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Original Article

Effect of bradykinin on cultured retinal pigment epithelial cells

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Abstract: Inflammation plays a key role in the development of proliferative vitreoretinopathy (PVR). This study aimed to detect the effects of bradykinin (BK) on retinal pigment epithelium (RPE) cells, especially regarding inflammation and collagen formation, and to investigate the possible pathway, including the receptors and downstream molecules. We found that BK induced an increase in Ca^{2+} and the levels of COX-2 and iNOS. The collagen formation was decreased with 100 nM BK treatment on the ARPE-19 cells (0.840 ± 0.030, P = 0.011). Moreover, in the B1R and B2R antagonistic groups, BK could improve the level of collagen compared with the BK-only-treated group (B1R antagonistic groups: 0.857 ± 0.021, P = 0.007; B2R antagonistic groups: 0.907 ± 0.015, P = 0.009). These results suggest the potential use of BK as a therapeutic agent to prevent the development of PVR.

Keywords: Bradykinin, proliferative vitreoretinopathy, cyclooxygenase, nitric oxide synthase, collagen

Introduction

Proliferative vitreoretinopathy (PVR) can occur after the rhegmatogenous retinal detachment or after major ocular trauma and its surgical repair. Surgical removal of the fibrotic membranes with retinal detachment repair is the primary treatment for PDR. Despite recent progress in surgical techniques, recurrent detachment can lead to irreversible damage and a poor visual outcome. The retinal pigment epithelium (RPE) is a highly specialized monolayer of cells with pigmented microvilli lining the basement membrane (Bruch's membrane) located between the neural retina and the choroid in the eye, which is a part of the blood-retinal barrier. Many studies have suggested that RPE cells are involved in the pathogenesis of PVR [1-3].

Recently, PVR was thought to be due to abnormal wound healing affected by various factors [4]. Inflammation plays a key role in the development of PVR [5]. In PVR, upon injury of the retina or choroid, RPE cells are exposed to the serum that comes through damaged blood-retinal barriers. Subsequently they are detached from Bruch's membrane, lose their epithelial morphology, migrate into the vitreous through a tear in the neural retina, and participate in the formation of an epithelial membrane with macrophages, fibroblasts and astrocytes on the surface of the neural retina [6, 7]. Many studies have supported chronic inflammation being important in PVR [8]. The proliferative membrane during PVR suggests that the extracellular matrix (ECM) might be important in the pathogenesis. The ECM is the filler of extracellular space during wound healing, which affects cellular structure and function [9]. In PVR progression, the collagen contracts with fibroblasts, causing retinal detachment. The collagen abnormal hyperplasia and deposition causes PVR progression [10]. There is a large amount of ECM in the neovascular membrane [11].

In our previous studies, the proteomic analysis of the vitreous fluid in PVR, DR and ERM showed that the complement and coagulation cascades were the key KEGG pathway [12-14], which suggested this pathway might be the common pathway in PVR. The complement and coagulation cascades included the kallikrein-kinin system

(KKS), coagulation system, and complement system. The KKS was the key regulator, which linked the other two systems, including kallikrein, kininogen, kinin, bradykinin B1 receptor (B1R), bradykinin B2 receptor (B2R), and kininase. Kininogen released the active kinin under the action of kallikrein. Bradykinin (BK) was the main kinin substance, which is the ultimate vasoactive substance of KKS.

A previous study using a PVR rat model showed that BK was expressed in the serum, vitreous body, and retina [15]. The expression was higher in the PVR group than the control group, which suggested the KKS system affected PVR pathogenesis [16]. Lim reported BK increased the RPE cellular absorptive capacity of glucose, through B1R and B2R, to reduce high glucose toxicity. The relationship between BK and DR needs to be demonstrated [17]. BK has been shown to be important in many diseases, but the effects of BK on RPE cells are still unclear. The purpose of this study was to investigate the effects of BK on RPE cells and collagen formation, determine the possible pathway and to clarify the effects of BK on PR.

Materials and methods

Cell cultures

The human retinal pigment epithelia cell line ARPE-19 was donated by Professor Guotong Xu from the Tongji Eye Institute. ARPE-19 cells were cultured in RPE medium (Dulbecco's modified Eagle's medium/Ham's F-12 1:1 medium; HyClone, Kogan, UT) containing 4% fetal calf serum (FCS), 2 mM L-glutamine, 0.1 mM nonessential amino acids at 37°C with 5% CO_2. The experiments were performed using cultured ARPE-19 cells at two to four passages that were grown until they were just confluent with hexagonal shape and without visible pigmentation. In our experience, ARPE-19 cells develop pigmentation only when they are cultured at confluence for an extended period of time (several weeks).

