Age-related reduction in calbindin-D28K expression in the Sprague-Dawley rat lens

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Abstract. Calcium levels in the lens rise with increasing age and increased intracellular calcium accumulation is known to be a risk factor for cataract formation. Calbindin-D28K (CALB1) is an intracellular calcium buffer. It is not clear whether CALB1 levels change in response to the Ca^{2+} accumulation in the lens that occurs with age. The present study investigated the distribution of CALB1 in the lenses of Sprague-Dawley (SD) rats and whether this changed with age. Lenses were isolated from SD rats at 1, 6, 12 and 18 months of age. CALB1 distribution was examined using immunohistochemistry. Lens epithelial cells were counted in median sagittal plane slices from the hematoxylin and eosin-stained lens and quantified using western blot analysis. *Calb1* gene expression was examined using reverse transcription-quantitative polymerase chain reaction. CALB1 was distributed in the epithelial and fiber cells of the lens. CALB1 levels declined significantly with increasing age, whilst there was no significant accompanying decrease in the number of lens cells. A similar reduction was noted in CALB1 mRNA levels. To the best of our knowledge, this is the first study to demonstrate that CALB1 expression and CALB1 protein levels in SD rat lens decrease with age. This reduction does not reflect a reduction in lens cell numbers but a genuine reduction in gene expression within these cells. Thus, CALB1 may be important in changes occurring in the lens in older age, in particular in the development of cataracts.

Introduction

Intracellular calcium accumulation is known to cause oxidative damage in the lens (1,2). Aberrant Ca^{2+} homeostasis is known to be involved in cataract formation (3), highlighting the importance of understanding the mechanisms by which Ca^{2+} is regulated in the lens. Elevated internal Ca^{2+} concentrations in the lens can be induced by a number of processes, including oxidation of external or internal sulfhydryl groups (4), removal of external glucose (5), reduced external Ca^{2+} levels (6) and aging itself (7).

Calbindin-D28K (CALB1) is a member of the EF-hand family of Ca^{2+}-binding proteins. It has a molecular weight of ~28,000 kDa (8) and was first isolated from chick intestinal mucosa (9). The function of CALB1 has not yet been fully established. CALB1 has been shown to be a carrier protein, which may act as a cytoplasmic Ca^{2+} buffer or facilitate transcellular Ca^{2+} transport (10), thereby preventing intracellular Ca^{2+} concentrations from reaching toxic levels (10,11). More recent studies have indicated that CALB1 may act as an inhibitor of apoptosis (12) and as a Ca^{2+} sensor (13). CALB1 levels in neurons are known to decrease with age and in neurodegenerative conditions (14). In the spinal cord, motor neurons do not express CALB1 and have an increased susceptibility to Ca^{2+}-induced injury (15). CALB1 is expressed widely within the central nervous system (16), including in the retina (17) and in non-neuronal tissue (18), including the kidney (19-21), bone (22) and pancreas (23). To date, however, to the best of our knowledge no data describing the expression or function of CALB1 in the lens has been reported.

In view of the potential role of CALB1 in Ca^{2+} regulation, this study aimed to establish the distribution and level of expression of CALB1 in the lens of Sprague-Dawley (SD) rats of varying ages. Median sagittal plane slices from the hematoxylin and eosin (H&E) stained lenses were used for quantifying lens epithelial cells. Western blot analysis was used to quantify CALB1 protein level changes with age, and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was employed to examine mRNA levels.

Materials and methods

Animals. All experiments were conducted with the approval of the Animal Ethics Committee of the Zhongshan Ophthalmic Center, Sun Yat-sen University, (Guangzhou, China). Experiments adhered to the ethical guidelines produced by the Laboratory Animal Care and Use Committee of the Association for Research in Vision and Ophthalmology (Zhongshan Opthalmic Center, Sun Yat-sen University, Guangzhou, China). SD rats were obtained from the Laboratory Animal Centre of the Zhongshan Ophthalmic Center and were examined at 1, 6, 12 and 18 months of age. Rats were anesthetized and killed by

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Table I. Reverse transcription-quantitative polymerase chain reaction primers.

