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The Expression of Calcium Sensing Receptor in Normal and Diabetic Rat Eyes

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Data Collection B
Statistical Analysis C
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Background: Calcium sensing receptor (CaSR) is widely expressed in many tissues of the body, but it is rarely reported to be expressed in the eyes. This research explored the expression and distribution of CaSR in eye tissues of normal and diabetic rats.





Material/Methods: Thirty male Sprague Dawley (SD) rats were randomly divided into a diabetic and a normal group. Diabetes mellitus (DM) models were successfully established by intraperitoneal injection of streptozotocin. The expression and distribution of CaSR in the rat eyeballs were detected by immunohistochemistry. Quantitative RT-PCR and western blotting were used to detect the presence of CaSR in normal and diabetic rats.

Results: CaSR was detected in the cornea, lens epithelium, and retina. CaSR was expressed the most in the cornea, followed by the lens epithelium, and the retina ($p < 0.05$). The expression of CaSR was decreased in the eye tissue of diabetic rats ($p < 0.05$).

Conclusions: In this study, CaSR was detected in rat cornea, lens, and retina. It was significantly decreased in the eyes of diabetic rats. This indicated that the downregulated expression of CaSR was associated with diabetic oculopathy.

MeSH Keywords: **Cornea • Diabetic Retinopathy • Lens Diseases • Receptors, Calcium-Sensing • Retina**

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Background

Calcium sensing receptor (CaSR) was first cloned from the parathyroid gland by Brown in 1993. CaSR belongs to the extended family of G protein-coupled heptahelical receptors. Extracellular calcium acts as a first messenger through CaSR in certain kinds of cells; this affects the diverse biological processes in the body [1]. It plays a pivotal role in calcium homeostasis and it also takes part in proliferation, differentiation, chemotaxis, cell survival fluid secretion, and absorption and membrane voltage [2–5]. CaSR is widely expressed in parathyroid, kidney, bone marrow, brain, central nervous system, intestine, lung, colon, epidermis, mammary gland, placenta, and lens epithelium [5–9]. In addition to lens epithelium, the expression of CaSR in other eye tissue has not yet been reported.

Studies have reported that activating CaSR mutations is associated with cataracts [9,10]. As we know, diabetes can cause cataracts, keratopathy, and retinopathy. Diabetic patients are more likely to have the following pathological changes: decreased sensitivity of the cornea and delayed healing of corneal injury [11,12]. Hyperglycemia has toxic effects on almost all organs in the body. It has been reported that the expression of CaSR was decreased in the brain and kidney in diabetic rats [13,14]. However, the relationship between CaSR and diabetic oculopathy is not clear.

This research explored the expression and distribution of CaSR in eye tissues of normal and diabetic rats. The research findings may pave the way for future research on the development and treatment of diabetic eye disease.

Material and Methods

Material and reagents

Reagents included mouse monoclonal anti-calcium sensing receptor antibody (anti-CaSR; Abcam, USA; ab19347), anti-mouse IgG (H+L) antibody (Kirkegaard & Perry Laboratories, USA; #074-1806), β -actin antibody (C4) (Santa Cruz Biotechnology, CA, USA; sc-4777), GAPDH antibody (G-9) (Santa Cruz Biotechnology, CA, USA; sc-365062).

Animals

Ten-week-old SPF male Sprague Dawley (SD) rats were handled according to our laboratory protocol. Our study was approved by the Institutional Animal Care and Use Committee of Tongji Medical College, Huangzhong University of Science and Technology. Briefly, SD rats were housed with free access to water and rodent laboratory food in standard conditions (controlled temperature was $24\pm 2^\circ\text{C}$ and a 12 hour light/dark cycle lights).