Immunofluorescence

The ARPE-19 cells were fixed with 4% paraformaldehyde at 4°C for 20 minutes, incubated with 10% FCS and 0.2% Triton PBS at 37°C for 1 hour, and then incubated with 1% sheep serum, 0.2% Triton and primary antibody at 37°C for 1 hour. Then, the cells were incubated overnight at 4°C. Thereafter, cells were incubated with 1% sheep serum, 0.2% Triton and secondary antibody at 37°C for 1 hour. Then cells were plated on microscope cover slides with glycerol and PBS (1:1).

Ca^{2+} change

ARPE-19 cells (1×10^4) were incubated for 30 minutes in the dark at 37°C with 5% CO_2 and 1 mL 10 μmol/L dye solution that contained the fluorescent dye fluo-3 AM (Fluo-3/AM) and 0.1% Pluronic F-127. They were detected with 1 mL DMEM under a laser focal scanning microscope (LCSM) with a 488 nm argon laser. LaserSharp2000 software was used with the TimeCourse program and the scanning interval was 30 seconds for 300 scan times. Changes in the image were recorded when the stimulation was BK 10 μM in 10 μl after the baseline was stable.

Western blot analysis

The cold radio-immunoprecipitation assay (RIPA) buffer and phenylmethylsulfonyl fluoride (PMSF: a protease inhibitor) were used to prepare the cells, which were washed twice with ice-cold PBS containing 1 mM EDTA (without Ca^{2+} and Mg^{2+}). A cell scraper was used to gather cell lysates, which were collected and transferred to a microcentrifuge tube and centrifuged for 30 minutes at 12,000 rpm (4°C). The supernatant was collected, and a BCA protein assay kit was used to measure the protein content of each lysate. Electrophoresis was performed (25 μg protein/well) with the use of a sodium dodecyl sulfate polyacrylamide gel, which was then transferred to a polyvinylidene difluoride membrane and analyzed by immunoblotting. Band densities were quantified by Image software.

Collagen measurements

Total soluble collagen in cell culture supernatants was quantified using the Sircol collagen assay (Biocolor, Belfast, UK). For these experiments, confluent cells in 25 cm^2 culture dishes were incubated for 24 hours with 1 mL DMEM-5% FBS. One milliliter of Sirius red dye, an anionic dye that reacts specifically with basic side chain groups of collagens under assay conditions, was added to 100 μL of superna-

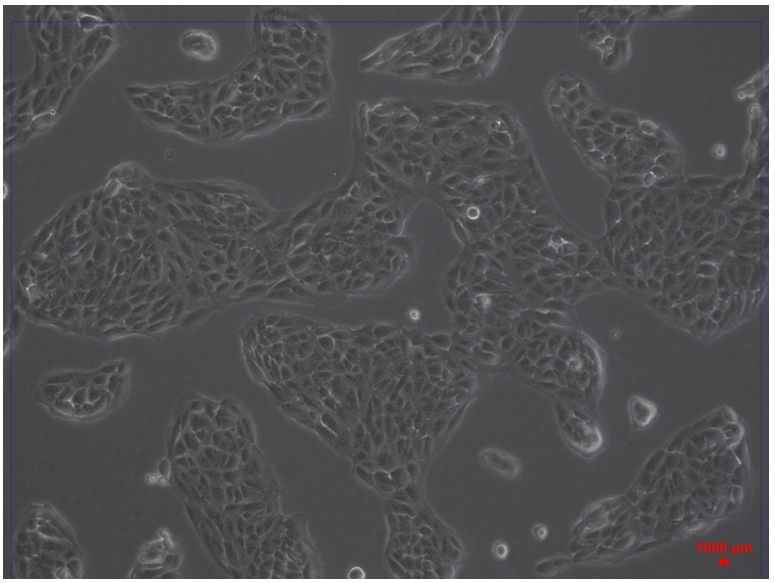

Figure 1. Morphology of the cultured ARPE-19 cells (magnified 100 times under the light microscope).

