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Apelin-13 induces proliferation, migration, and collagen I mRNA expression in human RPE cells via PI3K/Akt and MEK/Erk signaling pathways

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Purpose: Our previous study showed that apelin was increased in the vitreous and fibrotic membranes of patients with proliferative diabetic retinopathy (PDR) in vivo, which suggested that apelin may be involved in the development of PDR. In this study, we investigated whether the expression of apelin was upregulated in human retinal pigment epithelial (RPE) cells in vitro under high glucose conditions. Furthermore, to explore the role of apelin in RPE cells, we investigated the effect of exogenous recombinant apelin on proliferation, migration, and collagen I (a major component of extracellular matrix molecules, associated with PDR) expression and investigated the signaling pathways involved in these processes.
Methods: Real-time PCR and western blot were performed to determine the apelin expression in ARPE-19 cells under high glucose conditions. Exogenous recombinant apelin was used to study the effect of apelin on ARPE-19 cells in vitro. Cell proliferation, migration, and collagen I expression were assessed using an MTT assay, a transwell assay, and real-time PCR analysis. LY294002 (an inhibitor of phosphatidylinositol 3-kinase) and PD98059 (an inhibitor of mitogen-activated protein kinase) were used to help to determine the apelin signaling mechanism.
Results: High glucose upregulated apelin expression in RPE cells. Exogenous recombinant apelin activated protein kinase B (Akt) and extracellular signal-regulated kinase (Erk) phosphorylation and promoted proliferation, migration, and collagen I expression in RPE cells. Pretreatment with LY294002 and PD98059 abolished apelin-induced activation of Akt and Erk, proliferation, and collagen I expression. Apelin-induced migration was partially blocked by pretreatment with LY294002 and PD98059.
Conclusions: The expression of apelin was upregulated under high glucose conditions in RPE cells in vitro. Exogenous recombinant apelin increased the biologic activity of RPE cells, as well as the expression of collagen I. Apelin promoted proliferation, migration, and collagen I expression through the PI3K/Akt and MEK/Erk signaling pathways in RPE cells. From these results, we revealed the role of apelin in regulating proliferation, migration, and collagen I expression in RPE cells and the signaling mechanism under these processes, which suggested that apelin may play a profibrotic role in the development of PDR.

Diabetic retinopathy (DR) is a leading cause of blindness and vision loss in the working age population [1]. DR is a chronic, progressive, sight-threatening disease associated with prolonged hyperglycemia. PDR, an advanced stage of DR, is characterized by epiretinal outgrowth of fibrotic membranes at the vitreoretinal interface. RPE cells lie between Bruch's membrane and the retina. They normally form a monolayer of cells, which create the outer blood–retinal barrier. RPE cells are important for maintaining the visual system. Normal RPE cells are quiescent without proliferation or migration [2-4]. Breakdown of the outer blood–retinal barrier can expose RPE cells to various growth factors, neurotransmitter compounds, and cytokines in the subretinal space and in the vitreous [5-7], which can trigger the activation of RPE cells. The activated RPE cells can initiate proliferation and migration and secrete extracellular matrix molecules in diseases such as PDR [8], proliferative vitreoretinopathy (PVR) [9], and age-related macular degeneration (AMD) [10]. RPE cells were detected in combined traction rhegmatogenous retinal detachment membrane from patients with PDR [11].

The major components of the fibrotic membrane are extracellular matrix molecules in combination with some cell types. Collagen I is the major component of extracellular matrix molecules, and its expression is modulated by various cytokines [12]. RPE cells [13] and other cell types contribute to the formation of fibrotic membranes [14]. Formation of fibrotic membranes often leads to serious visual loss.

The apelin peptide, an endogenous ligand for the angiotensin-1-like receptor APJ, was first identified from bovine stomach extracts in 1998 [15]. The preproapelin contains 77 amino acids. It can be cleaved into several biologically active forms of apelin peptides in vivo, such as apelin-13 (apelin

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65–77), apelin-17 (apelin 61–77), and apelin-36 (apelin 42–77) [15-18]. Apelin-13 is the most bioactive of the three, followed by apelin-17 and then apelin-36 [16,19,20]. Apelin can be detected in various tissues and organs, such as the heart, stomach, brain, lung, uterus, and ovary [21-25]. Apelin, as a multibioactive peptide, participates in diseases such as heart failure, hypertension, metabolic syndrome [26], and retinal disease [27].