Type 1 diabetes mellitus animal model was created using ten-week-old SD rats (250–300 g, $n=15$, selected randomly) with a single intraperitoneal injection of 60 mg/kg streptozotocin (STZ, catalog no.S0130, Sigma-Aldrich, USA). STZ was freshly dissolved in 0.1 M citrate buffer (pH 4.5). Three days later, blood was collected from the tail vein for first glucose measurement. Plasma glucose levels ≥ 16.7 mM (twice contiguously) was considered to be a successful diabetic rat model. Normal control rats ($n=15$) were only injected with citrate buffer. Subsequently, both normal control (NC) rats and diabetes mellitus (DM) rats were housed for 20 weeks with adequate water and food in the barrier system of our animal laboratory.

Tissue preparation

The rat eyeballs were isolated after anesthesia, and were dissected to collect cornea, lens, and retina tissue samples. A portion of the dissected tissues was fixed in 4% paraformaldehyde solution. The rest was stored at -70°C for later protein and RNA extraction.

Detection of CaSR expression by immunohistochemistry

The fixed tissues were embedded in paraffin and cut into 3.5 μm thick slices. These slices were incubated in an oven at 60°C for two hours and then deparaffinized and rehydrated. After 15 minute pretreatment at 95°C with 10 mmol/L sodium citrate (pH 6.0) for antigen retrieval, the tissue slices were incubated with 3% H_2O_2 diluted in methanol for 15 minutes. The slices were incubated with 5% BSA diluted in PBS at room temperature for two hours to block nonspecific binding sites. The sections were then incubated overnight at 4°C with mouse monoclonal anti-CaSR antibody (catalog no. ab19347; Abcam, Cambridge, UK) at a dilution of 1: 100. The sections were exposed to the anti-mouse IgG (H+L) antibody (catalog no. 074-1806, KPL, USA) at room temperature for one hour. For the controls, PBS was used instead of primary antibody. The ABC complex and DAB solution in PBS were prepared as per manufacturer's protocol, and then coverslips were applied with permanent mounting medium.

Determination of CaSR by western blotting

Total proteins were extracted from cornea, lens, retina, and kidney tissue. A Bradford assay was conducted to determine protein concentrations. Equal concentrations of total proteins from the tissue specimens were separated by SDS-PAGE and transferred to PVDF membranes (0.45 μm , Millipore, Bedford, MA, USA) and then anti-CaSR antibody at 1: 800, GAPDH antibody (sc-365062, Santa Cruz, CA, USA) and secondary antibody (catalog no.074-1806KPL, USA) were applied. The membranes were washed with TBST, the CaSR protein were developed with Supersignal West Pico (Pierce, Rockford, IL, USA) for two to five

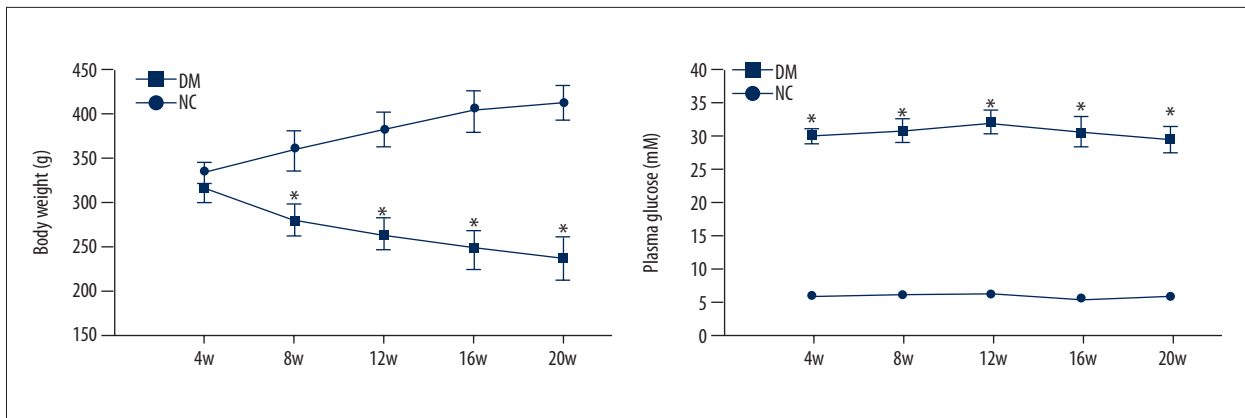


Figure 1. Hyperglycemia and growth retardation in diabetic mellitus (DM) rats. DM rats were intraperitoneally injected with STZ. Age-matched normal control (NC) rats received citrate buffer injection only. Body weight was measured weekly and there was a significance different between DM rats and NC rats,* $p < 0.001$. Plasma glucose was measured weekly,* $p < 0.001$.

