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Effect of cAMP and cGMP on Connexin43 Expression in Isolated Human and Bovine Ciliary Epithelium

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Abstract

The aim of the study was to assess the distribution of connexin43 (Cx43) and connexin40 (Cx40) in human and bovine ciliary bodies. The effect of the second messengers cAMP and cyclic cGMP on Cx43 protein expression was also investigated. Enucleated human eyes (remnant after corneal transplantation) and bovine eyes were used. Tissue preparations of the anterior segments of the eyes have proceeded for immunohistochemical staining with polyclonal antibodies of Cx43 and Cx40. Isolated ciliary bodies of human and bovine eyes were incubated with cAMP analog 8-Bromo-cAMP or the cGMP analog 8-Bromo-cGMP, the expression of Cx43 protein in the tissues was then assessed by Western blot assay. Both in human and bovine ciliary bodies, strong immunoreactivity of Cx43, but not Cx40, was observed predominantly in the apical cytoplasmic portions of the pigment ciliary epithelial cells and non-pigmented ciliary epithelial cells. In human ciliary body both cAMP and cGMP up-regulated Cx43 expression, while in the bovine ciliary body, cGMP increased Cx43 expression but cAMP decreased it. Cx43 is the major component of human and bovine gap junctions in the ciliary epithelium. The regulation on the Cx43 expression by cAMP and cGMP in human and bovine ciliary bodies suggests the possibly different roles of these signal messengers in the intracellular communication.

Keywords

Gap Junction, Connexin43, Connexin40, cAMP, cGMP, Aqueous Humor

1. Introduction

The ciliary processes of the eye are the site to produce aqueous humor (AH)

which is responsible for 1) Supply of nutrients and oxygen to the tissues inside the eye and removal of metabolic wastes; 2) Regulation of an adequate intraocular pressure (IOP) for maintenance of morphological and functional properties of the eyeball [1] [2]. Current models of AH secretion propose that the solutes, mainly NaCl, are transported into the pigmented epithelial (PE) layer which faces the stroma of the ciliary body, diffuse through the gap junctions to the non-pigmented epithelial layer, and finally exit to the posterior chamber [2] [3] [4] [5] [6]. The ion transport generates an osmotic gradient which draws the water pass from the stroma to the posterior chamber [7] [8].

Gap junctions are complexes of intercellular plasma membrane channels that act as conduits for the direct cell-to-cell communication by allowing the passage of small molecular less than 1 kDa, such as second messengers, current carried-ions and metabolites [9] [10]. Gap junctions are composed of connexins which have the same basic molecular structure and are named after their molecular weight in kDa. To date, more than 21 isoforms of connexin have been reported with distinct tissue-specific distributions in human [9] [11]-[16]. The expression of a limited number of connexin isoforms, such as connexin43 (Cx43) and connexin40 (Cx40), has been identified in the ciliary body in several species [17]-[22]. However, the expression, distribution, and functional modulation of connexins in human ciliary epithelium has not yet reported.

The second messengers cyclic adenosine monophosphate (cAMP) and cyclic guanylyl monophosphate (cGMP) play crucial roles in many ocular physiological procedures including the aqueous humor production [23]-[35], although there exists difference between different animal species including human being. The effect of cAMP and cGMP on connexin expression are not clear. In this study, we determined the distribution of two main structural connexins (Cx43 and Cx40) in human and bovine ciliary processes, and the influence of cAMP and cGMP on connexin expression was also tested.

2. Materials and Methods

2.1. Tissue Preparation

A total of ten human eyes were used in our study. Eyes were obtained from the eye bank of Sir Run Run Shaw Hospital, Hangzhou, China as the remnant after cornea transplantation. Human eyes were treated in adherence with the Declaration of Helsinki and the experiments was approved by the ethics committee at the Xiamen University Affiliated Eye Center. Bovine eyes were got from a local abattoir immediately after the animals were slaughtered. The use of bovine eyes in the study conformed to the ARVO Statement for the Use of Animals in Ophthalmology and Vision Research.

For immunohistochemistry, anterior segment the eye including the iris and the ciliary body were dissected, and proceeded for fixation in 4% paraformaldehyde for 24 h followed by paraffin embedding. Serial sections (3 μm thickness) were made for the tissues, and stained by 1) Hematoxylin and eosin; 2) Immu-

nostaining of Cx43 and Cx40.