Rat gene	Forward primer	Reverse primer
Calb1 (417 bp)	5'-ACACTGACCACAGTGGCTTC-3'	5'-GTTCGGTACAGCTTCCCTCC-3'
GAPDH (321 bp)	5'-GGACCAGGTTGTCTCCTGTG-3'	5'-GGCCCCTCCTGTTGTTATGG-3'

Calb1, calbindin-D28K.

intraperitoneal injection of 10% chloral hydrate after which the eyes were immediately enucleated.

Immunohistochemistry. For immunohistochemistry, rat eyes were placed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) containing 5% glacial acetic acid and 10% acetone (pH 7.4) for 18 h at 4˚C. They were serially cryoprotected in 10, 20 and 30% sucrose in 0.1 M PBS for 1-2 days at 4˚C, respectively. The eyes were then embedded in optimum cutting temperature compound. Cryostat sections (5 μm) were placed in an incubator chamber at 50˚C for 2 h, and then stored at room temperature for subsequent use. Frozen tissue sections were initially rehydrated in 0.01 M PBS (pH 7.2) and then incubated in 3% hydrogen peroxide solution to block endogenous peroxidase for 30 min at room temperature. After three 5-min rinses with 0.01 M PBS, sections were incubated in 5% normal goat serum for 30 min at 37˚C, and then with rabbit-anti-rat polyclonal antibody against CALB1 (1:3,000; Merck Millipore, Billerica, MA, USA) overnight at 4˚C. Following three 5-min rinses with 0.01 M PBS containing 0.1% Tween-20 (PBST), polymer helper (Zhongshan Golden Bridge Biotechnology Co., Ltd., Beijing, China) was added to the slides and the slides were incubated for 30 min at 37˚C. After three 5-min rinses with 0.01 M PBST, a polyclonal horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (Zhongshan Golden Bridge Biotechnology Co., Ltd) was added to the slides, which were then incubated for 30 min at 37˚C. After three 5-min rinses with 0.01 M PBST, diaminobenzidine was added. To label cell nuclei, H&E staining was conducted. Sections were cover-slipped, dehydrated in a graded ethanol series and cleared with dimethylbenzene. Control images were obtained using the same procedure, with the exception that the sections were not exposed to the CALB1 antibody.

Cell counts. Lenses were dissected from eyeballs following immersion fixation for 18 h in a solution of 4% paraformaldehyde in PBS (pH 7.0-7.4). Lenses were embedded with paraffin following dehydration with varying concentrations of ethanol (70 -> 75 -> 80 -> 85 -> 90 -> 95 -> 100% x 2)and cleared with dimethylbenzene. Paraffin sections (5 μm) were made and stained with H&E. Epithelial cell nuclei were counted in median sagittal plane slices, taken from the H&E stained lenses of rats aged 1, 6, 12 or 18 months, under x400 magnification. A total of 12 lenses from different animals was analyzed at each age. For each specimen, two slides from the aequator lentis and lens subcapsular epithelium were analyzed.

RT-qPCR. After enucleation, the lenses were isolated and stored in an RNAsafer stabilizer reagent (Omega Scientific, Tarzana, CA, USA) prior to use. Total RNA was isolated with TRIzol reagent (Takara Bio, Inc., Shiga, Japan), according to the manufacturer's instructions. Contaminated DNA was removed using the TURBO DNA-free™ kit (Applied Biosystems, Inc., Foster City, CA, USA) and cDNA was synthesized from 2 μg total RNA using the PrimeScript™ RT Reagent kit (Takara Bio, Inc.) in 20 μl reaction mixture. The cDNA was then used as a template for PCR amplification. PCR was conducted using the TProfessional thermocycler (Biometra GmbH, Göttingen, Germany). All primers were designed online using Integrated DNA Technologies SciTools software (Integrated DNA Technologies, Inc., Coralville, IA, USA). Primer sequences are listed in Table I and were designed according to the cDNA sequences of rat *Calb1* listed in the GenBank™ database (http://www.ncbi.nlm.nih.gov/genbank/). GAPDH was used as the internal control for each reaction. All primers were tested for their specificity by conventional PCR prior to being used for the RT-qPCR quantitative studies. The following PCR scheme was used: 5 min at 94˚C, 32 cycles of 60 sec at 94˚C; 45 sec at 60˚C; 60 sec at 72˚C, 10 min at 72˚C, and then 4˚C thereafter. Following the PCR reaction, the expression levels of the genes were observed using agarose gel electrophoresis. The change of *Calb1* expression was recorded as the fold change of the densitometric ratio between *Calb1* and *GAPDH* for each lens.

