• Basic Research •

Activation of the ATP-P2X pathway by TRPV4 in acute ocular hypertension

Li Sun, Ke Yao, Hong Zhang, Wei Chen

Department of Ophthalmology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430000, Hubei Province, China

Co-first authors: Li Sun and Ke Yao

Correspondence to: Wei Chen and Hong Zhang. Department of Ophthalmology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430000, Hubei Province, China. 450741814@qq.com; dr_zhanghong@vip.163.com

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Abstract

● **AIM:** To measure the expression of transient receptor potential cation channel subfamily V member 4 (TRPV4) in the rat cornea and determine whether it is related to adenosine triphosphate (ATP) generation in a rat model of acute ocular hypertension (AOH).

● **METHODS:** Immunofluorescence staining of TRPV4, P2X2 receptor, P2X3 receptor, and β3-tubulin in rat corneal longitudinal sections and paved was performed to clearly display histological structures. Rat models of AOH and agonist/antagonist-treated groups were established and corneal ATP was measured using an ATP assay. The independent *t*-test and simple linear correlation model were adopted for statistical analyses.

● **RESULTS:** Immunofluorescence staining of rat cornea sections revealed that epithelial and endothelial membranes showed strong immunoreactivity for TRPV4 and P2X2 receptor and coexpression with β3-tubulin in the rat corneal epithelial layer. Corneal ATP was significantly higher in the AOH rat model than in the control (P<0.05) and apparently lower after pretreatment by applying eyedrops of TRPV4 antagonist RN1734 with 30-40 mm Hg intraocular pressure (IOP; P<0.05). A simple linear regression model showed a positive correlation between rat corneal ATP and IOP values (R^2=0.996, P=0.0134) from the normal IOP (113 mm Hg) to 40 mm Hg. At 10-40min after anterior chamber injection of GSK1016790A (0.01 mL, 50 nmol/L in 0.9% NaCl), corneal ATP was significantly higher than in the control group (P<0.05), which peaked at 10min. The ATP concentration of the normal epithelium was higher than that of the endothelium in the AOH rat model and after anterior chamber injection of GSK1016790A (P<0.05).

● **CONCLUSION:** The ATP concentration in the AOH rat cornea is increased by TRPV4 activation.

● **KEYWORDS:** TRPV4; cornea; adenosine triphosphate; acute ocular hypertension

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INTRODUCTION

Glaucoma is an optic neuropathy, specifically a neurodegenerative disease characterized by loss of retinal ganglion cells and their axons[1-3]. It was predicted that 80 million people will suffer from glaucoma worldwide by 2020[4]. Increased intraocular pressure (IOP) is a major risk factor for glaucoma, and elevation of IOP usually causes ocular pain, a typical symptom of glaucoma[1]. Our previous studies showed that the steady state of regulation is provided by several nuclei located in multiple brain areas[5-6]. However, the mechanism underlying transduction of pressure signals remains unclear. Therefore, in this study, we investigated the transduction of such pressure signals.

The transient receptor (TRP) superfamily comprises a large number of cation channels that can be divided into seven subfamilies[7-8]. The transient receptor potential vanilloid 4 (TRPV4) ion channel, a member of the vanilloid subfamily of TRP channels, is activated by membrane stretch, non-noxious warm temperatures, and a range of chemical activators[9-12]. TRPV4 purinergic signaling plays an important role in mechanosensory transduction of mammalian organs[13-16]. For example, when bladder filling activates TRPV4 in epithelial cells, released adenosine triphosphate (ATP) acts on P2X2/3 receptors in the nerve fiber end. TRPV4−/− mice exhibit a lower frequency of voiding contractions and a higher frequency of non-voiding contractions[17]. Additionally, decreased intravesical stretch evokes ATP release in the whole bladder isolated from TRPV4−/− mice[14]. TRPV4 is expressed in the cornea, and sensory nerves are rich in the cornea[18-20].

Increased IOP leads to increased levels of extracellular ATP in the anterior chamber of patients with primary acute angle-closure glaucoma[21], but the original site of ATP is not yet clear.

We hypothesized that TRPV4 in the cornea may participate in mechanosensory transduction and pain sensation through ATP. Therefore, we investigated changes of corneal ATP in a rat model of acute ocular hypertension and after TRPV4 agonist/antagonist treatments.

