

Latanoprost Promotes Neurite Outgrowth in Differentiated RGC-5 Cells via the PI3K-Akt-mTOR Signaling Pathway

Jun Zheng · Xuemei Feng · Lina Hou ·
Yongyao Cui · Liang Zhu · Jian Ma ·
Zheng Xia · Wei Zhou · Hongzhan Chen

Received: 24 September 2010 / Accepted: 12 January 2011 / Published online: 29 January 2011
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Abstract Latanoprost, a synthetic derivative of the natural prostaglandin F_{2a} (PGF_{2a}), is a powerful antiglaucoma agent with ocular hypotensive and neuroprotective effects. However, the neuroregenerative effect and signaling pathway of latanoprost in retinal ganglion cells (RGCs) are still unknown. The purpose of this study is to investigate the regenerative effect of latanoprost in differentiated RGC-5 cells and its underlying mechanisms. Cell viability was determined by Cell Counting Kit-8 (CCK-8) assay and neurite length was examined by ArrayScan HCS Reader and Neurite outgrowth BioApplication. Expressions of Akt phosphorylation (p-Akt) and mammalian target of rapamycin phosphorylation (p-mTOR) were investigated by Western blot analysis. The results indicated that 0.1 μM latanoprost (at a clinically therapeutic concentration) significantly increased cell viability as compared with control. Meanwhile, 0.1 μM latanoprost resulted in the obvious promotion of neurite outgrowth similar to ciliary neurotrophic factor (CNTF) and simultaneously increased the levels of p-Akt and p-mTOR expression. The effects of latanoprost were blocked by the Prostaglandin F receptor (FP receptor) inhibitor AL8810, the phosphoinositide 3-kinase (PI3K) inhibitor LY294002 and the mTOR inhibitor rapamycin. This study presents novel *in vitro* evidence that latanoprost could promote neurite outgrowth through

an FP receptor-mediated modulation of the PI3K-Akt-mTOR signaling pathway. This finding may provide insight into a better understanding of a new mechanism of latanoprost for glaucoma therapy and into the physiological-modulating activities of prostaglandins.

Keywords Latanoprost · Neurite outgrowth · Phosphatidylinositol 3-kinase · Mammalian target of rapamycin · Retinal ganglion cells

Introduction

Glaucoma is a group of diseases characterized by progressive optic nerve degeneration that leads to visual field loss and irreversible blindness. The common characteristics of all types of glaucoma are the death of retinal ganglion cells (RGCs) and degeneration of optic nerve. The current standard therapy for glaucoma is the lowering of ocular pressure using medications and/or surgery. Neuroprotection is considered a therapy to prevent the death of RGCs (Morquette and Di Polo 2008). However, detailed studies investigating glaucoma therapies for neuroregeneration have rarely been undertaken.

Prostaglandin analogs are the most potent of the currently available glaucoma and ocular hypotensive medications. Prostaglandin analogs act on the Prostaglandin F receptor (FP receptor) to generate changes in the extracellular matrix and intermuscular space. Latanoprost is a first-line prostaglandin-based treatment for glaucoma because it effectively lowers intraocular pressure (IOP) and has fewer systemic side effects than β-blockers. In addition to its potent ocular hypotensive effect, latanoprost has neuroprotective effect on RGCs in stressed-damaged retina. Latanoprost may induce endogenous prostaglandin E₂ in the retina, which can protect

J. Zheng · X. Feng · L. Hou · Y. Cui · L. Zhu · J. Ma ·
Z. Xia · W. Zhou (✉) · H. Chen (✉)
Department of Pharmacology, Institute of Medical Sciences,
Shanghai Jiao Tong University School of Medicine, 280 South
Chongqing Road, Shanghai 200025, China
e-mail: sweetzw@hotmail.com

H. Chen
e-mail: hongzhan_chen@hotmail.com

neurons against excitotoxic and anoxic injury in the central nervous system (CNS). The neuroprotective activity of latanoprost may be mediated through the mitogen-activated protein kinase (MAPK) signaling pathway and a blockade of excessive Ca^{++} influx (Drago et al. 2001; Kudo et al. 2006; Nakanishi et al. 2006; Hernández et al. 2008; Kanamori et al. 2009). And it was also reported to rescue from apoptosis by stimulation of GSH and decreasing ROS generation (Osborne et al. 2010). A recent study demonstrated that animals treated with latanoprost had the lowest percentage loss of RGCs after IOP normalization (Vidal et al. 2010), which suggests that latanoprost exerts neuroprotection under stress and may play a role under physiological conditions.