Our previous study demonstrated that vitreous concentrations of apelin were significantly higher in the PDR group compared with the control group, and apelin mRNA expression in fibrotic membranes from patients with PDR was also higher compared with the control group. These data suggested that apelin may be involved in the development of PDR [28]. In addition, apelin has been reported to promote the phosphorylation of extracellular signal-regulated kinases (ERKs), protein kinase B (Akt), and p70S6 kinase in umbilical endothelial cells [29]. Apelin can promote proliferation and migration in retinal Müller cells [30] and retinal endothelial cells [31]. However, there is no information about apelin in RPE cells. In this study, we investigated the response of apelin under high glucose conditions in vitro. We also investigated the effect of exogenous recombinant apelin on the proliferation, migration, and collagen I mRNA expression and the signaling mechanism of apelin involved in these processes.

METHODS

Reagents: Synthetic apelin-13 peptide was purchased from Sigma (St. Louis, MO). The amino acid sequence of apelin-13 is Gln-Arg-Pro-Arg-Leu-Ser-His-Lys-Gly-Pro-Met-Pro-Phe. Anti-p-Akt, anti-Akt, anti-p-Erk, and anti-Erk were obtained from Cell Signaling Technology (Danvers, MA). Anti-apelin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-GAPDH was obtained from Abmart (Shanghai, China). LY294002 and PD98059 were obtained from Sigma.

Cell culture: The human retinal pigment epithelial cells (ARPE-19; CRL-2302) were obtained from the American Type Culture Collection (ATCC; Manassas, VA) and cultured in Dulbecco's Modified Eagle Media (DMEM; Gibco, Invitrogen, Grand Island, NY) with 10% fetal bovine serum (FBS, Gibco, Invitrogen), 100 units/ml penicillin, and 100 µg/ml streptomycin (Sigma) at 37 °C under 5% CO_2 and 95% ambient air. The ARPE-19 cells were used for each experiment at 80%–90% confluence and were passaged after being grown for 3 days in cultured media. The cells were used between passages 3 and 6. RPE cells were seeded in a 25 cm^2 flask at a density of 3×10^6.

Cell proliferation assay: Cell proliferation was examined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetracolium bromide (MTT; Roche Molecular Biochemicals, Mannheim, Germany) assay. The 1×10^4 cells grown in a 96-well plate for 24 h were partially starved in DMEM supplemented with 1% FBS for 12 h and then stimulated with various concentrations of recombinant apelin with or without other test substances for an additional 48 h. MTT was added to the culture medium, and the cells were incubated for an additional 4 h. The formed formazan crystals were then dissolved by the addition of dimethyl sulfoxide (100 µl/well). Absorbance at 490 nm was measured using a microplate reader (Model 550; Bio-Rad, Tokyo, Japan).

Cell migration assay: Cell migration was determined using a transwell assay: 4×10^3 cells were placed in the upper chamber (Costar, Cambridge, MA) with a volume of 200 µl serum-free medium. Next, 10% FBS with various concentrations of apelin, with or without other test substances, were placed in the bottom chamber with a volume of 600 µl per well. After 6 h incubation, the cells were fixed in 4% paraformaldehyde for 15 min, stained with crystal violet for 15 min, and washed in Dulbecco's Calcium and Magnesium free PBS (10010023, Gibco, Invitrogen). The remaining cells on the upper surface of the filter were removed by wiping with a cotton swab. Cells migration was quantified by the number of cells that migrated across the filter toward the lower surface in five random fields per filter under microscope.

Real-time PCR analysis: Total RNA was extracted from RPE cells using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After washing with 75% ethanol, the final RNA were eluted in 20 µl distilled diethylpyrocarbonate-treated water. The purity and concentration of RNA were measured using the Gene Quant pro device (Nanovue Spectrophotometer, GE Healthcare, London, UK). cDNA was synthesized with 2 µg total RNA using a RevertAid First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Roth, Germany). Real-time PCR was performed using a detector (Bio-Rad, Munich, Germany). The PCR solution contained specific primers (0.3 µM each), 12.5 µl Maxima SYBR Green qPCR Master Mix (Fermentas), and 2.5 µl cDNA with a final volume of 25 µl. PCR primers were as follows: human apelin: forward 5′-TGC TGC TCT GGC TCT CCT T-3′, reverse 5′CCG TCT TCC AGC CCA TTC C-3′; human GAPDH: forward 5′-TGT TCG ACA GTC AGC CGC AT-3′; reverse 5′-ACT CCG ACC TTC ACC TTC CC-3′; human collagen I: forward 5′-TGG TGG TTA TGA CTT TGG TTA CGA T-3′, reverse 5′-TGT GCG AGC TGG GTT CTT TCT A-3′. The reaction conditions for amplifying DNA were 95 °C for 10 min, followed by 40 cycles of 95 °C for 15

s, 61 °C for 30 s, and 72 °C for 30 s. The mRNA expression was normalized to the expression level of GAPDH and was calculated using the following equation: Fold change=$2^{-\Delta\Delta CT}$.