MATERIALS AND METHODS

Ethical Approval Six- to eight-month-old male Sprague-Dawley rats, weighing 150-200 g, were obtained from the Experiment Animal Center of Tongji Medical College, Huazhong University of Science and Technology, China. All animal experiments were conducted in accordance with the U.S. National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Acute Ocular Hypertension Rat Models Established with Various IOPs Intraperitoneal injection of 0.3-0.4 mL/100 g of 10% chloral hydrate was used to anesthetize rats. The rats (right eyes) were randomly divided into various groups. Rats were placed on their left side and their right eyes were fully exposed. Corneas were anesthetized topically with eyedrops of 2% proparacaine. To avoid damage to the lens and iris, the anterior chamber of the right eye was cannulated at the 6 o'clock position to the corneoscleral limbus with a 30 G needle parallel to the iris. After addition of a balance salt solution, IOPs in the various groups were increased and maintained at 30, 40, 50, or 70 mm Hg for 40min. Left eyes of the rats served as controls and only received an anterior chamber puncture. Perfusion pressure and duration were adjusted according to groups.

Immunofluorescence Rats were sacrificed by intraperitoneal injection of 1 mL/100 g of 10% chloral hydrate. Eyeballs were fixed with 4% paraformaldehyde for 24h. The cornea was obtained and gently washed thrice with phosphate-buffered saline (PBS) and then treated with 0.2% Triton-2% BSA for more than 1h to block non-specific binding. Sections were incubated with a rabbit anti-TRPV4 antibody (1:200, BA28762, Bosterbio, CA, USA), mouse anti-P2X3 antibody (1:200, A7078, Abclonal, China), and mouse anti-β3 tubulin antibody (1:200, sc8005, Santa Cruz Biotechnology, USA) at 4°C overnight. Cornea sections were washed again with PBS and subsequently incubated with secondary antibodies, donkey anti-mouse Alexa Fluor 488 and donkey anti-rabbit Alexa Fluor 594 (1:200, Thermo, Rockford, USA), at room temperature for 2 while protected from light. After counterstaining with DAPI (1:100, BP0740, Bosterbio, China) for 10min, the sections were imaged under a fluorescence microscope (Olympus Co. Ltd., Japan).

Anterior Chamber Injection Rats were anesthetized and maintained as described above. Then, 0.01 mL of a 50 nmol/L GSK1016790A solution was injected slowly into the anterior chamber. The rats' left eyes served as controls and only received a normal saline injection.

Measurement of Corneal Adenosine Triphosphate After establishment of the rat models, they were euthanized by intraperitoneal injection of 1.5 mL/100 g of 10% chloral hydrate. Corneal tissue was obtained and separated into two pieces (epithelial layer+Bowman's membrane+stroma layer and the endothelium+Descemet membrane). A standard curve of the ATP concentration change was constructed using Graphpad Prism 7.0 (GraphPad Software, USA). ATP measurement was conducted using an ATP assay kit (S0026, Beyotime Biotechnology, China), according to the manufacturer's procedure. Each sample (30 μL) and diluted ATP standard (30 μL) were added to the assay plate with a blank well. Relative light units (RLUs) were measured by a microplate reader (Fluoroskan Ascent, Thermo Scientific, FL, USA). The ATP concentration (nmol/L) of each sample was calculated by the standard curve according to their RLUs. Protein concentrations of samples were determined using a BCA kit (AR0146, Bosterbio, CA, USA). Corneal ATP was standardized using the unit nmol/L/mg protein.

Statistical Analysis Results are presented as the mean±standard deviation. Data were analyzed and plotted using SPSS software package version 20.0 (SPSS Inc., Chicago, USA) and GraphPad Prism 7.0, respectively. To determine statistically significant differences, one-way ANOVA or the independent two sample *t*-test were used to compare differences among multiple or two groups, respectively. Differences with *P*-values of less than 0.05 were considered as significant.

RESULTS

Expression of TRPV4 in the Membranes of the Rat Corneal Epithelium and Endothelium To confirm TRPV4 localization in the rat eye, we performed immunofluorescence staining of rat corneal longitudinal sections, revealing that epithelial and endothelial membranes had strong immunoreactivity for TRPV4, while the corneal stroma did not express TRPV4 (Figure 1).

Expression of P2X2 Receptor in the Rat Cornea without Coexpression of P2X3 To confirm localization of P2X2 and P2X3 in the rat eye, we conducted immunofluorescence staining of P2X2 and P2X3 in rat corneal longitudinal sections. The cornea did not show immunoreactivity for P2X3 compared with the positive retinal tissue (Figure 2). The corneal epithelium and endothelium were positive for P2X2 staining and little positive distribution was found in the stroma (Figure 3).