Although numerous studies have defined various pharmacological and neuroprotective effects of latanoprost, the neuroregenerative effect of latanoprost in RGCs has not been investigated. Neurite outgrowth is crucial component in neuroregeneration and remodeling injury, which are mediated by activation of the MAPK-extracellular signal-regulated kinase (ERK), phosphoinositide 3-kinase (PI3K)-Akt, and phospholipase C (PLC)-gamma 1 signaling pathways (Lindsley 2010). Recently, much attention has been directed to the PI3K-Akt-mammalian target of rapamycin (mTOR) signaling pathway that plays a fundamental role in axonal and dendritic growth (Li et al. 2010).

The RGC-5 cell line is a transformed retinal ganglion cell line that has certain biochemical characteristics of RGCs including the expression of Thy-1, Brn-3c, Neuritin, NMDA receptor, GABA receptor, and synaptophysin (Krishnamoorthy et al. 2001). Succinyl Concanavalin A (sConA) is a nontoxic derivative of the lectin concanavalin A, which induces differentiation of RGC-5 cells including a slowed growth rate, neurite outgrowth and the development of a sensitivity to glutamate toxicity (Ju et al. 2007; Ju et al. 2009; Wood et al. 2010). Differentiated RGC-5 cells appear to be a suitable model system to study a variety of biochemical and pharmacological mechanisms involved in axon and dendrite formation (Rock et al. 2008; Bergen et al. 2009). So we employed the model of differentiated RGC-5 to observe the neuroregenerative effect of latanoprost.

In this study, we demonstrate that latanoprost, at a clinical concentration of 0.1 μM , significantly promoted neurite outgrowth of differentiated RGC-5 cells via the PI3K-Akt-mTOR signaling pathway.

Materials and Methods

Treatment of Cell Cultures

Rat retinal ganglion cells (RGC-5 cells) were purchased from the American Type Culture Collection (Manassas,

VA). Cells were cultured in DMEM (Invitrogen Life Technologies, Carlsbad, CA) containing 1 mM glucose, 100 U/ml penicillin/streptomycin (Invitrogen Life Technologies, Carlsbad, CA) and 10% of heat-inactivated fetal calf serum (Invitrogen Life Technologies, Carlsbad, CA) in a humidified incubator with 95% air and 5% CO_2 at 37°C. Cells were then incubated with DMEM without heat-inactivated fetal calf serum and supplemented with 50 $\mu\text{g}/\text{ml}$ sConA (Sigma-Aldrich, St. Louis, MO) for 48 h to induce differentiation. After 48 h, the medium was changed to DMEM containing 10% heat-inactivated fetal calf serum. Chelerythrine Chloride (CC) (Sigma-Aldrich, St. Louis, MO, 1 μM , a protein kinase C (PKC) inhibitor), U0126 (Sigma-Aldrich, St. Louis, MO, 5 μM , an MAPK pathway inhibitor), LY294002 (Sigma-Aldrich, St. Louis, MO, 10 μM , a PI3 kinase inhibitor), SB216763 (Sigma-Aldrich, St. Louis, MO, 5 μM , a glycogen synthase kinase 3 (GSK-3) inhibitor), rapamycin (Sigma-Aldrich, St. Louis, MO, 0.5 μM , an mTOR inhibitor) and AL8810 (Sigma-Aldrich, St. Louis, MO, 10 μM , an FP receptor inhibitor) were used to observe their inhibitory effects on RGC-5 cells treated with latanoprost (Sigma-Aldrich, St. Louis, MO). The inhibitors were added 1 h prior to latanoprost.

Cell Viability Assay

Differentiated RGC-5 cells were treated with latanoprost at different concentrations (0.1–10 μM). After treatment with latanoprost and/or inhibitors for 24 h, cell viability was evaluated using a cell counting kit-8 (CCK-8) (WST-8, Dojindo, Japan). The CCK-8 was used to count living cells by combining WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] and 1-methoxy PMS. According to the supplier's recommendations, 10 μl of kit reagent was added to the cells, treated as described above, in 96-well plates and incubated for 3 h. Cell viability was assayed by reading the absorbance at 450 nm on a 96-well plate reader. The absorbance reading was subtracted from background control. All experiments were performed in three wells on five separate experiments.