Western blot analysis: Western blot analysis was performed as described previously [30]. Briefly, protein samples were analyzed on 10% or 12.5% SDS–PAGE gels, transferred to PVDF membranes (Millipore, Billerica, MA), and processed for analysis using an enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL). The primary antibodies were used at the following dilutions: anti-p-Akt (#4060; 1:2,000), total anti-Akt (#4691; 1:1,000), anti-p-Erk (#9106; 1:2,000), total anti-Erk (#4696; 1:1,000), anti-apelin (sc33804; 1:500), and GAPDH (M20006, 1:5,000).

Statistical analysis: The statistical analysis was performed using software SPSS version 17.0 (SPSS, Chicago, IL). Data are expressed as means±standard deviation (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA) and the Student *t* test. $p<0.05$ was considered statistically significant.

RESULTS

Induction of apelin mRNA and its protein expression in high glucose conditions: To examine the effect of high glucose on apelin expression, RPE cells were cultured in Dulbecco's Modified Eagle Media containing either 5.5 mM (normal glucose, NG) or 25 mM (high glucose, HG), and were exposed for 48 h. Real-time PCR data revealed an increased apelin mRNA level in the cells under the 25 mM condition (Figure 1A). An increased protein level was also observed in the cells under the 25 mM condition (Figure 1B,C).

Apelin activates PI3K/Akt and MEK/Erk signaling pathways in RPE cells: To investigate whether apelin promotes Akt and Erk phosphorylation in RPE cells, various concentrations of apelin were added to these cells. We found that apelin at concentrations of 10^{-8} M, 10^{-7} M, and 10^{-6} M significantly increased phosphorylation of Akt and Erk compared with the control group (Figure 2A–C). The activation of Akt and Erk by apelin was blocked by pretreatment with LY294002 or PD98059 (Figure 2D–G).

Apelin enhances RPE cell proliferation, migration, and collagen I expression: Experiments were performed to evaluate whether apelin had any effect on RPE cell proliferation with MTT. Cells were incubated with apelin at concentrations of 10^{-8} M, 10^{-7} M, and 10^{-6} M for 48 h. Among the various concentrations of apelin tested, apelin at a concentration of 10^{-8} M or 10^{-7} M was observed to significantly increase RPE cell proliferation compared with the control group (Figure 3A). In the cell migration assay, cells were measured in a modified Boyden Chamber in which RPE cells migrated through a porous membrane. The mean number of migrated cells in the apelin-treated RPE cells (10^{-8} M, 10^{-7} M, 10^{-6} M) was significantly higher than the mean number of migrated control cells (Figure 3B,C). We also evaluated whether collagen I levels might be affected in RPE cells treated with apelin (10^{-7} M). Collagen I mRNA expression was measured using real-time PCR. Apelin induced a high increase in collagen I mRNA expression, reaching a maximum after 3 h (Figure 3D).

PI3K/Akt and MEK/Erk signaling pathways mediate the effect of apelin on proliferation, migration, and collagen I expression in RPE cells: Having found that apelin treatment activated the PI3K/Akt and MEK/Erk signaling pathways and induced proliferation, migration, and collagen I expression in RPE cells, we examined whether the activation of the PI3K/Akt and MEK/Erk signaling pathways plays a vital role in apelin-induced proliferation, migration, and collagen I expression in RPE cells. Using MTT assay, pretreatment of RPE cells with LY294002 and PD98059 blocked apelin-induced proliferation (Figure 4A). In addition, we found that apelin-induced migration was significantly inhibited by pretreatment with LY294002 and PD98059 in RPE cells (Figure 4B,C). Pretreatment with LY294002 and PD98059 also blocked apelin-induced collagen I expression (Figure 4D).

DISCUSSION

Prolonged hyperglycemia is an important risk factor for the pathogenesis of PDR [32]. Sustained hyperglycemia leads to increased vasoactive factors and growth factors. Apelin and other molecules have been detected in vitreous fluid and fibrotic membranes from patients with PDR [28,33-36]. In this study, we found that high glucose (mimicking the hypoglycemia diabetic condition) upregulated apelin expression in RPE cells in vitro. RPE cells' contribution to the PDR membrane has been confirmed with an ultrastructural investigation [13]. In addition, patients with PDR typically have RPE cells that are contained (5%–20%) in combined traction rhegmatogenous retinal detachment membrane [11]. These observations indicate that RPE cells may migrate through the retinal breaks and contribute to the formation of PDR membrane by secreting important factors.