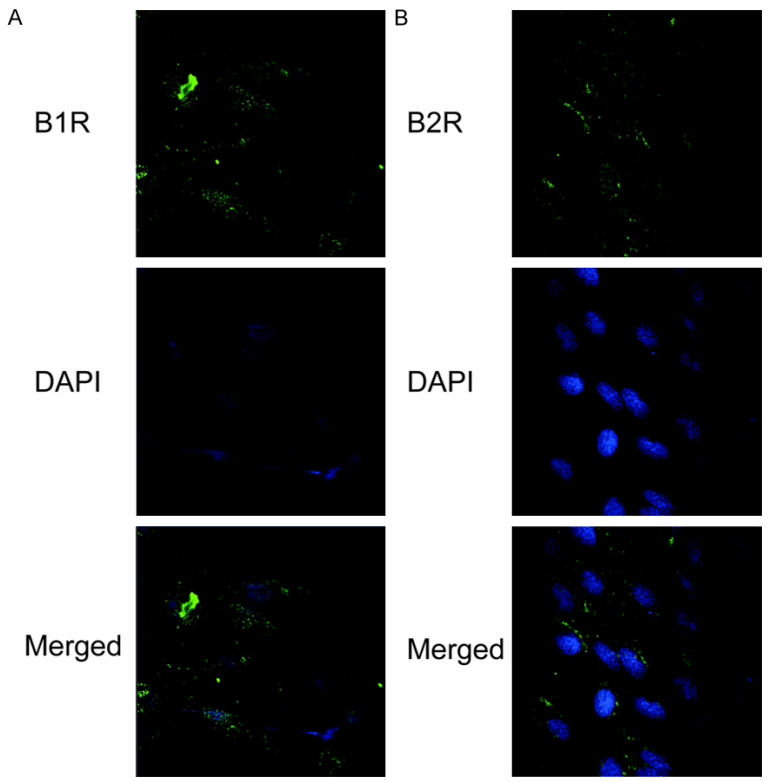

Figure 2. Location of BK receptors on ARPE-19 cells. A. B1R. B. B2R.

tant and incubated with gentle rotation for 30 minutes at room temperature. After centrifugation at 12,000 rpm for 10 minutes, the collagen-bound dye was redissolved with 1 mL of 0.5 M NaOH, and the absorbance at 555 nm was measured by enzyme-linked immunosorbent assay (MRX; Dynex, Chantilly, VA, USA). The absorbance was directly proportional to the amount of newly formed collagen in the cell culture supernatant.

Statistical analysis

Statistical analyses were performed using SPSS 19.0 Software. All measurement data are expressed as the mean ± standard deviation (SD) and were compared between two groups using Student's t test. *P*-values less than 0.05 were considered significant. All data are the average of the three times experiments.

Results

BK receptors are located in ARPE-19 cells

The APRE-19 cells were transparent and flat polygons with a small amount of pigment particles in the cytoplasm under optical microscopy (**Figure 1**). B1R and B2R were detected on the cell membrane and in the cytoplasm and nucleus by immunofluorescence (**Figure 2A**, **2B**).

The effect of BK on intracellular Ca^{2+}

The intracellular Ca^{2+} remained stable at baseline without BK (**Figure 3A**). After adding 100 nM BK, the intracellular Ca^{2+} expression was significantly higher and maintained the high level for a period of time, then decreased to the baseline (**Figure 3B**). The peak declined when cells were incubated with B1R antagonist Leu-8 or B2R antagonist HOE-140, and the inhibition was more significant with HOE140 (**Figure 3C**,