Measurement and Analysis of Neurite Length

Cellomics' Neurite Outgrowth High-Content Screening (HCS) Reagent Hit Kit (Thermo, USA) was used to assay the mean total neurite length of differentiated RGC-5 cells. The Kit product provides a more effective solution for quantifying neurite length by combining HCS-quality fluorescence reagents with a validated, automation-compatible protocol. After the RGC-5 cells were treated, the medium was removed and the cells were fixed in

pre-warmed Fixation/Hoechst Dye Solution (em. = 350/461 nm) for 20 min. The cells were incubated for 1 h with a neurite outgrowth primary antibody. Next, cells were incubated with DyLight 488-conjugated (em. = 495/519 nm) Secondary Antibody for 1 h. Sample plates are automatically quantitated using the ArrayScan HCS Reader and Cellomics' neurite outgrowth BioApplication (Thermo, USA). The BioApplication distinguishes and quantifies outgrowth of neurites from neurons and neuron-like cells. Using a 20× objective lens, a sufficient number of fields (1 field = 660 μm × 660 μm) were acquired for the analysis of at least 200 cells per well. CNTF (50 ng/ml) was used as a positive control.

Western Blot Analysis

Cells were washed twice in phosphate-buffered saline and lysed in ice-cold SDS buffer (2% SDS, 30 mM Tris-HCl pH 6.8, 10% glycerol, 2 mM EDTA). Lysates were sonicated and protein concentrations were determined using the BCA Protein Assay Kit (Pierce Chemical, Rockford, IL, USA). Prior to loading, 2.5% 2-mercaptoethanol and 0.0125% bromophenol blue were added and lysates were boiled for 5 min. Proteins were separated by SDS-PAGE and transferred onto PVDF membranes (Millipore Billerica, MA). The membranes were incubated overnight at 4°C with the following primary antibodies: anti-p-Akt antibody (1:1000, Santa Cruz Biotechnology, Santa Cruz CA, USA), anti-Akt antibody (1:1000, Cell Signaling Technology, USA), anti-p-mTOR antibody (1:500, Cell Signaling Technology, USA), anti-mTOR antibody (1:500, Cell Signaling Technology, USA), and anti-β-actin antibody (1:1000, Santa Cruz Biotechnology, Santa Cruz CA, USA). The membranes were incubated with the corresponding horseradish peroxidase-coupled secondary antibodies (1:10000, Pierce Chemical, Rockford, IL, USA) at room temperature for 1 h. The immunoblots were visualized using an enhanced chemiluminescence detection kit (Pierce Chemical, Rockford, IL, USA). Relative levels of protein were quantified by optical density analysis. To avoid interassay variability, values were normalized to the control value in each experiment.

Data Analysis

The data are presented as the mean ± SEM. Data were analyzed with a one-way analysis of variance (one-way ANOVA) and the post hoc Dunnett's test for multiple comparisons where $P < 0.05$ indicated a significant difference.

Results

Effect of Latanoprost on the Viability of Differentiated RGC-5 Cells

RGCs viability is a prerequisite for ensuing neuroregeneration (Kretz et al. 2005). To determine the effect of latanoprost on the viability of differentiated RGC-5 cells, cells were cultured with various concentrations (0.1–10 μM) of latanoprost for 24 h. CNTF (50 ng/ml) was used as positive control. CCK-8 assay showed that treatment with latanoprost remarkably increased differentiated RGC-5 cell viability at lower concentration of 1 and 0.1 μM, whereas 10 μM latanoprost had no effect on cell viability as compared with control (Fig. 1a). Exposure to 0.1 μM latanoprost for 24 h, which significantly improved cell viability by approximately 30.5% ($P < 0.01$) compared with the control, was used in subsequent experiments. And treatment with 10 μM AL8810 (FP receptor inhibitor) significantly abolished the effect of latanoprost (Fig. 1b). These results indicated that latanoprost promoted cell viability via the FP receptor.