minutes, and then exposed to film (Kodak, Rochester, NY, USA). Films were visualized using Kodak Medical X-ray processor 102 (Kodak, Rochester, NY, USA) to visualize the reactive proteins followed by densitometric quantification using Image-Pro Plus software (Media Cybernetics, Rockville, MD, USA).

Detection of CaSR mRNA by quantitative reverse-transcriptase polymerase chain reaction

Total RNA was isolated and purified from tissue samples using TRIzol (Invitrogen, Carlsbad, CA, USA), and then reverse transcribed into cDNA by RevertAid First Strand cDNA Synthesis Kit (Thermo). SYBR Premix Ex Taq (Takara, Shiga, Japan) was used in a 7500 Real-time PCR system (Applied Biosystems, California, USA). The cycling condition of the CaSR gene was 40 cycles of denaturation (15 seconds at 95°C), annealing (one minute at 58°C, the specific primer-optimized annealing temperature), and extension (one minutes at 72°C). The primers used for amplification were as follows:

CaSR-Forward: TGACGAGCCTCAGAAGAATGC;
CaSR-Reverse: CCTCCACCACTAATGACGAAGC;
 β -actin-Forward: CCGTAAAGACCTCTATGCCAACA;
 β -actin-Reverse: CCGACTCATCGTACTCCTGCTT.

Amplification of the housekeeping gene β -actin was used as an internal control to normalize the CaSR mRNA level. The fluorescence threshold cycle (Ct) value was determined for each gene and normalized with β -actin. All values were compared and normalized to normal control tissues.

Statistical analysis

All data were analyzed using SigmaPlot12.0 software. The results were expressed as the mean \pm SD. The difference in each tissue was determined using one-way analysis of variance (ANOVA). Statistical comparisons between NC and DM

groups were performed by Student's *t*-test. Values of $p \leq 0.05$ were considered statistically significant.

Results

The features of experimental rats

The DM rats exhibited polydipsia, polyphagia, polyuria, diarrhea, adipose, and muscle tissue wasting. DM rats started showing lens opacity around eight weeks of hyperglycemia and by 16 weeks nearly all the DM rats developed cataracts, while the lens of the NC rats remained clear. The weight of the DM rats decreased significantly after injected with high dose of STZ ($p < 0.001$). While the NC rats kept a stable weight gain (Figure 1). Compared with the NC rats, the blood glucose level of DM rats were approximately 30 mM, which were 5.13- to 5.64-fold higher than the control rats (Figure 1).

The localization and distribution of CaSR in NC and DM rat eyes

In NC rats, CaSR was detected in the cornea, lens and retina (Figure 2A–2C). In addition, the expression of CaSR was more significant in the epithelium and endothelium of the cornea, lens epithelium, and retina pigment epithelium (RPE). And CaSR in the epithelial basement membrane was more deeply staining than the surface layer in corneal epithelium. However, CaSR was nearly absent in corneal stroma and lamellar fiber of the lens. And in the retina, CaSR was not observed in the outer nuclear layer, inner nuclear layer, the ganglion cell layer, or the choroid (Figure 2C).

Compared with the NC group, the expression of CaSR was decreased in all the eye tissues in the DM group (Figure 2D–2F).