Apelin can promote proliferation and migration in retinal Müller cells and endothelial cells. In addition, the proliferation and migration of RPEs cell are associated with PDR [11]. However, the role of apelin in RPE cells has not been understood. We found that when RPE cells were incubated with various concentrations of apelin, cell proliferation was

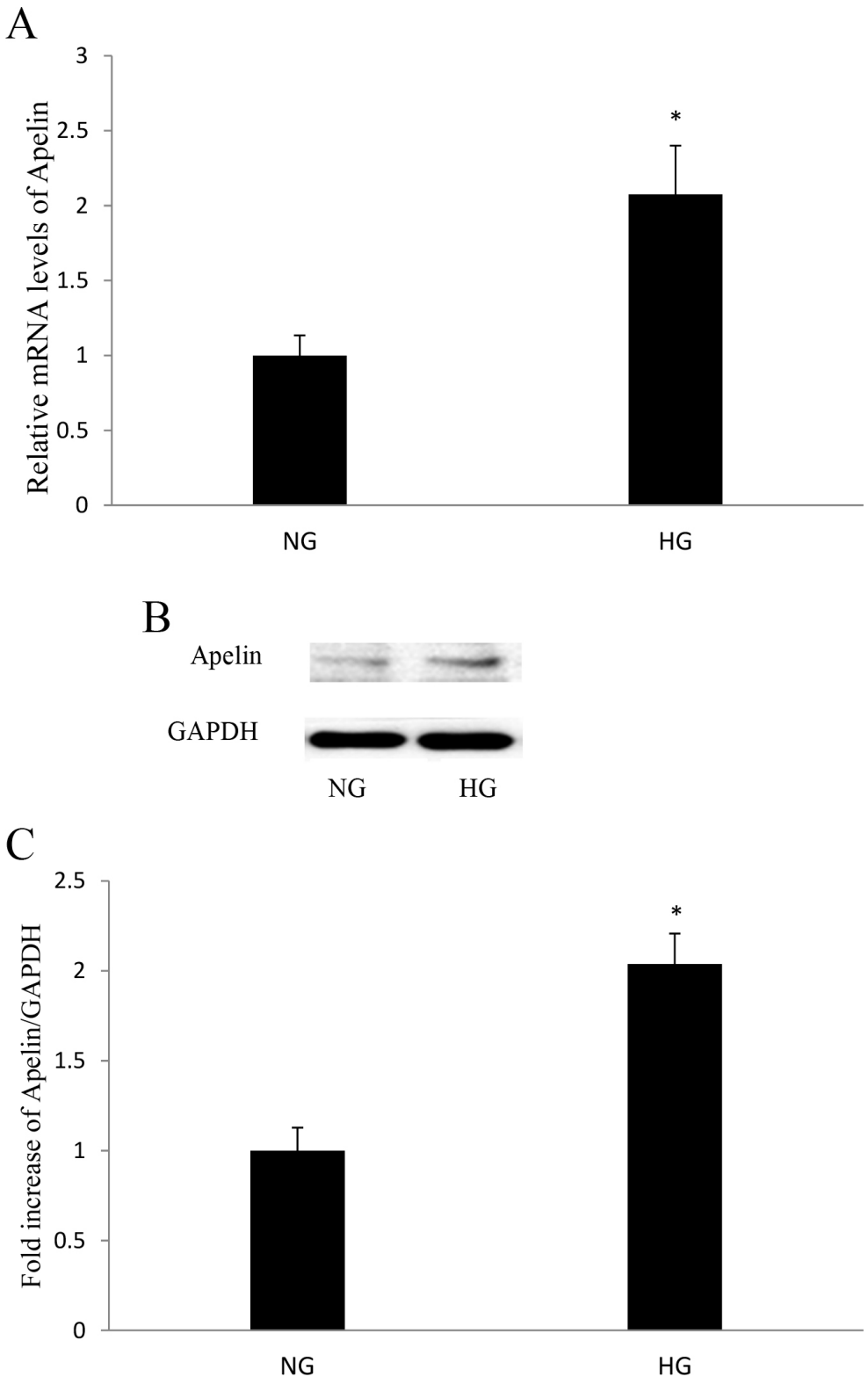

Figure 1. Effect of high glucose concentration on apelin expression in RPE cells. **A**: Real-time PCR analysis of apelin mRNA expression. RPE cells were exposed to normal glucose (NG) and high glucose (HG) for 48 h. Compared with the NG, the expression of apelin was upregulated in response to HG. **B**: Western blot analysis of apelin protein expression. RPE cells were exposed to NG and HG for 48 h. **C**: The expression of apelin on protein increased under HG conditions compared with NG. NG and HG represent the normal glucose group and the high glucose group. The data shown represent the mean±standard deviation (SD) of three independent experiments, *p<0.05 versus NG.