Western blot analysis. Individual rat lenses were homogenized in ice-cold cell lysis buffer (20 mM Tris-HCl buffer, pH 7.5, 150 mM NaCl, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM EDTA, 1 mM Na_3VO_4 and 1 μg/ml leupeptin) according to the manufacturer's instructions (Beyotime Institute of Biotechnology, Haimen, China). Homogenates were centrifuged at 12,000 x g for 5 min at 4˚C, and the clear supernatants were stored at -80˚C until use. Protein concentrations were determined using a bicinchoninic acid kit (Comwin Biotech, Co., Ltd., Beijing, China). Samples (20 μg protein/well) were loaded and electrophoresed on 12% sodium dodecyl sulfate-polyacrylamide gels for 60 min at 100 V. Proteins were then transferred to polyvinylidene fluoride (PVDF) transfer membranes (Bio-Rad, Hercules, CA, USA) for 80 min at 200 mA. Following transfer, the PVDF membranes were blocked with blocking solution containing Tris-buffered saline with 0.1% Tween-20 (TBST) and 5% non-fat milk for 60 min at room temperature. Membranes were incubated with rabbit-anti-rat polyclonal antibody against CALB1 (1:3,000: Merck Millipore), overnight at 4˚C. Membranes with GAPDH protein were incubated with rabbit-anti-rat monoclonal antibody against GAPDH (1:1,000, Abcam, Cambridge, UK). Blots were then washed three times (5 min each) with TBST. All membranes were then incubated with secondary antibodies (HRP-labeled goat-anti-rabbit polyclonal IgG, 1:500; Abcam) for 1 h at room

