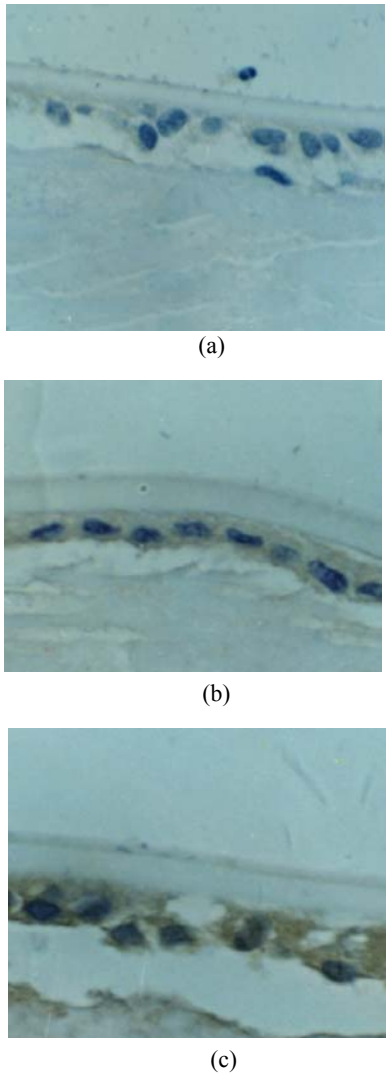

Fig.3 Expression of m-calpain in rat LECs of trial group (a) at 6 h; (b) at 12 h; (c) at 24 h; (Immunohistochemistry ×400)

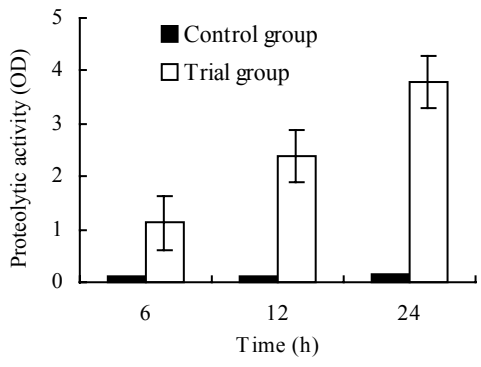

Fig.4 Proteolytic activity of calpain in rat LECs of 2 groups

many cellular functions such as cytoskeletal remodeling process, cell differentiation, apoptosis and signal transduction (Reverter *et al.*, 2001; Churchill-dagger and Louis, 2002). Cells that contain calpain also contain its endogenous inhibitor – calpastatin. However, in normal condition, calpastatin is present in concentrations sufficient to completely block the over-expression of calpain. It is clear that most mammalian cells contain the two distinct calpains referred to as μ-calpain and m-calpain. The expression of calpain was detected in many tissues of rat eyes including cornea, iris, ciliary body and retina (Persson *et al.*, 1993). Calpain

proteins in normal lenses of bovine, rat, mouse, pigeon, rabbit, chick and human were isolated by several authors. In Varnum *et al.*(1989)'s report, m-calpain concentrations decreased in the rat lens with age, whereas levels of calpastatin were maintained (Varnum *et al.*, 1989). David *et al.*(1989) showed that both enzymatic and immunologic assays indicated human lenses contained m-calpain activity was highest in the cortex of lenses from young donors, and lowest in the nucleus of aged lenses, even sometimes undetectable (David *et al.*, 1989). Andersson *et al.*(1994) reported that m-calpain was the most important calpain in human epithelium. They found that calpains occurred in decreasing amounts from the epithelium to the cortex to the nucleus in lenses from different species; and that the amount of m-calpain in lens decreased with increasing age, while the amount of calpastatin did not decrease. Shih *et al.*(2001) observed that many lens proteins including α-crystallin and β-crystallin were ideal substrates of calpain. Sanderson *et al.*(2000) found that vimentin in human lens was substrate of calpain too.

Many researches showed that Ca^{2+} concentration in lens with almost all forms of cataract was higher than that of normal people, though the extent of increment was different according to the type of cataract (Kadoya *et al.*, 1993). A high concentration of Ca^{2+} can induce cataractogenesis in vitro (Duncan and Wormstone, 1999).

Our study showed that the expression of m-calpain protein and proteolytic activity of calpain were very low in cytoplasma of normal rat LECs and increased obviously after cataract induced by H_2O_2. We propose that the endogenous inhibitor calpastatin inhibited the over-expression of m-calpain and pumping-out of calcium by Ca^{2+}-ATPase on the membrane of cell maintained low concentration of intracellular calcium. The action of H_2O_2, oxidative stress caused increasing cytoplasmic calcium concentration and then activated the over-expression of m-calpain. Our research observed that Ca^{2+} concentration of H_2O_2-induced cataract increased 50% after 6 h and 200% after 24 h. This result showed that high Ca^{2+} concentration induced by H_2O_2 could provide the essential prerequisite for activation of m-calpain.