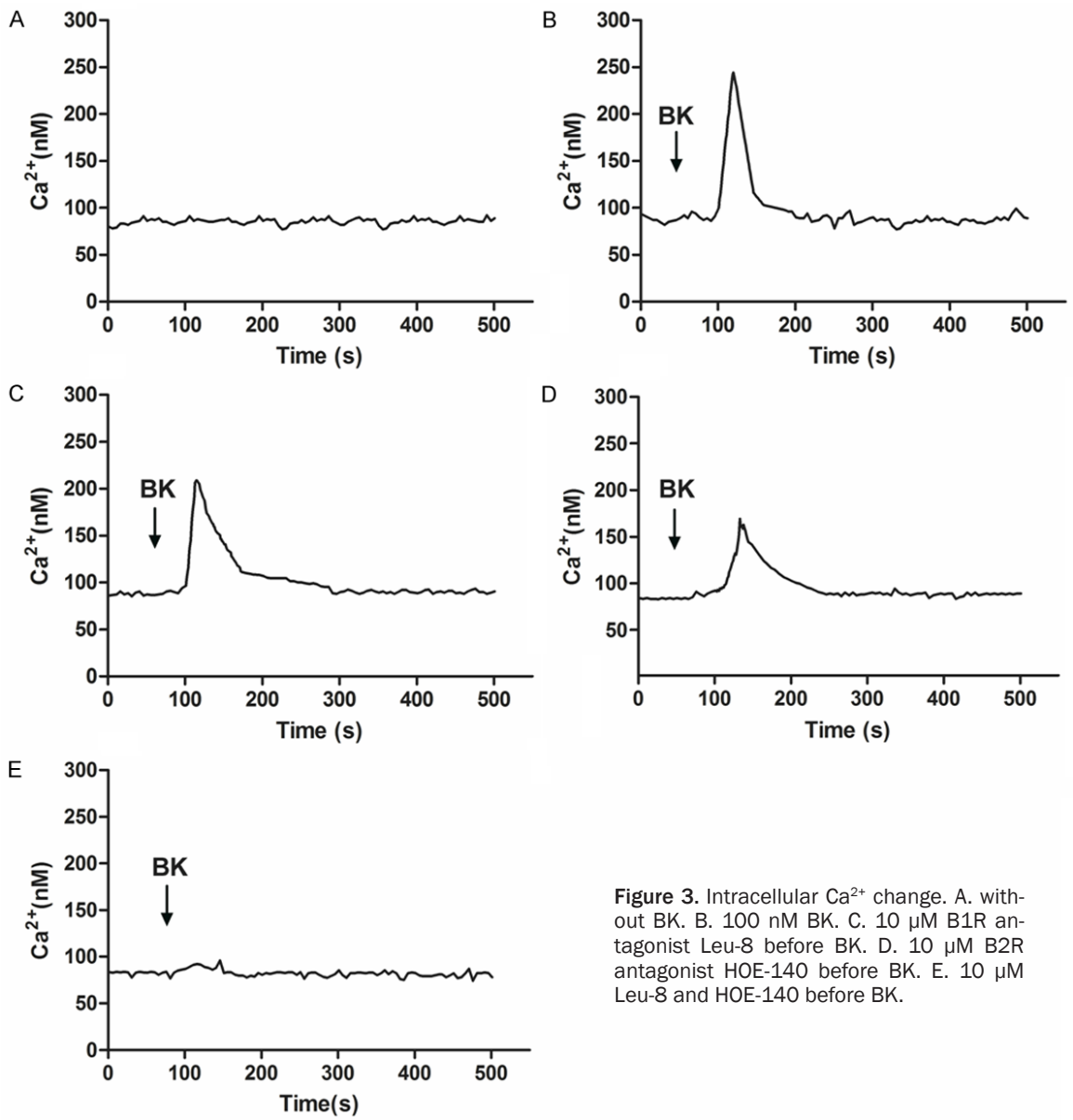

Figure 3. Intracellular Ca^{2+} change. A. without BK. B. 100 nM BK. C. 10 μM B1R antagonist Leu-8 before BK. D. 10 μM B2R antagonist HOE-140 before BK. E. 10 μM Leu-8 and HOE-140 before BK.

3D). When cells were incubated with both Leu-8 and HOE-140, the intracellular Ca^{2+} did not change after BK stimulation (**Figure 3E**).

The effect of BK on downstream inflammatory proteins

There was low expression of COX-1 in the control group (without BK), and the level did not change at 24 hours after BK stimulation (**Figure 4A**). The ARPE-19 cells in normal conditions seldom expressed COX-2. However, COX-2 was increased significantly at 24 hours after BK stimulation (**Figure 4B**, $P < 0.001$).

The ARPE-19 cells in normal conditions expressed eNOS and there was no change at 24 hours after BK stimulation (**Figure 5A**). There was low expression of iNOS in the control group (without BK) and iNOS was increased significantly at 24 hours after BK stimulation (**Figure 5B**, $P < 0.001$).