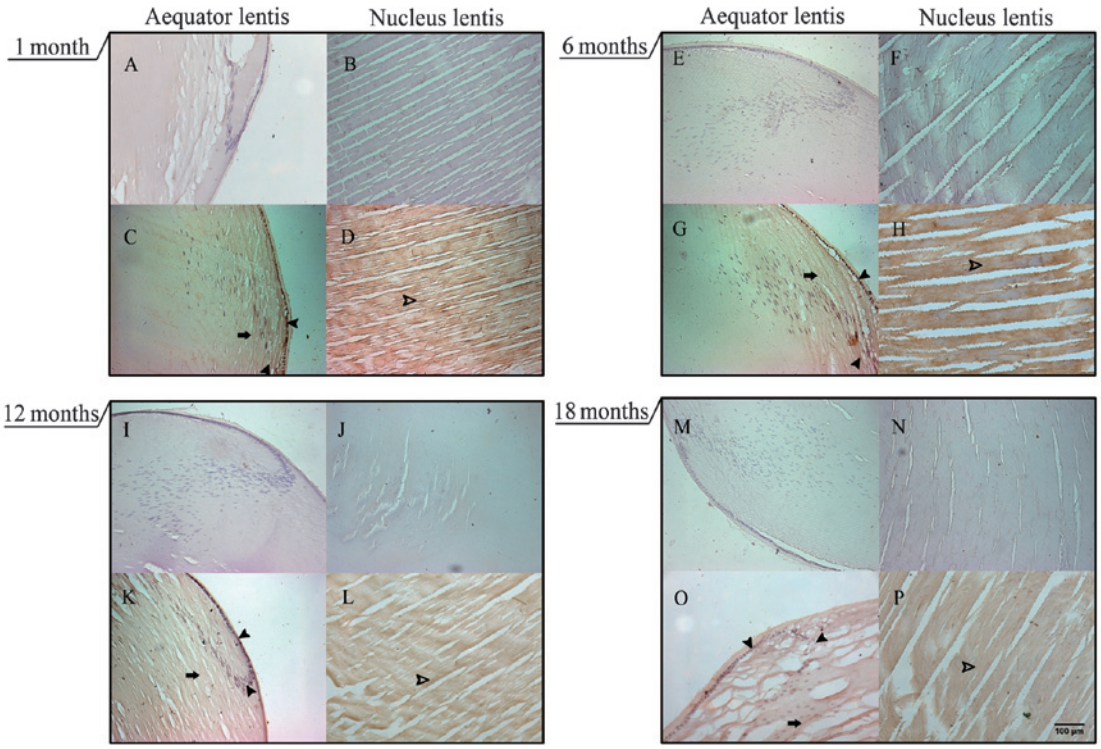

Figure 1. Localization of CALB1 in the aging SD rat lens. Immunohistochemistry was performed to show CALB1 protein in sections of lenses obtained from SD rats at the ages indicated. A-D, 1 month; E-H, 6 months; I-L, 12 months; and M-P, 18 months, by diaminobenzine staining. At each age, the upper pair of images (A, B, E, F, I, J, M and N) were obtained from sections prepared without exposure to the CALB1 primary antibody (control images). Left and right columns show cross-sections through the aequator lentis or nucleus lentis, respectively. Arrows, CALB1-positive resions in the lens cortex; filled arrowheads, CALB1-positive cells in the lens epithelial cells; open arrowheads, CALB1-positive lens fiber cells in the lenticular nucleus. CALB1, Calbindin-D28K; SD, Sprague-Dawley. The scale bar indicates 100 μm.

temperature whilst being shaken. Membranes were rinsed between incubations. Finally, blots were developed by incubating in enhanced chemiluminescence reagent (Comwin Biotech, Co., Ltd) and subsequently exposed to Biomax™ Light film (Eastman Kodak, Rochester, NY, USA) for 20-30 min. Signal specificity was confirmed by blotting in the absence of primary antibody, and bands were normalized to GAPDH-immunoreactive bands visualized in the same membrane after stripping. Density measurements for each band were performed with ImageJ version 1.41o software (National Institutes of Health, Bethesda, MA, USA). Background samples from an area near each lane were subtracted from each band to obtain the mean band density. Densitometric ratios between CALB1 and GAPDH were calculated to determine the relative levels of CALB1.

Statistical analysis. All data are presented as the mean ± standard error. Data were evaluated by one-way analysis of variance, and the least significant difference post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Distribution of CALB1 in the rat lens. An antibody specific for CALB1 was used to investigate age-related changes in CALB1 levels in the SD rat lens. Immunohistochemical staining showed that CALB1 is widely distributed in the SD rats lens, including in lens epithelial cells and lens fiber cells in the cortex lentis and nucleus lentis. At all ages, CALB1 is predominantly expressed in the lens epithelial cells, although the density of CALB1 labeling appeared lower in lenses obtained from older rats (Fig. 1).

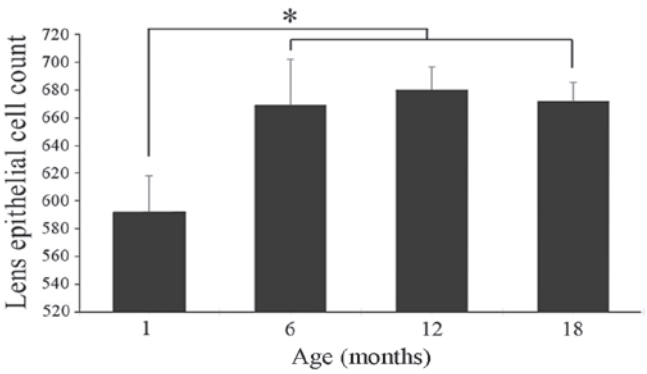

Figure 2. Numbers of lens epithelial cells. The number of epithelial cells increased significantly from 1 month to a plateau, which was maintained at 6, 12 and 18 months. Data are presented as the mean ± standard error of 12 lenses. *$P<0.01$.