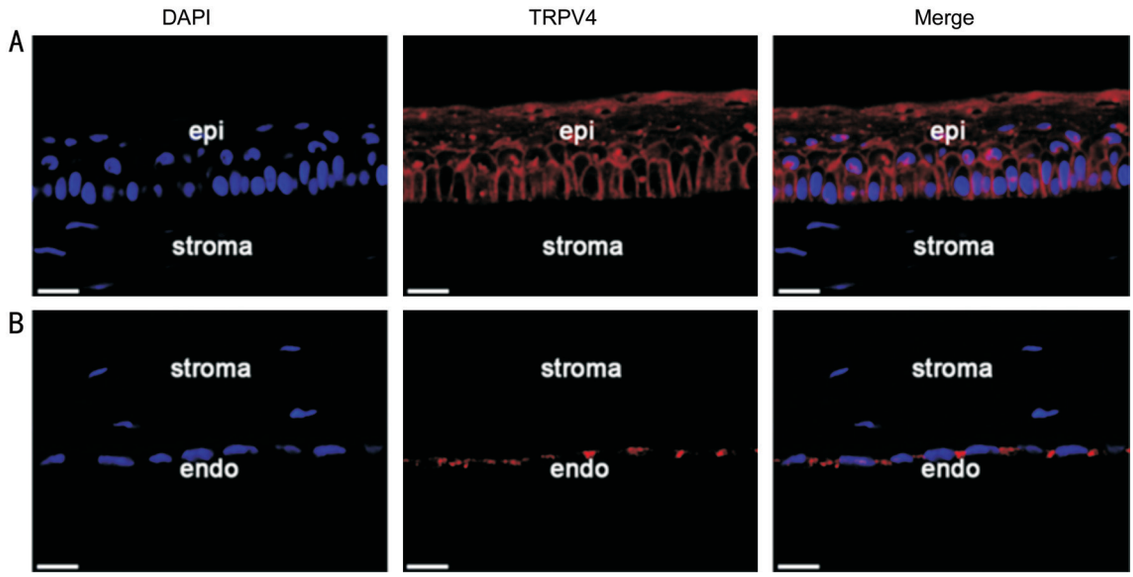

Figure 1 Detection of TRPV4 in the normal rat cornea by immunofluorescence A: Corneal epithelium and partial stromal layer; B: Partial corneal stroma and corneal endothelium. Epi: Epithelium; endo: Endothelium. Red: TRPV4; Blue: DAPI nuclear counterstaining. Scale bars=10 μm.

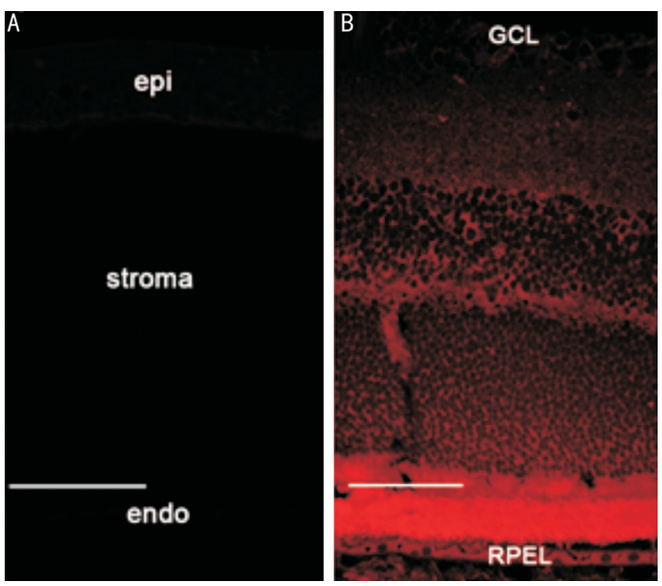

Figure 2 Immunolocalization of P2X3 in the normal rat cornea A: Negative immunostaining of P2X3 in the cornea; B: Positive staining of P2X3 in the retina. Epi: Epithelium; endo: Endothelium. Red: P2X3. Scale bars=50 μm.

Coexpression of P2X2 and β3-tubulin in the Rat Cornea Next, we explored coexpression of P2X2 and β3-tubulin, a marker of never fibers, in the paved cornea. Never fibers were distributed erratically around the cornea, entered the cornea through the stroma, and crossed into Bowman's membrane with formation of a compact never plexus, ending at epithelial cell spaces (Figure 4). Double immunofluorescence staining showed that the corneal epithelium was marked by β3-tubulin and P2X2 immunoreactivity with an overlap of both markers (Figure 5).