Effect of BK on collagen formation in ARPE-19 cells

The collagen concentration was measured 24 hours after stimulation with different BK concentrations (1 nM, 10 nM, 100 nM, and 1000

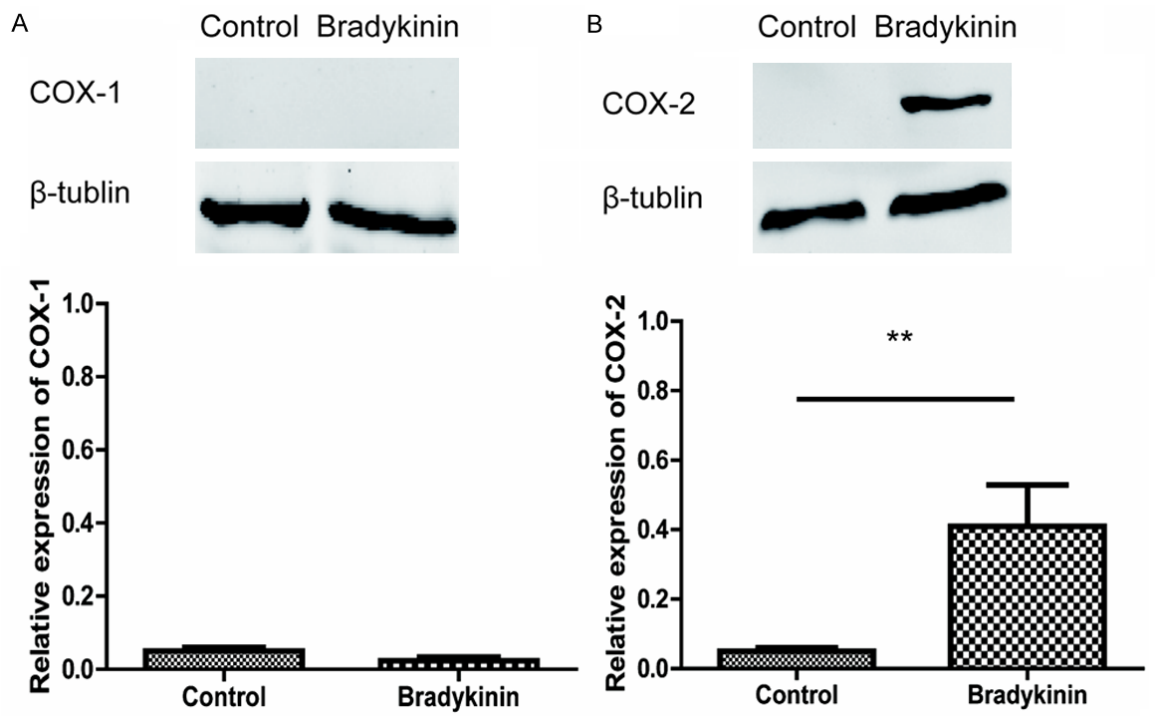

Figure 4. Effects of BK on COX-1 and COX-2. A. On COX-1. B. On COX-2. It was the average of three experimental results of grey scale scanning result of COX-1 and COX-2 protein expression after internal correcting. **: $P < 0.01$ vs. the control group.