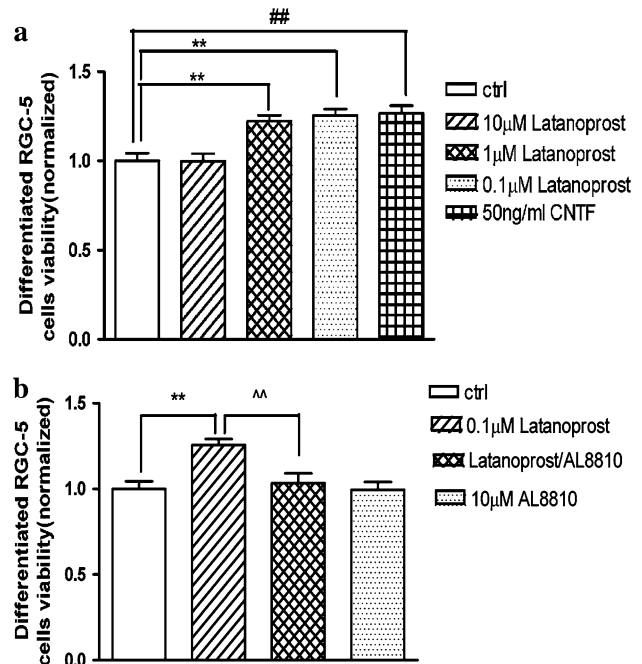


Fig. 1 Effects of latanoprost and FP receptor inhibitor AL8810 on cell viability of differentiated RGC-5 cells **a** Differentiated RGC-5 cell cultures were treated with different concentrations of latanoprost and 50 ng/ml CNTF for 24 h. **b** After pretreatment with 10 μM of the FP receptor inhibitor AL8810 for 1 h, RGC-5 cell cultures were exposed to 0.1 μM latanoprost for 24 h. Data were normalized to values of the control group. The data are reported as the mean ± SEM of five independent experiments performed in triplicate. ** $P < 0.01$ compared with the control. ### $P < 0.01$ CNTF group compared with the control

Effect of Latanoprost on Neurite Outgrowth of Differentiated RGC-5 Cells

To investigate the neuroregenerative effect of latanoprost, the mean total neurite length (an average of all neurite outgrowth of neurons within each well) of differentiated RGC-5 cells was quantified after the application of 0.1 μM latanoprost for 24 h. Neurite Outgrowth HCS Reagent Hit Kit was widely used to assay the neurite outgrowth of variety neural cells (Hansen et al. 2007; Radio et al. 2008). By using the kit, cells were immunostained using the Hoechst dye and primary antibody with corresponding DyLight 488-conjugated secondary antibody. Image acquisition using two fluorescence channels was performed using the ArrayScan HCS Reader for the nuclei (blue) and the cell body and neuritis (green) (Harrill et al. 2010). Neurite outgrowth BioApplication software analyzed the field to determine morphological parameters of interest in

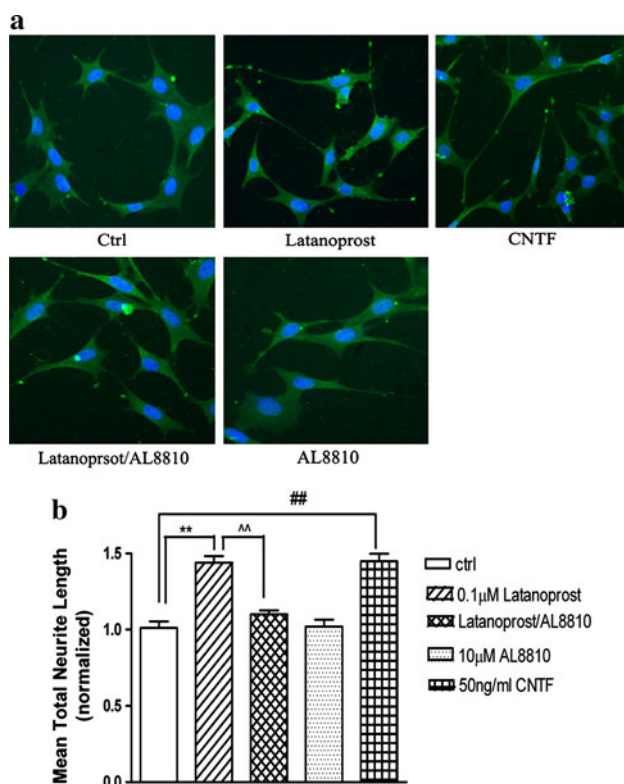


Fig. 2 Effect of latanoprost on neurite outgrowth of differentiated RGC-5 cells **a** Images of differentiated RGC-5 cells were acquired with a 20 \times objective lens with Neurite Outgrowth HCS reagents. A sufficient number of fields (1 field = 660 μm \times 660 μm) were acquired for the analysis of at least 200 cells per well. Cell nuclei were labeled with Hoechst Dye. The neurons and their neurites were identified by immunofluorescence. **b** Quantification of the mean total neurite length of differentiated RGC-5 cells. Data were normalized to values of the control group. The data are the mean \pm SEM for three different experiments. ** $P < 0.01$ compared with the control. ### $P < 0.01$ CNTF group compared with the control. ^^ $P < 0.01$ compared with the latanoprost group