Changes of Corneal ATP Caused by AOH and TRPV4 Antagonist RN1734 We divided rats into various groups randomly (n=6 eyes) and established acute ocular hypertension (AOH) rat models with IOPs of 30, 40, 50, and 70 mm Hg for 40min. After applying eyedrops of TRPV4 antagonist RN1734 (50 μmol/L, five times every 10min), the same AOH rat models were also established. Control rats were only subjected to an anterior chamber puncture. Their corneas were then obtained and ATP concentrations were determined (Table 1). From 30 to 70 mm Hg IOP, corneal ATP was significantly higher than in the control group (P<0.05; Figure 6A). A simple linear regression model showed a positive correlation between rat corneal ATP and IOP values (R^2=0.996, P=0.0134) from the normal IOP (about 13 mm Hg) to 40 mm Hg (Figure 6B). After pretreatment with RN1743-containing eyedrops, the corneal ATP concentration at IOPs of 30 or 40 mm Hg was similar to that in the control group (P=0.3529, 0.6095, respectively). However, from 50 to 70 mm Hg IOP, corneal ATP was significantly higher than in the control group (P=0.0005, 0.0066, respectively; Figure 6C). Compared with RN1743 eyedrop and control groups, the corneal ATP concentration at IOPs of 30 and 40 mm Hg showed significant differences, but there were no significant differences at IOPs of 50 and 70 mm Hg (Table 1; Figure 6D).

Significant Increase of corneal ATP Caused by TRPV4 Agonist GSK1016790A Rats were divided into various groups randomly (n=6 eyes) and injected with a solution containing TRPV4 agonist GSK1016790A (0.01 mL, 50 nmol/L) through the anterior chamber. Control rats were injected with a 0.9% NaCl solution in the same manner. Corneas were acquired at various times after injection of the GSK1016790A-containing solution and then the ATP concentration was measured. At 10-40min after injection of the GSK1016790A solution into the anterior chamber, corneal ATP was

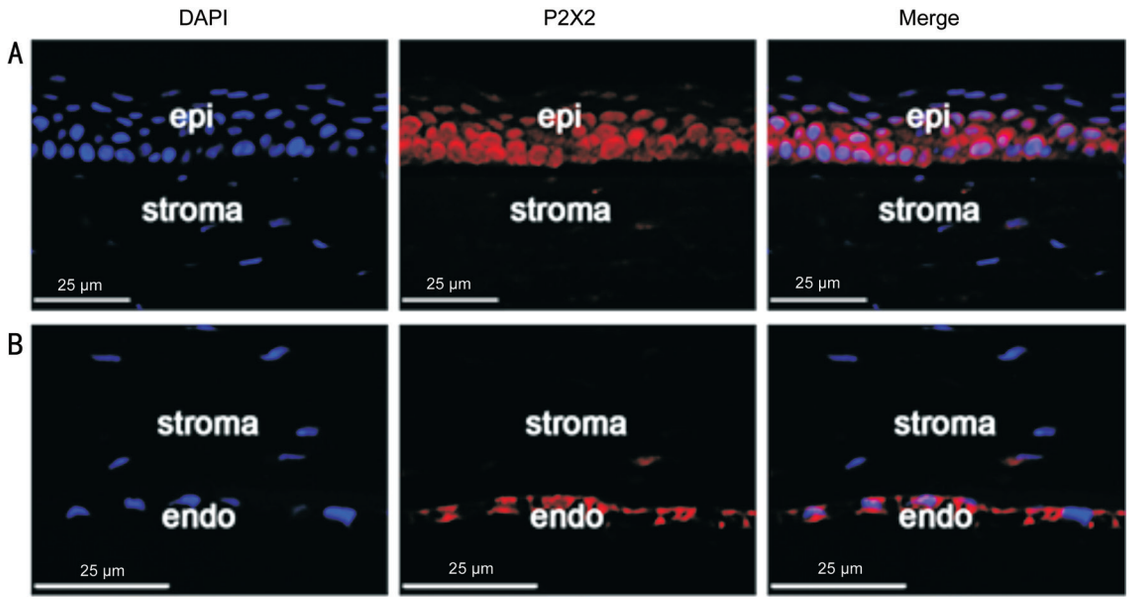

Figure 3 Immunolocalization of P2X2 in the normal rat cornea A: Corneal epithelium and partial stromal layer; B: Partial corneal stroma and corneal endothelium. Epi: Epithelium; endo: Endothelium. Red: P2X2; Blue: DAPI nuclear counterstaining. Scale bars=25 μm.