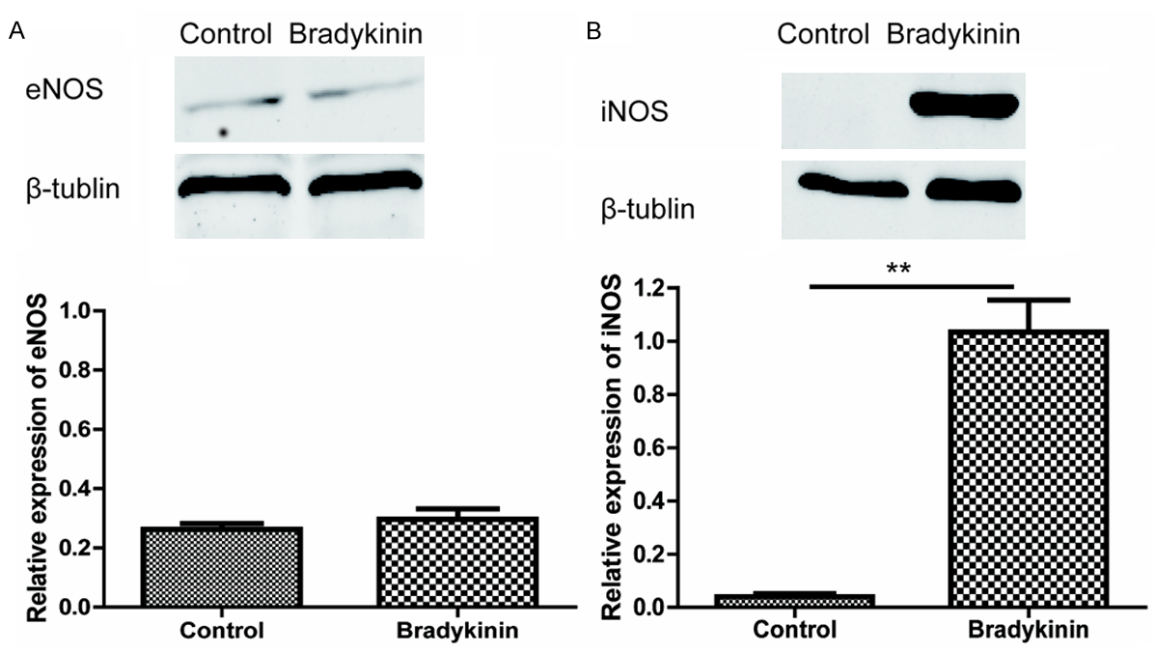

Figure 5. Effects of BK on eNOS and iNOS. A. On eNOS. B. On iNOS. It was the average of three experimental results of grey scale scanning result of eNOS and iNOS protein expression after internal correcting. **: $P < 0.01$ vs. the control group.

nM) in 1×10^6 ARPE-19 cells. The collagen concentration in the control group (without BK) was defined as 1, and there was no significant change in the 1 nM group (1.013 ± 0.035, P =