the image (Liu et al. 2007). The acquired images were quantified using a mathematical algorithm to evaluate the mean total neurite length. As shown in Fig. 2, latanoprost and CNTF had a similar effect on neurite length in differentiated RGC-5 cells. The effect of latanoprost was approximately 1.45-fold stronger than the control ($P < 0.01$). Pretreatment with 10 μM AL8810 completely blocked the effect of latanoprost, suggesting the involvement of the FP receptor. The result implicated a potential neuroregenerative activity of latanoprost. And neurite outgrowth promotion by latanoprost depended on the activation of the FP receptor.

Effect of Latanoprost on the Modulation of the PI3K-Akt-mTOR Signaling Pathway

There are several cellular signaling pathways that play critical roles in neurite outgrowth, such as MAPK-ERK signaling pathway and PI3K-Akt signaling pathway. To elucidate the signaling pathway involved in the neurite outgrowth-promoting effect of latanoprost, we applied four signaling pathway inhibitors (MAPK inhibitor U0126, PI3K inhibitor LY294002, PKC inhibitor CC, and GSK-3 inhibitor SB216763) with or without latanoprost to retinal cultures. The data presented in Fig. 3 indicated that only the PI3K inhibitor, LY294002, completely inhibited the neuroregenerative effect of latanoprost. In addition, the mTOR (the PI3K-Akt downstream target) inhibitor rapamycin also markedly reduced the effect of latanoprost ($P < 0.01$).

In support of previous results, Western blot analysis indicated that latanoprost significantly increased the levels of Akt phosphorylation (p-Akt) compared with the control. Consistent with changes in Akt activity, latanoprost resulted in an increase of mTOR phosphorylation (p-mTOR). Moreover, pretreatment of the cells with AL8810 and LY294002 markedly prevented the latanoprost-induced increase in the levels of p-Akt and p-mTOR (Figs. 4, 5). The results suggested that latanoprost stimulated neurite outgrowth of differentiated RGC-5 cells in a PI3K-Akt-mTOR signaling pathway-dependent fashion.

Discussion

This study presents the first evidence that latanoprost can significantly promote neurite outgrowth of differentiated RGC-5 cells. And latanoprost exerted its neurite outgrowth-promoting effect through an FP receptor-mediated modulation of the PI3K-Akt-mTOR signaling pathway. This was supported by our major findings: (i) treatment with latanoprost promoted neurite outgrowth in differentiated RGC-5 cells; (ii) neurite outgrowth promotion by