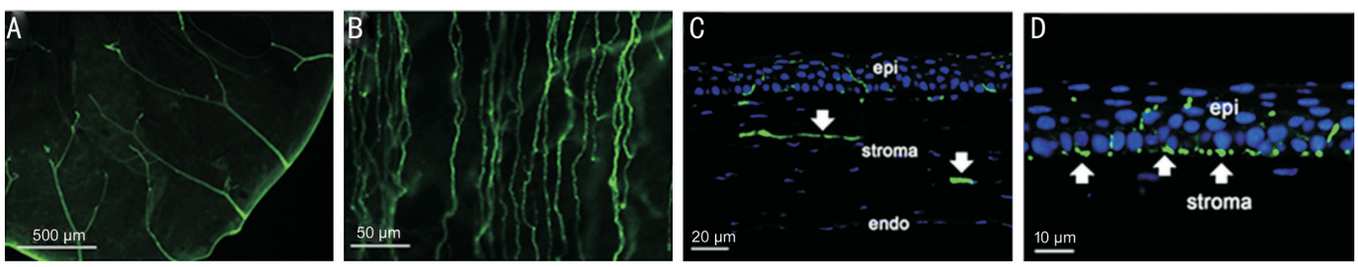

Figure 4 Immunolocalization of innervation in the normal rat cornea A, C: Stromal nerve (downward white arrows: green fluorescence); B: Epithelial nerve (green fluorescence); D: Upward course of the subepithelial nerve through Bowman's membrane (upward white arrows: green fluorescence). Epi: Epithelium; endo: Endothelium. Green: β3-tubulin (nerve); Blue: DAPI nuclear counterstaining.

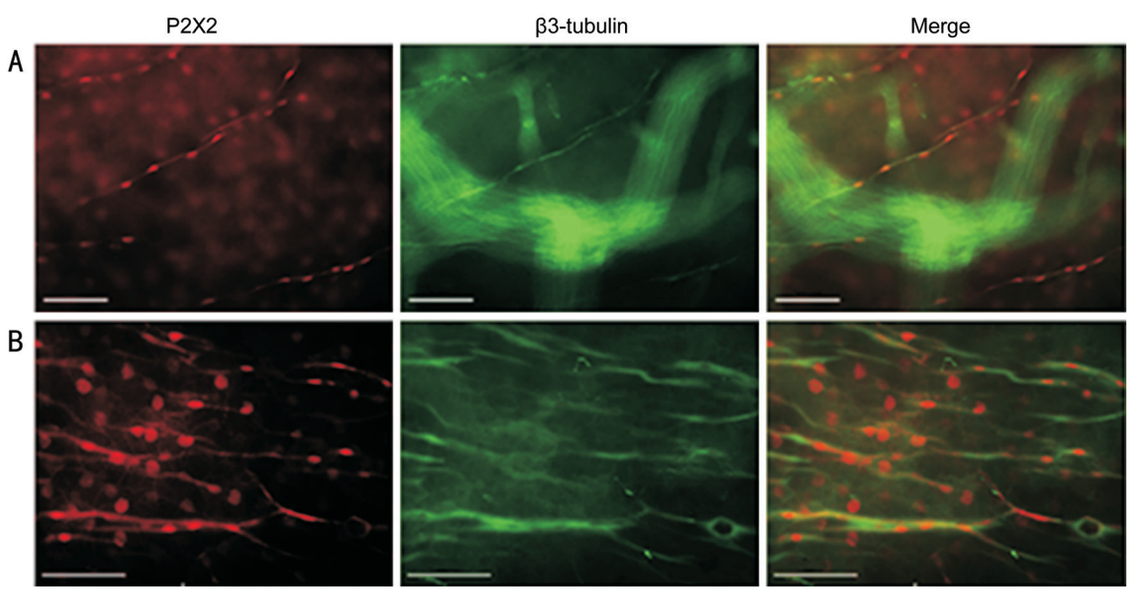

Figure 5 Immunolocalization of P2X2 and β3-tubulin coexpression in the rat cornea A: Cornea of a normal rat; B: Cornea of a rat AOH model. Red: P2X2; Green: β3-tubulin. Scale bars=50 μm.