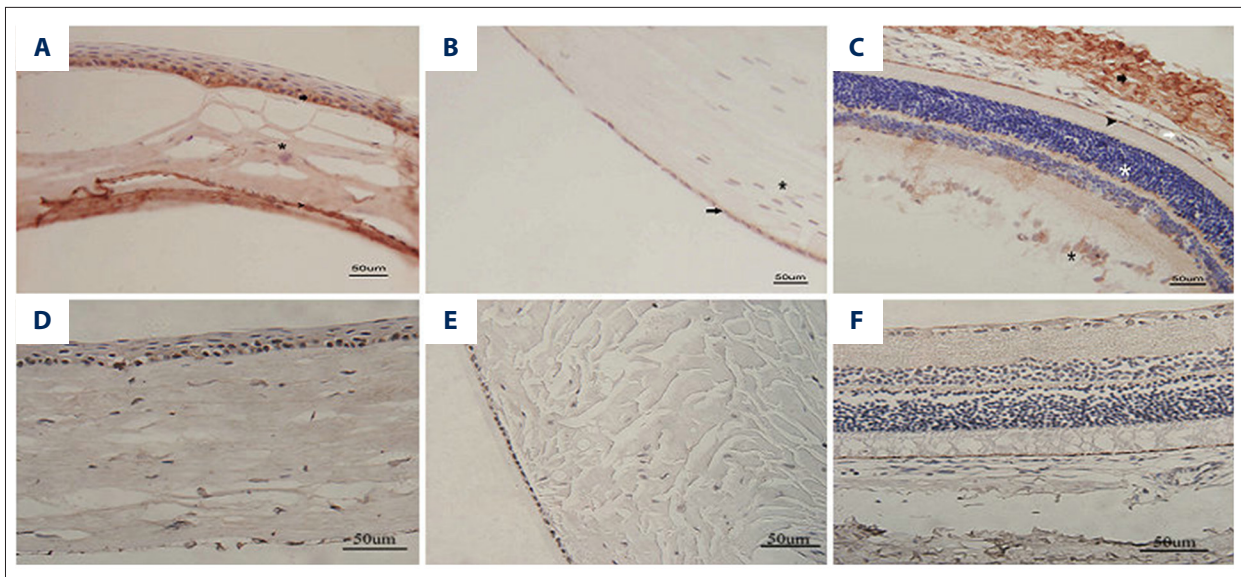


Figure 2. Localization of CaSR in rat eyes. Optical micrographs show the distribution and expression of CaSR (brown) in different eye tissues. CaSR was detectable in the epithelium (arrow) and endothelium of the cornea (A), lens epithelium (B, arrow), retina pigment epithelium (C) in normal control rats. The expression of CaSR was decreased in the epithelium and endothelium of the cornea (D), lens epithelium (E), and retina pigment epithelium (F) in diabetic mellitus rats.

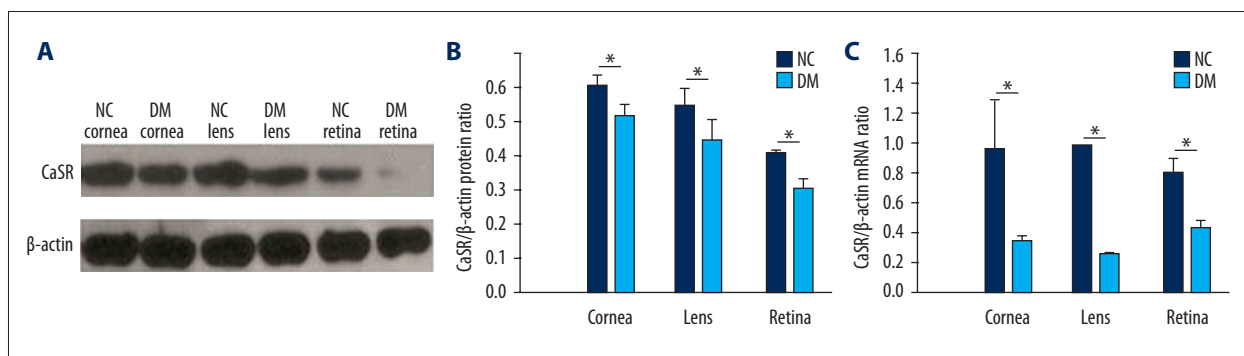


Figure 3. The expression of CaSR protein (A, B) and mRNA (C) in different eye tissues of normal control (NC) rats and diabetes mellitus (DM) rats. The levels of CaSR protein and mRNA were significantly declined in DM rats than NC rats ($p < 0.05$).