For Western blot, the ciliary body was carefully dissected free of iris and rinsed with PBS. Ciliary bodies were either left quiescent, or incubated with the cAMP analogue 8-Bromo-cAMP (250 μM), or cGMP analogue 8-Bromo-cGMP (500 μM) in 24-well culture plates containing Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum, 2 mmol/l L-Glutamine, penicillin (100 U/ml) and streptomycin (100 μg/ml), at 37˚C in 5% CO_2 for 36 hours. Tissues were then taken out of the culture medium for Western blot assay.

2.2. Immunohistochemistry Staining of Cxs

Tissue sections in paraffin were dried in a 55˚C - 60˚C oven and deparaffinized in xylems, dehydrated with graded alcohols. The tissue preparations were then immersed in 0.25% potassium permanganate for 1 h and 1% oxalic acid for 30 min to eliminate the pigment. Tissues were subsequently treated with 0.3% hydrogen peroxide in PBS for 10 min to block endogenous peroxidase activity. After this, sections were incubated with 10% normal goat serum for 10 min and exposed to rabbit polyclonal antibody against Cx43 (1:500) and Cx40 (1:25) for 2 h at room temperature. Rinsed with PBS for 3 times, the appropriate secondary antibody against rabbit and mouse immunoglobulins were added subsequently for 1 h incubation. Diaminobenzidine peroxidase reaction was conducted. Negative controls were achieved by replacing the primary antibody with PBS. Controls and experimental preparations were handled in a similar manner.

2.3. Cx43 Protein Determination by Western Blot Assay

Total protein of the ciliary body was extracted as follows. Tissue homogenization and lysis were conducted in a buffer containing 0.5% Nonidet P-40, 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride and 5 mM aprotinin. Protein extraction was performed with the RIPA lysis buffer according to manufacturer's instruction and quantified with the BCA Protein Assay Kit. Protein samples were electrically separated by SDS/PAGE (Bio-Rad, USA), and electro-transferred onto 0.4-μM polyvinylidene difluoride membranes. After blocking with 5% nonfat milk for 1 hour, the membranes were incubated with anti-Cx43 primary antibody (1:5000) containing 1% bovine serum albumin overnight. After being rinsed with PBS-0.1% Tween20, the membranes were probed with HRP-conjugated goat anti-rabbit IgG for 1 hour. Immunoreactivity was detected by the enhanced chemiluminescence by Bio-RAD ChemiDox XRS (Bio-Rad, USA). Mouse monoclonal anti-β-actin antibody was used as loading control primary antibody for all samples. The membranes were treated with 30% hydrogen peroxide for 15 min at 37˚C and blocked with 5% nonfat milk for 18 hours, then reprobed for β-actin. Bound antibody was determined with goat anti-rabbit IgG (horseradish peroxidase-linked whole antibody) and developed by chemiluminescence reaction kit (Amersham Pharmacia Biotech, UK). Optical density of each protein band was assessed with the Quantity One software (Bio-Rad Sys-

tems, USA) and normalized to β-actin.

2.4. Antibodies and Chemical Reagents

The primary rabbit polyclonal anti-Cx43 and anti-Cx40 antibodies were got from Abcam (UK). Mouse monoclonal anti-β-actin antibody and horseradish peroxides-conjugated goat anti-rabbit IgG were obtained from Beyotime Institute of Biotechnology (China). The second antibodies and DAB for immunostaining were bought from Dako company (Denmark). 8-Bromo-cAMP and 8-Bromo-cGMP were from Beyotime Institute of Biotechnology (China). Enhanced chemiluminescence was purchased from Amersham Bioscience (UK). Other reagents for immunohischemistry and Western blot were purchased from Maxim-Bio (China).

3. Results

3.1. Immunohistochemical Localization of Cx43 and Cx40 in the Ciliary Body

In human and bovine eyes studied, immunoreactivity of Cx43 was observed in the epithelial layers of the ciliary body, but was not seen in the connective tissue and muscles of the ciliary body, trabecular meshwork or Schlemm's canal (Figure 1 and Figure 2). In the epithelial cells, specific immunostaining of Cx43 could be observed largely along the apical cytoplasm of the PE and NPE (Figure 1 and Figure 2).