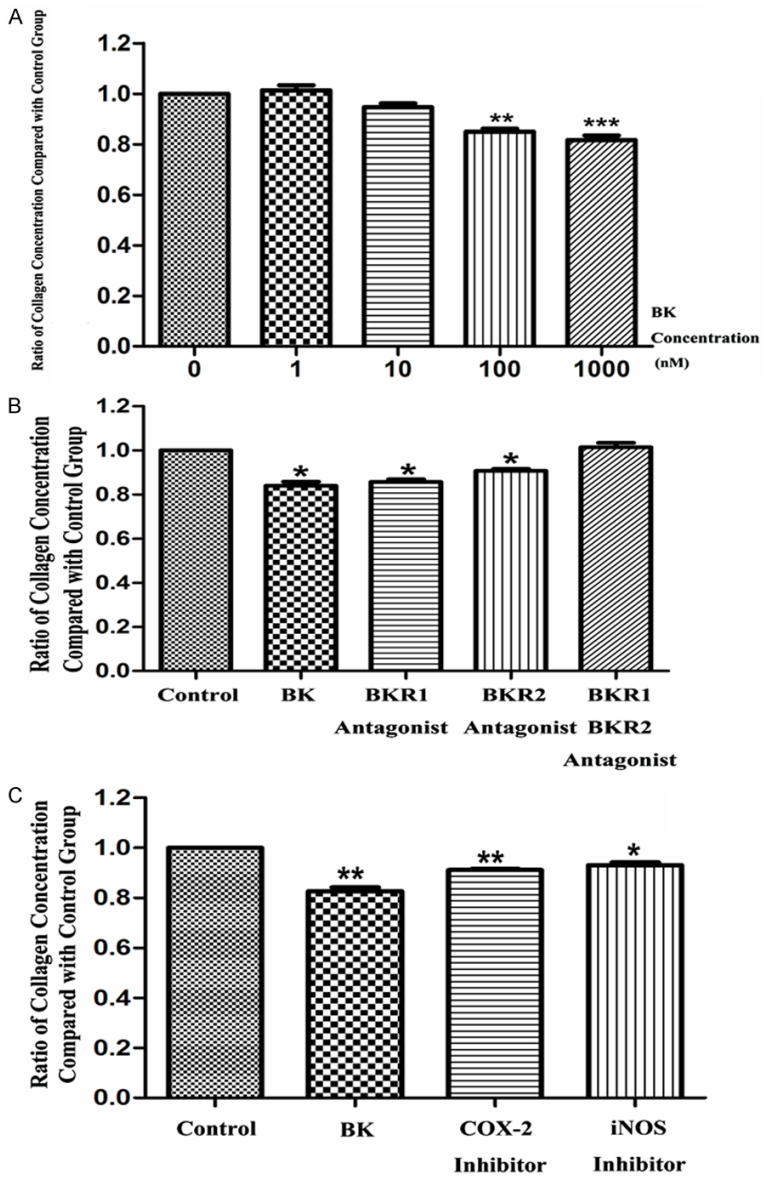

Figure 6. Effects of BK on collagen formation in ARPE-19 cells. A. Effects of different concentration of BK on ARPE-19 cells collagen formation. **: P < 0.01 vs. the control group; ***: P < 0.001 vs. the control group. B. Effects of BK receptors antagonist on inhibition collagen formation of BK. *: P < 0.05 vs. the control group. C. Effects of COX-2 and iNOS inhibitors on inhibition collagen formation of BK. *: P < 0.05 vs. the control group; **: P < 0.01 vs. the control group.

0.578). The 10 nM group levels were decreased, but not the difference was not significant (0.947 ± 0.025, P = 0.067). However, the collagen was decreased significantly in the 100 nM group (0.850 ± 0.020, P = 0.006) and in the 1,000 nM group (0.817 ± 0.031, P < 0.001; **Figure 6A**).

The collagen concentration in the control group was defined as 1 and the concentrations in the BK group (0.840 ± 0.030), B1R antagonist group (0.857 ± 0.021), and B2R antagonist group (0.907 ± 0.015) were reduced significantly (P = 0.011, P = 0.007, and P = 0.009, respectively). There was no significant difference between the B1R/B2R antagonist group and the control group (1.013 ± 0.035, P = 0.578). Compared with the BK group, there was no significant change in collagen in the B1R antagonist group (0.857 ± 0.021 vs 0.840 ± 0.030, P = 0.464), but collagen was increased significantly in the B2R antagonist group (0.907 ± 0.015 vs 0.840 ± 0.030, P = 0.017). The collagen was increased in the B1R/B2R group (1.013 ± 0.035 vs 0.840 ± 0.030, P = 0.035; **Figure 6B**).

The collagen concentration in the control group was defined as 1, and the concentrations in the BK group (0.827 ± 0.025), COX-2 inhibitor group (0.910 ± 0.010), and iNOS inhibitor group (0.930 ± 0.021) were decreased significantly (P = 0.007, P = 0.041, and P = 0.026, respectively). Compared with the BK group, there were no significant changes in the COX-2 inhibitor group (0.910 ± 0.010 vs 0.827 ± 0.025, P = 0.042) and iNOS inhibitor group (0.930 ± 0.021 vs 0.827 ± 0.025, P = 0.042; **Figure 6C**).

Discussion

In recent years, sub-clinical chronic low-grade inflammation has been suggested to be associated with PDR. Abnormal expressions of inflammatory factors, such as interleukin, play a role in PDR [18]. Anti-inflammatory therapy decreases the incidence of PDR [19]. Our previous studies showed that the complement and coagulation cascades were common in different prolif-

erative retinal diseases, and BK was the final effector of KKS. However, the effect and pathway were still unclear. This study aimed to investigate the relationship between BK and RPE cells.

Previous studies have reported that the intracellular Ca^{2+} levels changed in response to BK application as Ca^{2+}-induced Ca^{2+} release [20, 21], which was consistent with the results of this study. In several cell types, BK elicits an abrupt rise in Ca^{2+} to a peak that depends on Ca^{2+} recruitment from internal stores, which then declines to a plateau that is sustained by Ca^{2+} entry from the extracellular compartment. The persistent elevation in Ca^{2+} observed after the initial Ca^{2+} peak probably results from a receptor- or second messenger-operated Ca^{2+} current activated by BK rather than via a capacitive pathway, which was supported by results showing that the peak declined when cells were incubated with BK receptors antagonists.