significantly increased at concentrations of 10^{-8} M and 10^{-7} M, as determined with MTT. The effect of apelin on RPE cell migration was determined using a transwell assay. Treatment with apelin significantly increased RPE cell migration

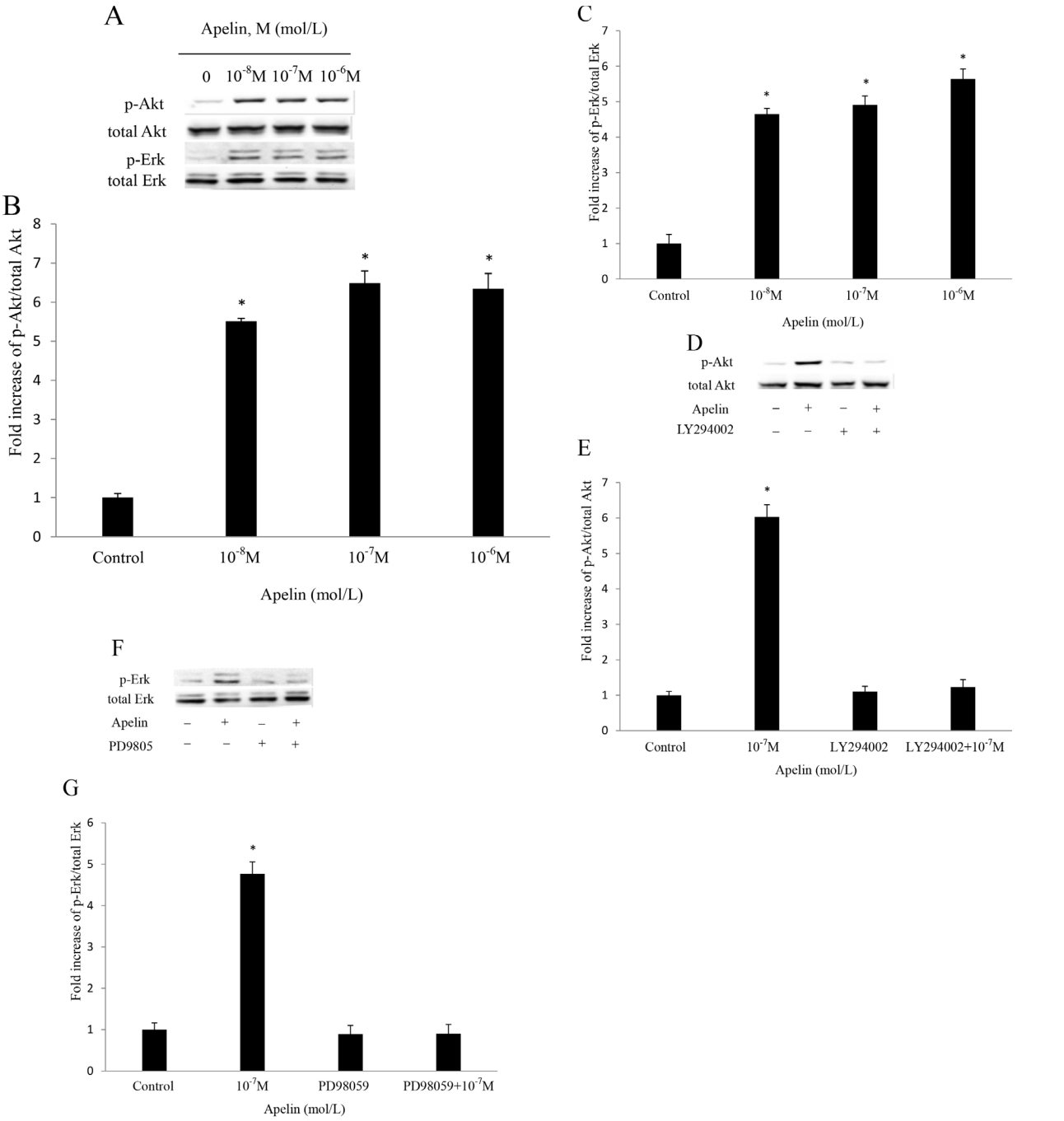

Figure 2. Apelin-induced phosphorylation of Erk and Akt. **A**, **B**, **C**: RPE cells were incubated with 10^{-8} M, 10^{-7} M, and 10^{-6} M apelin for 30 min for assay of Erk and Akt phosphorylation, levels of phosphorylated and total Akt and Erk were determined with western blot analysis, respectively. RPE cells were pretreated with 10 μM LY294002 (**D**, **E**) or 20 μM PD98059 (**F**, **G**) for 30 min and then incubated with 10^{-7} M apelin for 30 min for assay of Akt and Erk phosphorylation. Levels of phosphorylated Akt and Erk were determined with western blot analysis. The data represent the mean±standard deviation (SD) of three independent experiments, *p<0.001 versus untreated control.

at concentrations of 10^{-8} M, 10^{-7} M, and 10^{-6} M. The present study indicates that apelin can promote proliferation and migration in RPE cells in vitro. In addition, a recent study suggested that the RPE cell coverage of the lesion was delayed in choroidal neovascularization induced by laser photocoagulation of apelin-deficient mice [37], which suggested that apelin may play a role in RPE cells in vivo.