Positive staining of Cx40 could not be noticed either in human or bovine ciliary body (Figure 3).

3.2. Effect of cAMP and cGMP on Cx43 Protein Expression in Human and Bovine Ciliary Bodies

Baseline expression of Cx43 protein was detected in human and bovine ciliary bodies by Western blot assay (Figure 4, control). When pre-incubated with

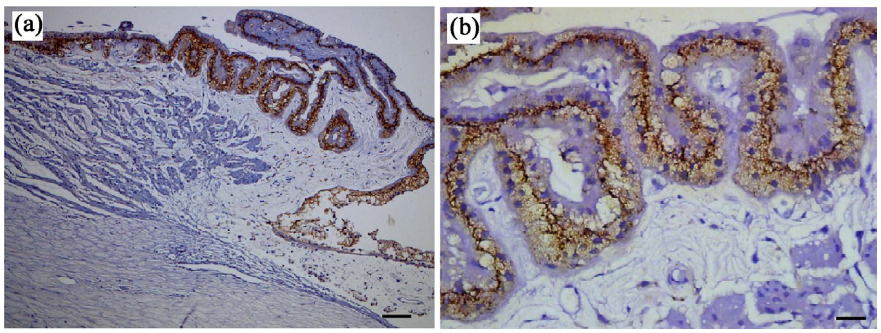

Figure 1. Immunohistochemical localization of Cx43 in human ciliary body. (a) ×4. Distribution of positive Cx43 staining was seen in the ciliary epithelial layers, but not in the ciliary stroma, ciliary muscle, trabecular meshwork, or the Schlemm's canal. Scale bar: 100 μm. (b) ×40. Positive Cx43 immunostaining distributes predominantly along the apical cytoplasm of the pigmental epithelial and the non-pigmental epithelial cell layers. Scale bar 100 μm.

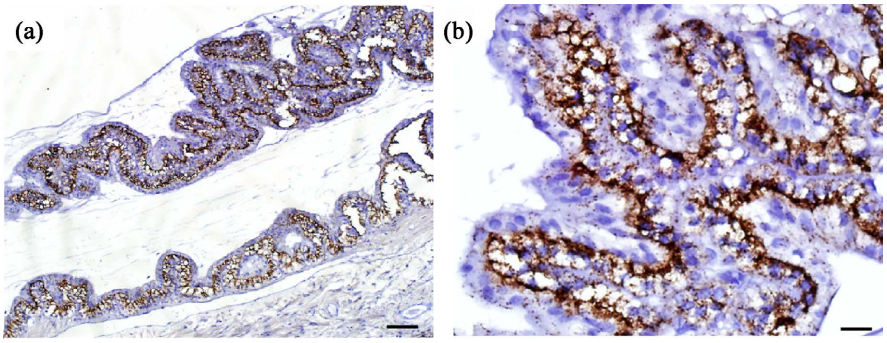

Figure 2. Immunohistochemical localization of Cx43 in bovine ciliary body. (a) ×4. (b) ×40. Distribution of positive Cx43 staining was seen in the ciliary epithelial layers, predominantly along the apical cytoplasm of the pigmental epithelial and the non-pigmental epithelial cell layers. Scale bar: (a), 100 μm; (b), 10 μm.

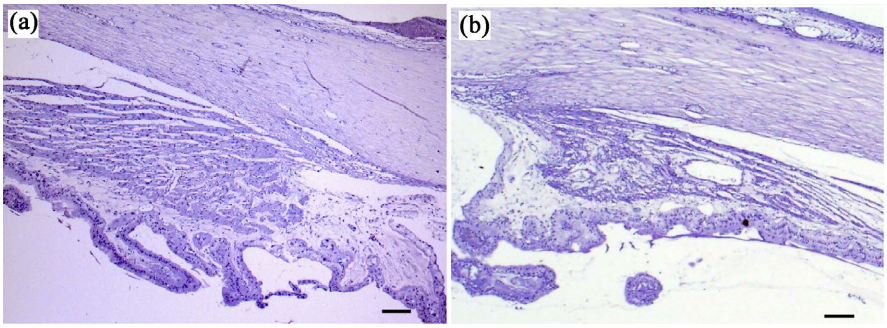

Figure 3. Immunohistochemical localization of Cx40 in human (a) and bovine (b) ciliary bodies. Positive Cx40 immunostaining could not been observed in the ciliary bodies (×4). Scale bar: 100 μm.