Table 1 ATP content variation under various anterior chamber perfusion pressure gradients and comparison of statistical differences with or without RN1734 pretreatment

ATP (nmol/L/mg)	AOH	RN1734+AOH	*P*
Control	424.0±85.8	424.0±85.8	-
30 mm Hg	909.3±190.5	381.5±63.8	<0.0001
40 mm Hg	1208.7±257.2	393.5±111.6	<0.0001
50 mm Hg	1089.7±195.7	1046.4±291.4	0.7687
70 mm Hg	1024±254.6	1085.9±320.4	0.7207
P	<0.0001	<0.0001	-

ATP: Adenosine triphosphate; AOH: Acute ocular hypertension.

significantly higher than in the control group (P<0.05), which peaked at 10min (Figure 7).

Higher ATP Concentrations in the Epithelium than it in the Endothelium Rats were divided into various groups randomly (n=6 eyes) and injected with a TRPV4 agonist GSK1016790A-containing solution (0.01 mL, 50 nmol/L) through the anterior chamber and then subjected to AOH model establishment with an IOP of 7 mm Hg. The cornea was then obtained and separated into two parts: the epithelium+Bowman's and stromal layers) and the endothelium+Descemet's membrane. At 10-40min after injection of the GSK1016790A solution into the anterior chamber, epithelial ATP was significantly higher than in the endothelium (P<0.05; Figure 7B). A simple linear regression model did not show a correlation between the corneal ATP concentration and the duration after anterior chamber injection of the GSK1016790A solution, and epithelial ATP was significantly higher than endothelial ATP (P<0.05). Another simple linear regression model did not show a correlation between the corneal ATP concentration and the duration of anterior chamber perfusion pressures (P>0.05). ATP concentrations were always higher in the epithelium than in the endothelium of rat AOH models with anterior chamber injection of the GSK1016790A solution (P<0.05; Figure 7D).

DISCUSSION

Wang *et al*[17] observed that ATP was released from the bladder mucosa in response to filling or pressure by *in situ* rabbit experiments. Gevaert *et al*[14] found decreased intravesical ATP release evoked by stretch in bladders from TRPV4−/− mice compared with normal mice and a defect in the voiding reflex. Therefore, activation of purinoceptors by TRPV4-dependent ATP release is speculated to be important for initiation of the voiding reflex. In this study, immunofluorescence showed that TRPV4 was expressed in the membranes of the rat corneal epithelium and endothelium. Corneal endothelial cells have been reported to release ATP upon mechanical stimulation[22]. Zhang *et al*[21] found increases in the levels of extracellular ATP in aqueous humor samples of human patients with primary acute angle-closure glaucoma. In their study, this elevated ATP had a positive correlation with IOP and ocular pain.

Increases in IOP and ATP are associated with many changes including alterations in the vascular supply to the eye, compression of the lamina cribrosa, loss of retinal ganglion cells, corneal edema, and atrophy of structures in the anterior segment[21,23-24]. P2X is a ligand-gated ion channel that responds to ATP, ADP, uridine triphosphate (UTP), and uridine diphosphate (UDP). Seven subunits have been found (P2X1-7) and the receptor functions in the form of three or four identical or different subunits[25-26]. P2X receptors have high selectivity for calcium ions (Ca^{2+})[27-29]. Sensory nerves express P2X2, P2X3, P2X2/3, P2X4, and P2X6 receptors, among which P2X2 and P2X3 play an important role in pain transduction[27,30]. Nerve bundles enter the cornea at the periphery in a radial fashion parallel to the corneal surface towards the center below the anterior third of the stroma in human corneas. Stromal nerves in the cornea subsequently divide into smaller branches, most of which penetrate upward into the epithelium. Purinergic receptors may participate in mechanosensory transduction and pain pathways in visceral, cutaneous, and musculoskeletal tissues[27]. Immunofluorescence staining of rat corneal longitudinal sections and paved was performed, revealing P2X2 receptor, but not P2X3 receptor, expression in the rat corneal epithelium and endothelium, and P2X2 receptor and β3-tubulin coexpression in the rat cornea epithelium layer,

In our study, anterior chamber perfusion pressure of 30 to 70 mm Hg for 40min gave significantly higher corneal ATP concentrations than in the control group (P<0.05). From normal IOP to 40 mm Hg, there was a positive correlation between rat corneal ATP and IOP values (R^2=0.996, P=0.0134), and corneal ATP content in rats with 40 mm Hg anterior chamber perfusion pressure was significantly higher than that in rats with 30 mm Hg. However, the ATP content showed no significant changes when anterior chamber perfusion pressure was changed from 40 to 70 mm Hg.