Density of lens epithelial cells. In order to determine whether the age-related decrease in CALB1 labeling reflected a decrease in the number of lens epithelial cells, the number of epithelial cells in lenses obtained from rats aged 1, 6, 12 or 18 months was counted. Cell numbers were significantly lower in lenses obtained from 1-month-old rats than from older rats. No significant difference was identified in lens density between the 6-, 12- or 18-month-old rats (Fig. 2).

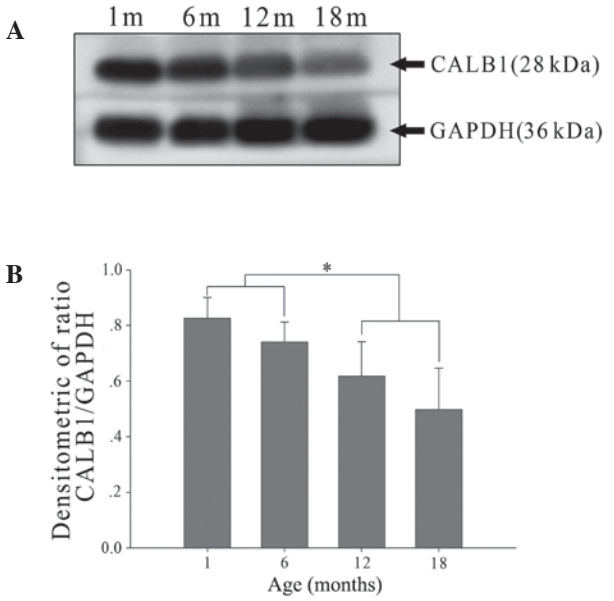

Figure 3. Western blot analysis of CALB1 protein levels. (A) Representative western blots for CALB1 (28 kDa) and GAPDH (36 kDa) obtained from lenses of rats at 1, 6, 12 or 18 months of age. (B) Ratio of CALB1 to GAPDH protein levels for lenses obtained from rats aged 1, 6, 12 or 18 months of age. Data are presented as the mean ± standard error of 12 lenses obtained from 12 separate rats at each age. There was no statistically significant difference between the rats at 1 and 6 months, and between the rats at 12 and 18 months. However, the CALB1 expression levels were lower in the 12 and 18 months old rats, as compared with the 1 and 6 month old rats. *P<0.01. CALB1, Calbindin-D28K.

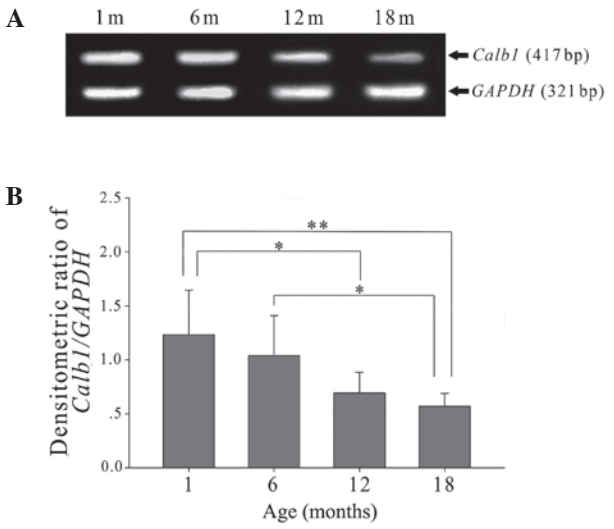

Figure 4. Densitometric analysis of CALB1 mRNA expression by reverse transcription-quantitative polymerase chain reaction. (A) Representative polyvinylidene fluoride membranes labeled for CALB1 (471 bp) or GAPDH (321 bp) from lenses obtained from rats aged 1, 6, 12 or 18 months. (B) Ratio of CALB1 to GAPDH expression for lenses obtained from rats aged 1, 6, 12 or 18 months. Data are presented as the mean ± standard error of 10 lenses obtained from 10 separate rats at each age. *P<0.05 and **P<0.01. CALB1, Calbindin-D28K.