cGMP analogue 8-bromo-cGMP, the protein expression of Cx43 in both human and bovine ciliary bodies was significantly increased. Cyclic analogue AMP 8-bromo-cAMP significantly increased Cx43 protein expression in human ciliary body, but, decreased Cx43 protein expression in bovine ciliary body (**Figure 4**).

4. Discussion

Aqueous humor is produced in the ciliary processes and secreted to the posterior chamber of the eye. Aqueous humor formation is essential for the regulation of the IOP level to maintain the integrity of the globe. In addition, it provides nutrients and oxygen to tissues inside without blood supply, such as the lens and cornea. Attempt to reduce aqueous humor formation is also an important strategy to treat glaucomatous optic neuropathy [36]. The ciliary body epithelium (CBE) of the eye consists of a dual-layer of epithelial cells and is the site of aqueous humor production. The CBE is unique and complex with the PE rests on the outer side facing the connective tissue stroma while the NPE rests on the inner side facing the posterior chamber. Several animal models have been used for the study of aqueous humor secretion; however, significant species variations in the transport mechanisms across the ciliary epithelium were reported [37] [38]. For example,

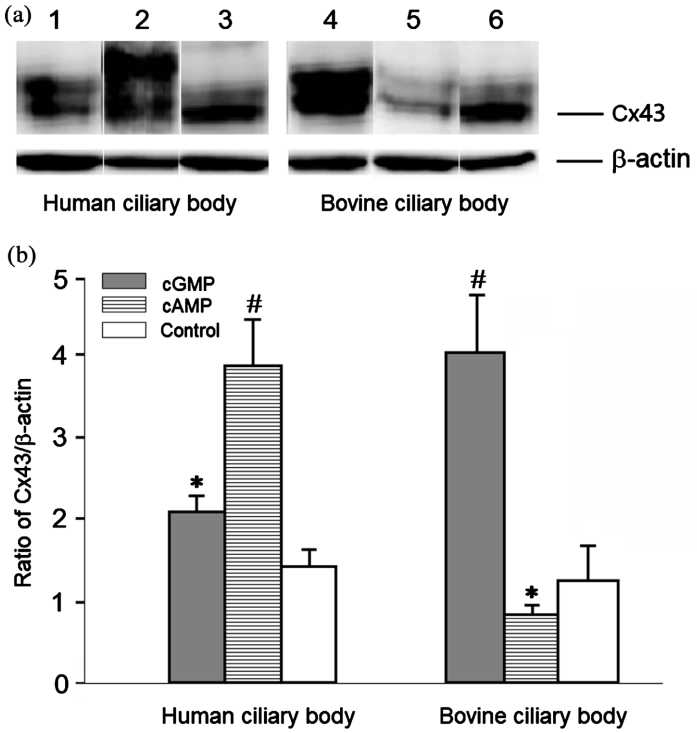

Figure 4. Effect of cAMP and cGMP on Cx43 protein expression in human and bovine ciliary bodies. (a) Lane 1 and 4: 8-bromo-cGMP, lane 2 and 5:8-bromo-cAMP, lane 3 and 6: control. Each experiment was repeated for 3 times and the results were summarized in (b). $^{*}p < 0.05$, $^{\#}p < 0.01$, vs. control.

it has been revealed that the rabbit's ciliary epithelium primarily secretes $HCO3^{-}$ to drive aqueous humor formation, whereas in bovine and porcine preparations, Cl^{-} secretion seems to be the major driving force [38] [39] [40]. Current models of AH secretion propose that the solutes, mainly NaCl, are taken up to the PE from the stroma via paired Na^{+}/H^{+} and HCO_3^{-}/Cl^{-} antiporters, diffuse to the NPE through the intracellular gap junctions, and then exit to the posterior chamber by basolateral Na^{+}/K^{+} ATPase and Cl^{-} channels [1]. The ion transport generates an osmotic gradient which draws the water pass from the stroma to the posterior chamber via basolaterally located aquaporin AQP1 and AQP4 channels [41].