TRPV4 is involved in many different cellular functions such as temperature sensation, tactile sense, and osmotic pressure. It is activated by various physical and chemical stimuli including hyposmolality, heat, mechanostimuli, arachidonic acid, and synthetic phorbol ester alpha-phorbol 12,13-didecanoate (4αPDD)[10,31-32]. Activation of the TRPV4 ion channel is enhanced by the serine/threonine kinases PKC and PKA[32-33]. GSK1016790A, which was discovered as a specific small molecule agonist of TRPV4, causes TRPV4-specific Ca^{2+} influx[9,12,34]. RN1734 is a small molecule antagonist of the TRPV4 channel, which completely antagonizes activation of TRPV4 mediated by 4αPDD and hyposmolality[35]. After applying TRPV4 antagonist RN1734 eyedrops (50 μmol/L, five times every 10min) with 40min of perfusion, rat corneal ATP at 30 or 40 mm Hg anterior chamber perfusion pressure

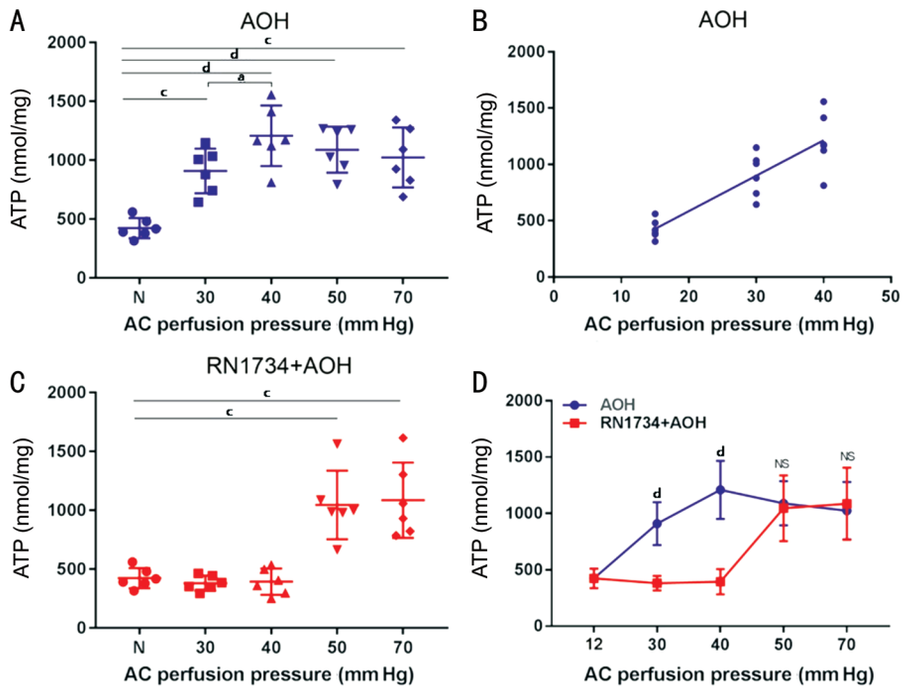

Figure 6 ATP content variation under different anterior chamber perfusion pressure gradients and comparison of the statistical differences with or without RN1734 pretreatment A: Rat corneal ATP content variation under different anterior chamber perfusion pressures; B: Correlation analysis of the rat corneal ATP content variation under different anterior chamber perfusion pressures; C: Rat corneal ATP content variation under different anterior chamber perfusion pressures with RN1734 pretreatment; D: Variation trend and comparison of the statistical differences in ATP content between groups with or without RN1734 pretreatment. N: Control group (IOP=11.98±0.93 mm Hg). [a]*P*<0.05; [b]*P*<0.01; [c]*P*<0.001, [d]*P*<0.0001; NS: No statistically significant difference.