Age-related changes in CALB1 protein and Calb1 mRNA expression. Western blot analysis was used to define the time course over which CALB1 levels vary with age. At all ages examined, CALB1 levels were detectable using the procedures described in the Materials and methods section (Fig. 3A). When CALB1 levels were analyzed relative to GAPDH levels, no difference was found between CALB1 levels in lenses from rats aged 1 or 6 months (Fig. 3B). By contrast, CALB1 levels were significantly reduced in lenses obtained from rats aged 12 and 18 months compared with those from the younger age groups (Fig. 3B). *Calb1* mRNA levels were also lower relative to GAPDH in lenses obtained from older rats than those from younger rats (Fig. 4A). In parallel with the protein measures, there was no significant difference between *Calb1* mRNA levels of lenses from 1- and 6-month old rats, whilst levels observed in lenses from 12- and 18-month-old rats were significantly reduced in comparison to their younger counterparts (Fig. 4B).

Discussion

The primary role of the ocular lens is to focus light onto the retina. To support this role, the lens has evolved into a highly specialized avascular tissue with a single layer of epithelial cells on the anterior surface. Cataracts, in which reduced lens optical homogeneity or transparency are observed (24), are linked to a number of factors, including genetics, diabetes, smoking, nutrition, radiation, ultraviolet exposure and changes in endocrine or enzymatic equilibria (25-28). In the lens, an increase in internal calcium can be induced by several processes. These include oxidation, either of external or internal sulphydryl groups (4); removal of external glucose (5); reduction of external calcium (29); and aging (7). Reactive oxygen species reduce lens homeostasis and elevate Ca^{2+} concentration (30,31), increasing calpain activity (32-35), which denatures lens proteins and reduces lens transparency (25,36). Consistent with this model, the Ca^{2+} content of cataractous lenses of inherited cataract/f rats is ~10-fold that of lenses from Wistar rats (37-39). In the lenses of UPL rats, a hereditary rat cataract model, decreased ATP levels lead to a reduction in Ca^{2+}-ATPase function, resulting in the elevation of lens Ca^{2+} levels. This process may contribute to cataract development (40). In human senile cataracts, an ionic imbalance in the lens with increased levels of Ca^{2+} has been suggested to be involved in cataract formation (25,41).

CALB1 is a member of the calcium-binding protein super family (42). CALB1 has high affinity for Ca^{2+}. It buffers Ca^{2+} quickly, preventing Ca^{2+}-induced impairment of mitochondria and also preventing the release of cytochrome *c* (43). The majority of the literature regarding CALB1 focuses on the nervous system. For example, spinal motor neurons that lack CALB1 are sensitive to Ca^{2+}-induced injury (15). When examined across the age range, neuronal expression of *Calb1* declines. It is also known to be reduced in neurodegenerative disorders. In these conditions, decreased or absent CALB1 may contribute to free radical stress altering the normal distribution of Ca^{2+} (44). However, the correlation between Ca^{2+} and CALB1 in the lens has not yet been reported.

The present study demonstrated that CALB1 is expressed in the SD rat lens, where it is primarily localized to the cortex lentis. In addition, it was shown that CALB1 levels decreased with age in the SD rat lens. This reduction did not reflect a reduction in the number of lenticular cells. In fact, although CALB1 levels declined between 1 and 6 months of age, the number of lens cells increased between these time points. Thus, the reduction in CALB1 reflected a true decrease in *Calb1* expression. There is no

earlier information concerning *Calb1* expression in the rat lens, although prior studies have focused on multiple tissues, including the retina (17). We hypothesize that the age-related reduction in CALB1 may contribute to the observed increases in Ca^{2+} levels in lenses obtained from older animals. This may in turn increase lenticular oxidative damage. However, the mechanisms underlying CALB1 downregulation remain unclear. In addition to addressing this issue, evaluation of changes in CALB1 levels and *Calb1* expression with age in the human lens is required. These studies are important in understanding the function of CALB1 in the lens and its possible role in cataract development.

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