The expression of CaSR protein and mRNA in normal and DM rat eyes

CaSR protein and mRNA were mostly expressed in the cornea, followed by the lens and the retina ($p < 0.001$, Figure 3). They were significantly decreased in the DM rat eyes compared with NC rat eyes (Figure 3).

Discussion

The expression of CaSR in the eye has been rarely reported. Our study aimed to make a primary exploration of the distribution and expression of CaSR in normal and diabetic rat eyes. The present study showed that CaSR was detected in the epithelium and endothelium of the cornea, lens epithelium, and

RPE in normal rat eyes. It had decreased expression in all the eye tissues of diabetic rats.

Keratopathy and retinopathy are most common in diabetic eye disease. Diabetic keratopathy includes recurrent erosions, delayed wound healing, ulcers, and edema. These changes undoubtedly related to epithelial dysfunction and alterations in epithelial basement membrane occur. Our study showed that CaSR was expressed in the epithelium (mainly in epithelial basement membrane) and the endothelium of the cornea in normal rats, and was significantly decreased in diabetic rats. This suggested that the decreased expression of CaSR was responsible for diabetic keratopathy. Corneal epithelium forms a barrier for eyes, and the proliferation of epithelial cells is associated with corneal epithelial defects [15]. Corneal epithelial reconstruction mainly depends on the epithelial basement

membrane [16]. The primary function of the endothelium is to maintain corneal transparency by regulating corneal hydration and nutrition through a high-permeability barrier and metabolic pump. The barrier function of the cornea endothelium is dependent upon a sufficient number of endothelial cells and intact tight junctions between the endothelial cells [16]. Furthermore, recent research has found that CaSR participates in the [Ca²⁺]-induced assembly of tight junctions through an intracellular mechanism in Madin-Darby canine kidney cells [17]. In this regard, reduced CaSR may cause the damage of tight junction in epithelial and endothelial cells, and ultimately led to corneal dysfunction.

Sixteen weeks after STZ injection, cataracts occurred in all the diabetic rats. We know that epithelial cells and their active transport mechanisms maintain lens homeostasis and clarity. In our study, the expression of CaSR was remarkably declined in the lens epithelium in these rats. This demonstrated that decreased expression of CaSR was related to cataracts. This result was consistent with previous research that reported that activating CaSR mutation in a mouse model was associated with cataracts [10].

RPE forms the outer blood retinal barrier and it controls the flow of solutes and fluid from the choroid vessels into the outer

retina. Apart from this, the other functions of RPE are the following: transport of nutrients, ions and water; phagocytosis of shed photoreceptor membranes; absorption of light and protection against photo oxidation; secretions of various factors and exosomes; stabilizes ion composition in the subretinal space [18,19]. Therefore, dysfunction of RPE could cause many kinds of retinopathy. CaSR was mainly detected in RPE in the retina in this study, and decreased in diabetic rats. Therefore, this may be an important reason for diabetic retinopathy.

Hyperglycemia is a stimulator of the synthesis of diacylglycerol (DAG) and free fatty acid (FFA); and increased DAG and FFA could activate PKC [20]. In addition, PKC is a negative regulatory factor for CaSR [21]. Finally, increased PKC inhibits the expression of CaSR.

Conclusions

This *in vitro* study provided new evidence for the expression of CaSR in normal rat eyes and significantly decreased expression in diabetic rat eyes. This demonstrated that CaSR is an important agent in the development and progression of diabetic eye diseases. Further studies need to explore the CaSR-induced signaling pathways in the eye.

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