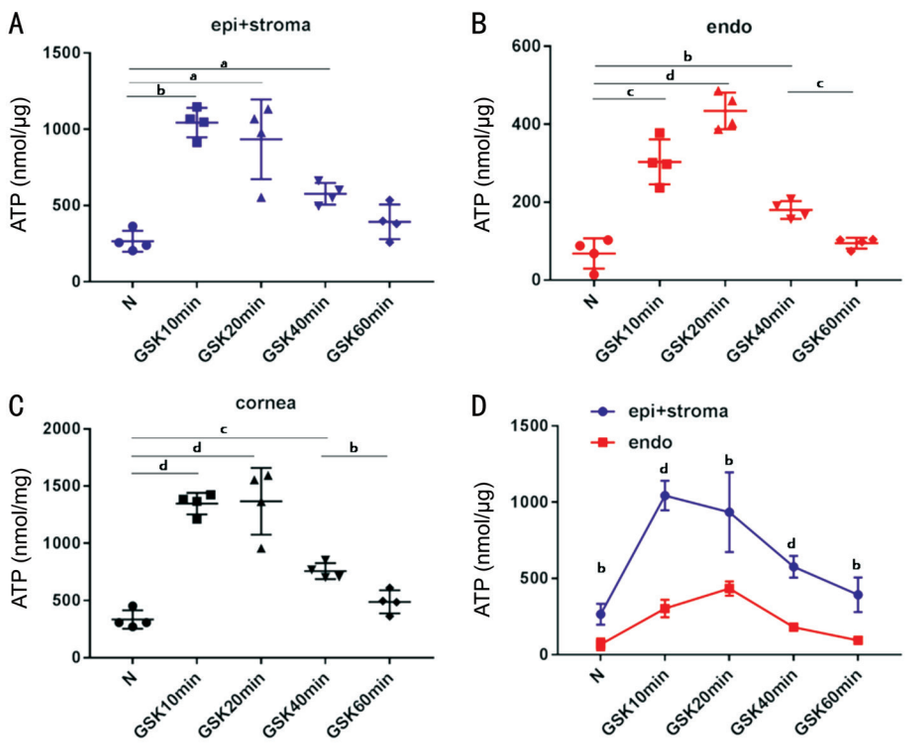

Figure 7 ATP content variation of the rat corneal epithelium+stroma, corneal endothelium, and full thickness cornea with GSK1016790A anterior chamber injections A: ATP content variation of the rat corneal epithelium+stroma after GSK106790A anterior chamber injections at various times; B: ATP content variation of the rat corneal endothelium after GSK106790A anterior chamber injections at various times; C: ATP content variation of the rat full thickness cornea after GSK106790A anterior chamber injections at various times; D: Variation trend and comparison of the statistical differences between rat epithelium+stroma and corneal endothelium ATP contents after GSK106790A anterior chamber injections at various times. [a]*P*<0.05; [b]*P*<0.01; [c]*P*<0.001, [d]*P*<0.0001; NS: No statistically significant difference.

was similar to that in the control group (P=0.3529, 0.6095, respectively). However, from 50 to 70 mm Hg, rat corneal ATP was significantly higher than in the control group (P=0.0005, 0.0006, respectively). This was close to the critical point of eye pain and we found that P2X2 and β3-tubulin were coexpressed in the rat cornea, which provides further evidence that an increase of corneal ATP is related to eye pain. We found that RN1734 eyedrops antagonized the correlation between rat corneal ATP and IOP values from the normal IOP to 40 mm Hg. Moreover, we found that anterior chamber injection of the TRPV4 agonist significantly increased rat corneal ATP concentrations for 10-40min compared with the control group. These results suggested that the rat cornea with a high IOP may increase ATP content through the TRPV4 channel.

At 70 mm Hg anterior chamber perfusion pressure, rat corneal ATP concentrations at 10-40min were significantly higher than in the control group. However, the simple linear regression model did not show a correlation between the rat corneal ATP concentration and the duration of anterior chamber perfusion pressure (P>0.05). After 40min of anterior chamber perfusion, corneal ATP was apparently lower, while ATP concentrations were always higher in the epithelium and stroma than that in endothelium (P<0.05). These results suggested that there was no significant correlation between the rat corneal ATP concentration and the duration of anterior chamber perfusion pressure. The epithelium and stroma might play a greater role than the endothelium in ATP generation, probably because TRPV4 expression was higher in the epithelium and stroma than in the endothelium. In humans, ATP translates stretch deformation of epithelial cells into an electrochemical signal[36], and P2X receptors play an important role in pain conductivity[37-38].

There are some limitations to this study. We intend to culture corneal epithelial and endothelial cells to examine differences in ATP generation. Additionally, GSK1016790A and RN1734 need to be applied at various concentrations.

In conclusion, the rat cornea has the histological structure of the TRPV4-ATP-P2X2 receptor pathway. Additionally, the rat cornea might increase ATP content by activation of the TRPV4 channel under the conditions of acute ocular hypertension. Rat corneal ATP had a positive correlation with anterior chamber perfusion pressure from the normal IOP to 40 mm Hg, but did not show a correlation with the duration of anterior chamber perfusion. Moreover, the epithelium might play a more important role than the endothelium in increasing ATP.

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Conflicts of Interest: Sun L, None; **Yao K,** None; **Zhang H,** None; **Chen W,** None.

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