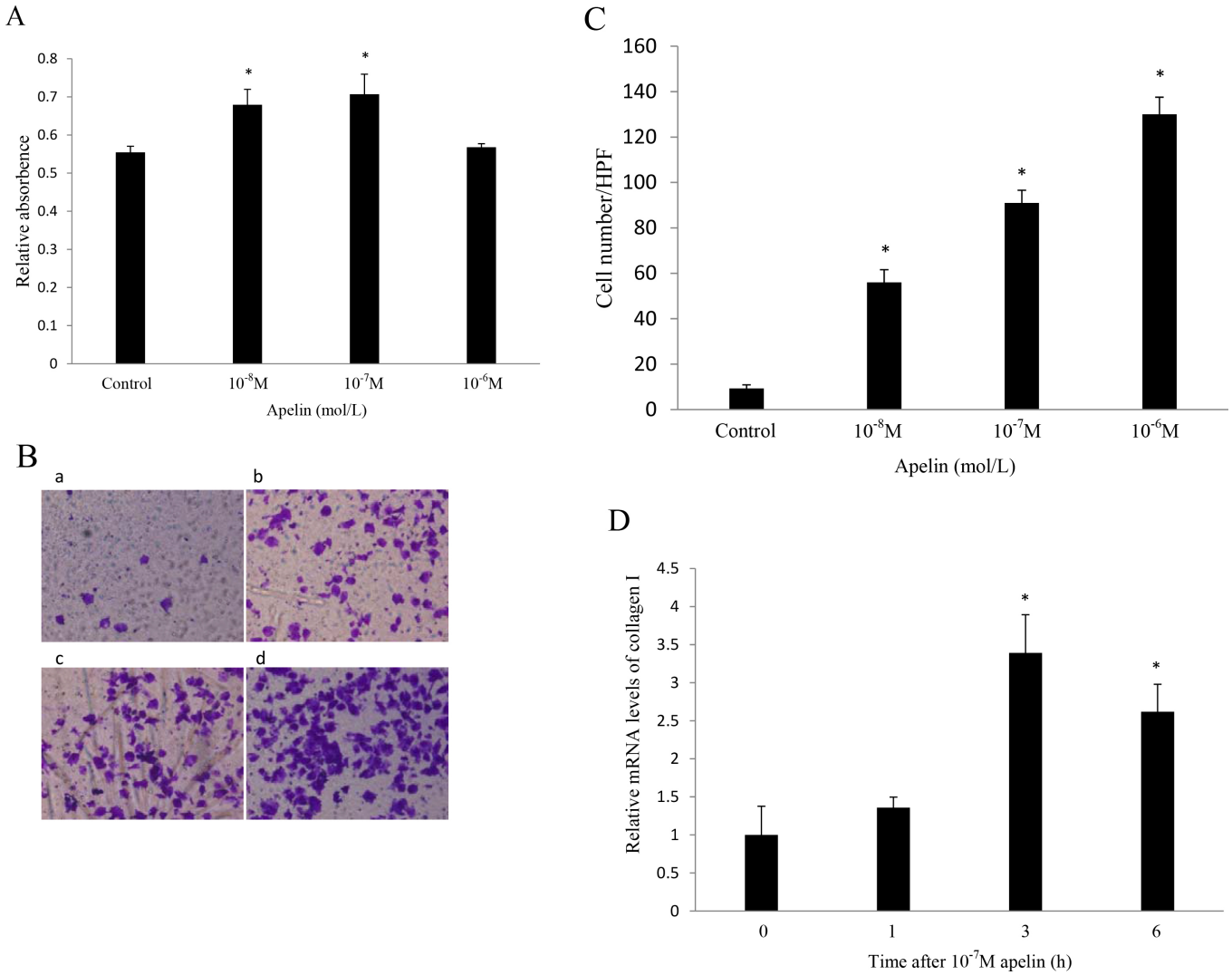

Figure 3. Apelin-induced proliferation, migration, and collagen I expression in RPE cells. **A**: RPE cell proliferation was determined with MTT after 48 h incubation with concentrations of 10^{-8} M, 10^{-7} M, and 10^{-6} M apelin. The 10^{-8} M and 10^{-7} M apelin treatment increased RPE cell proliferation. *p<0.05 versus untreated control. **B**, **C**: RPE cell migration in response to apelin treatment was measured using a transwell assay (a: control; b: 10^{-8} M apelin; c: 10^{-7} M apelin; d: 10^{-6} M apelin. 200X magnification). The values were assessed by the mean number of migrated cells. The number of migrated cells per high power field (HPF) is shown. *p<0.001 versus untreated control. **D**: Collagen I expression in RPE cells was determined with real-time PCR. Time-dependent induction of collagen I expression after stimulation with 10^{-7} M apelin. *p<0.05 versus untreated control. The data are expressed as means±standard deviation (SD), and the experiments were performed independently three times.

Fibrosis is an important but neglected worldwide health problem, which is involved in diseases such as post-infection fibrotic complications, atherosclerosis, and connective tissue disease. Fibrosis is an excessive deposition of extracellular matrix molecules in tissues and organs. Forming fibrotic membranes is a very important pathological feature of PDR. Collagen I, as a major extracellular matrix molecule, is involved in forming fibrotic membrane in PDR [38]. Previously, apelin has been observed to mediate collagen I expression in LX-2 cells [39]. However, the effect of apelin on collagen I expression in RPE cells remains unclear. Our study showed that administering synthetic apelin peptide induced collagen I at the transcriptional level.

Phosphorylation of Akt and Erk is involved in proliferation, migration, vascular remoding, and angiogenesis [40-42]. Furthermore, endothelial growth factor receptor (EGFR) ligand activation promotes RPE cell proliferation and survival, signaling through the ERK/MAPK and PI3K pathways [43,44]. Apelin can activate phosphorylation of Akt and Erk in umbilical endothelial cells [29]. However, the signaling