Gap junctional intercellular communication (GJIC) is thought to play a significant role in maintenance of metabolic homeostasis and control of various cellular physiologic activities including cell growth, differentiation, and embryonic development. Gap junctions are formed by amphipathic polypeptides connexins that consist of four transmembrane segments connected by one intracellular and two extracellular loops; the carboxyl and amino termini are located on the cytoplasmic side [42]. Identification of several connexin isoforms has been reported the ciliary body in several species. Coffey etc. reported that in rat CBE Cx40 and Cx43 were both localized to the PE-NPE interface where they formed discrete homomeric/homotypic gap junction complex [43]. In porcine [20] [44] [45] and bovine [22] [44] [46] [47] CBE, it is reported that Cx43, but not Cx40,

is the major structural and functional unit of PE-NPE gap junction. In our study, we confirmed the distribution of Cx43 PE-NPE interface in bovine ciliary processes. Furthermore, for the first time we reported the distribution of Cx43 in human ciliary epithelial bilayers both by immunostaining and protein assays. Cx43 immunostaining distributes predominantly along the apical cytoplasm between the PE and NPE. Cx40 expression was absent both in human and bovine CBE. These results suggest that, like in bovine and porcine CBE, Cx43 is a major gap junction component to form functional coupling between PE and NPE.

Many cellular signaling cascades are involved in the regulation of aqueous humor inflow. Among them, cAMP has long been suggested to play a pivotal role in this process. Through inhibition of cAMP formation in the ciliary body, beta-adrenergic antagonists (beta-blockers) have been used clinically to reduce IOP [43] primarily by decreasing the rate of aqueous humor production [48]. Interestingly, administration of beta-adrenergic agonists, which are capable to stimulate adenylate cyclase, and forskolin, a direct activator of adenylate cyclase, decrease the IOP in human, monkey, and rabbit [43] [48] [49]. Regulation of cAMP on gap junction in the ciliary epithelial cells has not been extensively studied. It is shown that in bovine CBE, cAMP inhibits PE-NPE gap junctional communication through protein kinase A activation [22]. In the present study, we observed cAMP-induced Cx43 expression in bovine ciliary body. In the contrast, cAMP significantly increased Cx43 expression in human ciliary body. This difference observed in human and bovine CB may reflect the different role of cAMP in aqueous humor formation in different animal species. In fact, previous studies have shown that cAMP may inhibit net Cl^- transport across the ciliary epithelium (considered as the major driving force to aqueous humor production in many animal species) in bovine [39], while in porcine ciliary process, cAMP induces an increase in stroma-to-aqueous Cl^- flux [50]. Similar to the effect on the porcine ciliary body, cAMP increase the Cl^- -related short-circuit current across isolated human ciliary body in vitro [51]. Taken these together, one may speculate that the effect of cAMP on the short-circuit current and Cl^- transport across the ciliary processes may, at least partially, be mediated by the modulation of PE-NPE gap junction.

Little is known about the effect of cGMP on aqueous humor formation in human and animal eyes. Our previous report has shown that cGMP may induce an increase of anionic (possibly Cl^-) transport across porcine ciliary processes [52]. There exist very few reports on the effect of cGMP on connexin/gap junction. Administration of cGMP analog to mesangial cells with up-regulated the expression of gap junction protein Cx43 [53]. In the present study, both in human and bovine ciliary body cGMP significantly increased Cx43 expression. This result strongly suggests the possible role of cGMP in the PE-NPE communication and aqueous humor formation. This hypothesis needs further investigation and the finding on this may benefit the understanding of the mechanisms underlying aqueous humor production and IOP control.

In summary, in human and bovine ciliary epithelial cells, Cx43, but not Cx40, is the predominant structural unit of the gap junction. The second messenger cAMP up-regulated Cx43 protein expression in human ciliary body, but down-regulated Cx43 expression in bovine. cGMP increased Cx43 expression both in human and bovine ciliary bodies. These results suggest that cAMP and cGMP may play different roles in the intracellular communication and aqueous humor formation, a hypothesis needs to be further tested.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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