COX and NOS are important inflammatory proteins in PR. Some reported that COX expression levels in the retina were higher in PVR and PDR than normal clinical samples or animal models [22], which increased the inflammation and promoted the disease. COX inhibition therapy was effective in PDR system management [23]. Previous studies suggested that increased iNOS produced nitric oxide, which injured the retina in diabetic patients and animal models [24], while iNOS inhibitor significantly inhibited the formation of the retinal microvasculature in PDR [25]. The significant increase of COX-2 and iNOS after BK stimulation suggested that they might be the downstream effectors. Inoue found COX-1 remained constant and prostaglandin E2 increased with the increase of COX-2 after BK stimulating the dorsal root ganglion cells of rats, which suggested that BK improved prostaglandin E2 formation by increasing COX-2 expression [26]. However, studies on arteriae interlobular renis showed the pathway was COX-1 rather than COX-2 [27]. Lim studied RPE cells and suggested that BK increased COX-2 but did not affect COX-1 [28], which was consistent with this study. The inconsistent influences of BK on COX-1 and COX-2 suggested the effect was different in different tissues and cells. When BK stimulates RPE cells, eNOS remains constant and iNOS increases, which suggests iNOS is an effector of BK. Catalán studied myocardial cells and TGF-1β was shown to induce cardiac fibroblasts, and found BK influenced COX-1, COX-2 and iNOS in myocardial cells but only influenced COX-2 in cardiac fibroblasts [29].

In this study, BK was found to promote the expression of COX-2 and iNOS, which are important inflammatory factors. BK is an important vasoactive substance that might be related to PDR. BK activates NOS expression, promotes NO formation, and dilates blood vessels to improve ischemia and hypoxia; meanwhile, BK increases the permeability of the retinal microvasculature, and growth factor extravasation promotes the development of PDR [30]. The relationship between BK and PDR needs further research. This study was based on no-stimulation RPE cells. However, downstream effectors might change with cell functional changes after growth factor stimulation. The mechanism of BK in different conditions should be studied in the future.

Collagen is an important ECM protein, which has been shown to be increased in PR. Collagen protein and fibrin are produced by various cells that form the proliferative membrane with other ECM proteins produced with retinal detachment. Previous studies have shown that KKS activation was common in the three proliferative retinal diseases. As the final effector of KKS, BK inhibited collagen formation in various cells [16, 17, 29]. However, the mechanism was still unclear. This study aimed to investigate the relation of BK and collagen formation. BK was found to inhibit collagen formation by affecting cultured ARPE-19 cells, which was dose dependent. Blaes's research on glomerular mesangial cells suggested that high glucose [16], epithelial growth factor (EGF), and transforming growth factor-β (TGF-β) induced a collagen formation increase, which could be inhibited significantly by BK through B2R. Wei injected BK in an animal model directly through atrial perfusion [17]. Collagen was decreased by 30% after 24 h. The effect was weakened visibly with the B2R antagonist, which suggested the pathway was BK binding to B2R.

The analysis of RPE cell collagen content showed that the capacity of BK inhibiting collagen formation still existed with either the B1R or B2R antagonist, while the capacity vanished with a combination of the two. The results sug-

gested both receptors affected collagen formation. Comparing the BK receptor antagonist group with the BK group, inhibiting B1R alone did not influence the collagen decrease, however, inhibiting B2R alone did reduce the collagen decrease, which suggested B2R was more important. BK receptors were distributed in the eyes, and B1R and B2R were detected in the retina and ciliary body [31]. Lim's study on RPE cells in a high glucose environment showed BK improved the glucose absorptive capacity of RPE through B1R and B2R reduced high glucose toxicity, which suggested the interaction of the two receptors [28]. Further research indicated that the expression and content of BK receptors changed according to different stimulating factors and time. This finding suggested BK receptors were involved in the progression of diseases. In this study, the collagen decrease declined with COX-2 and iNOS inhibitors, which indicated that COX-2 and iNOS affected BK inhibition of collagen formation. The mechanism might be BK promoting the expression of COX-2 and iNOS to affect cell function, which inhibited collagen secretion and formation, with the collagen content consequently decreased.

This study showed that the expression of COX-2 and iNOS increased with BK stimulation to promote the development of disease. In addition, BK inhibited collagen formation slightly by COX-2 and iNOS, which was dose dependent. Additional research is needed. First, BK is the main effector of KKS, but other substances might affect collagen formation. Second, this study was based on cultured RPE cells without animal model confirmation. What is more, the pathway of BK was unclear, which could be illuminated in subsequent studies.

Conclusions

BK worked through binding with either B1R or B2R in the cultured RPE cells, and BK induced the increasing expression of COX-2 and iNOS in the cultured RPE cells. Furthermore, BK mildly reduced the formation of collagen in the cultured RPE cells through COX-2 and iNOS.

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Disclosure of conflict of interest

None.

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