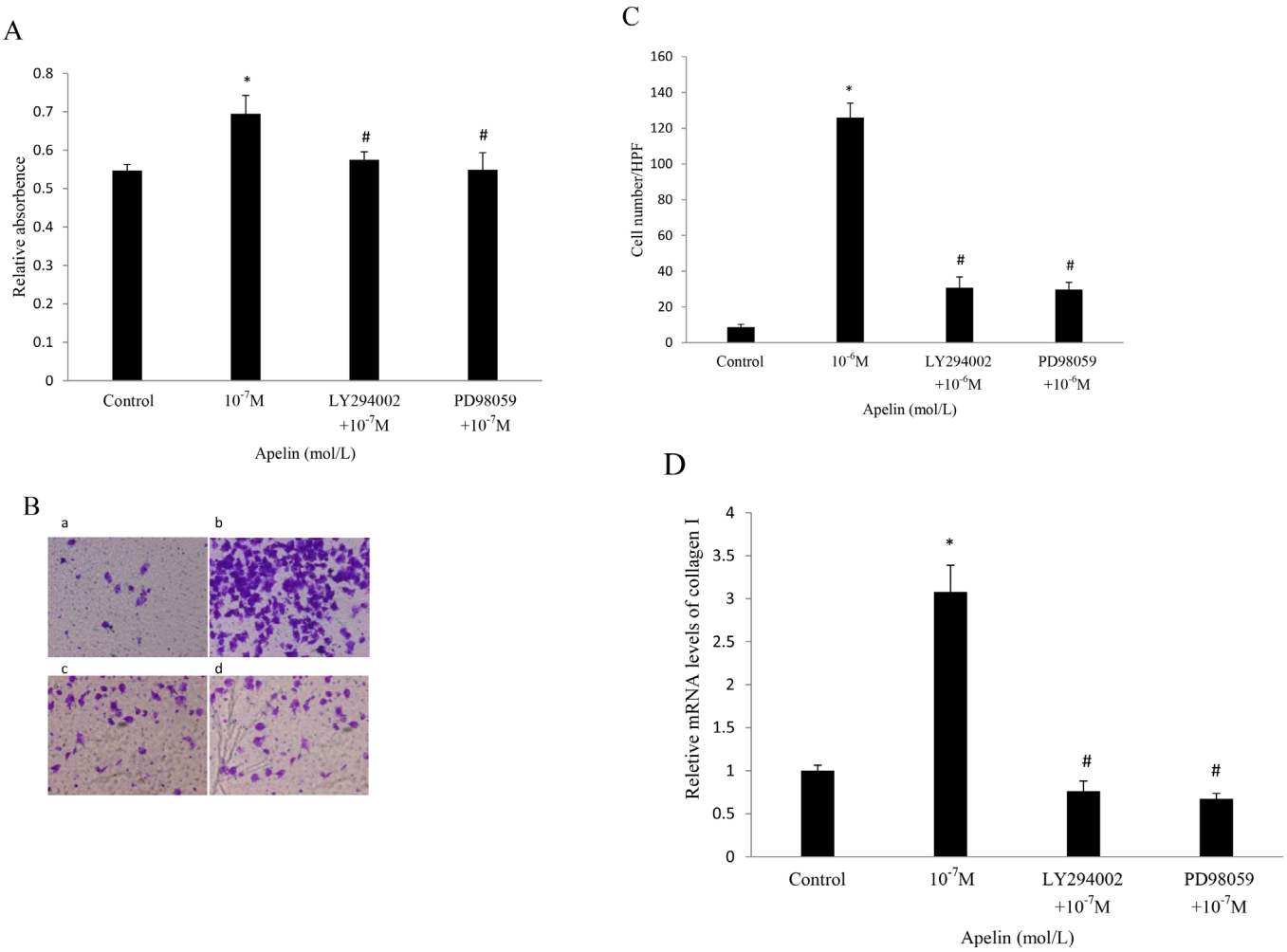

Figure 4. Distinct intracellular signaling pathways mediate apelin-induced proliferation, migration, and collagen I production. **A**: RPE cells were treated with various kinase inhibitors in the presence of 10^{-7} M apelin. Blocking apelin-induced RPE cell proliferation by pretreatment with 10 μM LY294002 and 20 μM PD98059 for 30 min, as measured with MTT assay after 48 h incubation. *p<0.05 versus untreated control. #p<0.05 versus apelin-treated alone. **B**, **C**: Inhibition of apelin-induced migration by pretreatment with 10 μM LY294002 and 20 μM PD98059 for 30 min, as measured with a transwell assay after 6 h incubation (a: control; b: 10^{-6} M apelin; c: LY294002+10^{-6} M apelin; d: PD98059+10^{-6} M apelin. 200× magnification). The number of migrated cells per HPF is shown. *p<0.001 versus untreated control. #p<0.01 versus apelin-treated alone. **D**: Complete inhibition of apelin-induced collagen I expression by 10 μM LY294002 and 20 μM PD98059. RPE cells were stimulated with 10^{-7} M apelin with or without pretreatment with kinase inhibitors for 30 min. After 3 h incubation, RNA was extracted, and collagen I expression was measured with real-time PCR. *p<0.05 versus untreated control. #p<0.05 versus apelin-treated alone. The data shown represent the mean±standard deviation (SD) of three independent experiments.

mechanism of apelin in RPE cells is unclear. To gain further insight into the molecular mechanisms by which apelin induces proliferation, migration, and collagen I expression in RPE cells, we examined intracellular signaling pathways. We found that treatment of RPE cells with various concentrations of apelin significantly increased phosphorylation of Akt and Erk. Treatment with the PI3K inhibitor LY294002 and MEK inhibitor PD98059 blocked the activation of Akt and Erk in RPE cells, indicating that the phosphorylation of Akt and Erk depends on PI3K and MEK. We next examined the functional involvement of the PI3K/Akt and MEK/Erk signaling pathways in apelin-induced proliferation, migration, and collagen I expression. We also found that treatment with LY294002 and PD98059 abolished apelin-induced proliferation and collagen I expression and partially blocked apelin-induced migration in RPE cells.

In summary, our previous study demonstrated that apelin was increased in the vitreous and fibrotic membranes in patients with PDR in vivo. Our results in this study showed that high glucose upregulated apelin expression in RPE cells

in vitro. Furthermore, exogenous recombinant apelin induced proliferation, migration, and collagen I mRNA expression through the PI3K/Akt and MEK/Erk signaling pathways in RPE cells. This study extends our knowledge of the role of apelin in the development of PDR.

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