Shearer *et al.*(1996) found that most rapid light-scattering occurred with total soluble proteins from young rat lenses, either after adding purified m-calpain or by activating endogenous lens m-calpain with calcium. McGinnis *et al.*(1999)'s research showed that procaspase-3 (32 kDa) was substrate of calpain and that procaspase-3 could convert into caspase-3 which was the key substance of the apoptotic process. Wang *et al.*(1998) reported that calpastatin was fragmented by caspases to various extents during the process of early apoptosis. Does proteolysis of proteins in lenses by m-calpain, or LECs apoptosis induced by caspase-3 activated by m-calpain, or both be attribute to the role of m-calpain in the mechanism of oxidative cataract? More experimental data are needed to confirm the role of m-calpain in oxidative cataractogenesis.

CONCLUSION

H_2O_2 can increase intracellular free Ca^{2+}, and then enhance the expression and proteolytic activity of calpain which may play a role in the mechanism of oxidative cataract of rat.

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References

Andersson, M., Sjtrand, J., Andersson, A.K., Andersen, B., Karlsson, J.O., 1994. Calpains in lens epithelium from patients with cataract. *Experimental Eye Research*, **59**(3):359-64.

Andersson, M., Sjtrand, J., Karlsson, J.O., 1996. Calpains in the human lens: relations to membranes and possible role in cataract formation. *Ophthalmic Research*, **28**(1): 51-54.

Churchilldagger, G.C., Louis, C.F., 2002. Ca^{2+} regulation in differentiating lens cells in culture. *Experimental Eye*

Research, 75(1):77-85.

David, L.L., Varnum, M.D., Lampi, K.J., Shearer, T.R., 1989. Calpain II in human lens. *Investigative Ophthalmology and Vision Science*, **30**(2):269-275.

Duncan, G., Wormstone, I.M., 1999. Calcium cell signaling and cataract: role of the endoplasmic reticulum. *Eye*, **13**(pt 3b):480-483.

Grynkiewicz, G., Poenie, M., Tsien, R.Y., 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *Journal of Biological Chemistry*, **260**:3440-3450.

Kadoya, K., Azuma, M., David, L.L., Shearer, T.R., 1993. Role of calpain in hydrogen peroxide induced cataract. *Current Eye Research*, **12**(4):341-346.

McGinnis, K.M., Gnegy, M.E., Park, Y.H., Mukerjee, N., Wang, K.K., 1999. Procaspase-3 and poly (ADP) ribose polymerase (PARP) are calpain substrates. *Biochemical Biophysical Research Communications*, **263**(1):94-99.

Persson, H., Kawashima, S., Karlsson, J., 1993. Immunohistochemical localizaton of calpains and calpastatin in the rabbit eye. *Brain Research*, **611**(2):272-278.

Potter, D.A., Tirnauer, J.S., Janssen, R., Croall, D.E., Hughes, C.N., Fiacco, K.A., Mier, J.W., Maki, M., Herman, I.M., 1998. Calpain regulates actin remodeling during cell spreading. *Journal of Cell Biology*, **141**(3):647-662.

Reverter, D., Strobl, S., Fernandez-Catalan, C., Sorimachi, H., Suzuki, K., Bode, W., 2001. Structural basis for possible calcium-induced activation mechanisms of calpains. *Journal of Biological Chemistry*, **382**(5): 753-766.

Sanderson, J., Marcantonio, J.M., Duncan, G., 2000. A human lens model of cortical cataract: Ca^{2+}-induced protein loss, vimentin cleavage and opacification. *Investigative Ophthalmology and Vision Science*, **41**(8): 2255-2261.

Shearer, T.R., Shih, M., Mizuno, T., David, L.L., 1996. Crystallins from rat lens are especially susceptible to calpain-induced light scattering compared to other species. *Current Eye Research*, **15**(8):860-868.

Shih, M., David, L.L., Lampi, K.J., Ma, H., Fukiage, C., Azuma, M., Shearer, T.R., 2001. Proteolysis by m-calpain enhances in vitro light scattering by crystallins from human and bovine lenses. *Current Eye Research*, **22**(6):458-469.

Tesfamariam, B., Frohlich, B.H., Gregg, R.E., 1999. Differential effects of pravastatin, simvastatin, and atorvastatin on Ca^{2+} release and vascular reactivity. *Journal of Cardiovascular Pharmacology*, **34**(9):95-101.

Varnum, M.D., David, L.L., Shearer, T.R., 1989. Age-related changes in calpain II and calpastatin in rat lens. *Experimental Eye Research*, **49**(6):1053-1065.

Wang, K.K., Posmantur, R., Nadimpalli, R., Nath, R., Mohan, P., Nixon, R.A., Talanian, R.V., Keegan, M., Herzog, L., Allen, H., 1998. Caspase-mediated fragmentation of calpain inhibitor protein calpastatin during apoptosis. *Achieves of Biochemistry and Biophysics*, **356**(2):187-196.