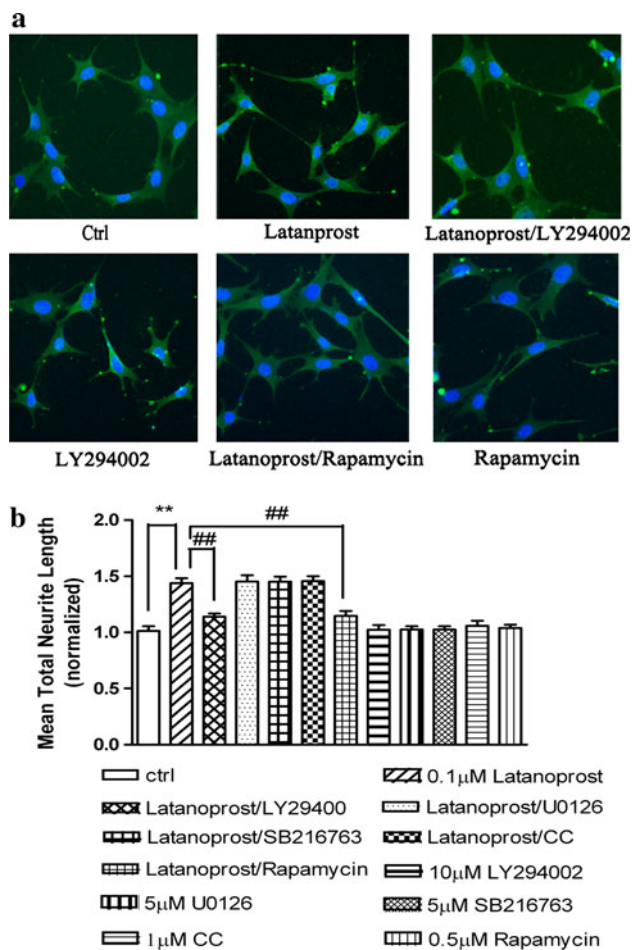


Fig. 3 Effects of several signaling pathway inhibitors on neurite outgrowth of differentiated RGC-5 cells. **a** Images of differentiated RGC-5 cells were acquired with a 20× objective lens with Neurite Outgrowth HCS reagents. A sufficient number of fields (1 field = 660 μm × 660 μm) were acquired for the analysis of at least 200 cells per well. Cell nuclei were labeled with Hoechst Dye. The neurons and their neurites were identified by immunofluorescence. **b** Quantification of the mean total neurite length of differentiated RGC-5 cells. Data were normalized to values of the control group. The data are the mean ± SEM for three different experiments. ** $P < 0.01$ compared with the control. ## $P < 0.01$ compared with the latanoprost group

latanoprost depended on the activation of the FP receptor; and (iii) treatment with latanoprost up-regulated the levels of p-Akt and p-mTOR expression.

Glaucoma is one kind of neurodegenerative diseases, which is characterized by progressive loss of RGCs and optic nerve damage. So far, glaucoma therapy mainly focused on reduction of IOP, whereas the development of neuroregenerative therapy for glaucoma is still at an early stage. Neurites, including axons and dendrites, play an important role in the formation and maintenance of the nervous system. In humans, the millions axons of RGCs join to form the optic nerve. The promotion of neurite

outgrowth is important for neuroregeneration strategies in the treatment of glaucoma (Dahlmann-Noor et al. 2010).

Prostaglandins are a group of biologically active compounds that play major roles in the physiology and pathology in the body. In recent years, prostanoids have been recognized to have an additional role in the central nervous system where they are involved in synaptic plasticity, memory, and neuronal protection (Chen and Bazan 2005; Villena et al. 2009). Using differentiated RGCs cultures, we showed that latanoprost had a neurite growth-promoting capability similar to CNTF and that this effect was directly mediated by the FP receptor. CNTF is a trophic molecule that promotes neurite outgrowth and supports the survival of all classes of peripheral nervous system neurons and many central nervous system neurons (Lingor et al. 2008; Müller et al. 2009; Ahmed et al. 2009). Our data suggest that latanoprost may exhibit a CNTF-like activity in RGC-5 cells and produce a neurite growth-promoting effect.

Accumulating evidence suggests that a single administration of eye drops may penetrate to the retrobulbar tissue or vitreous via the peribulbar route. And the amount is in the order of $1/10^4$ of eye drops in the vitreous (Mizuno et al. 2002). Usually, the volume of one drop is about 50 μl and 250 pg latanoprost can be expected in the vitreous. Given the molecular weight of latanoprost, about 0.2 μM latanoprost can be expected in the vitreous cavity (Alm and Stjernschantz 1995). In this study, 0.1 μM latanoprost showed a neurite growth-promoting effect, indicating that latanoprost at a clinical dose may exert sufficient neurite growth-promoting effects on RGCs.

Neuronal differentiation and neurite outgrowth are mediated by activation of the MAPK-ERK, PI3K-Akt, and PLC-gamma 1 signaling pathways. Using specific antagonists of signal transduction in this study, we found that only the PI3K inhibitor LY294002 compromised latanoprost-promoted neurite outgrowth in differentiated RGC-5 cells. Akt regulates a broad range of biological responses through the phosphorylation of distinct substrates (Read et al. 2008; Jover-Mengual et al. 2010). mTOR is one of the identified physiological targets of Akt, and its activity can be directly inhibited by the PI3K inhibitor LY294002 (McMahon et al. 2005). The activation of PI3K-Akt-mTOR signaling pathway induces the synthesis of cytoskeleton-associated protein, which plays a fundamental role in axonal and dendritic growth (Kwon et al. 2006; Narayanan et al. 2009). As a main modulator of cell growth and proliferation, mTOR controls the efficiency of protein translation via its downstream targets. The activation of mTOR could promote axonal regeneration in the adult CNS (Grider et al. 2009; Park et al. 2008; Park et al. 2010). In agreement with previous findings that the presence of LY294002 abolished

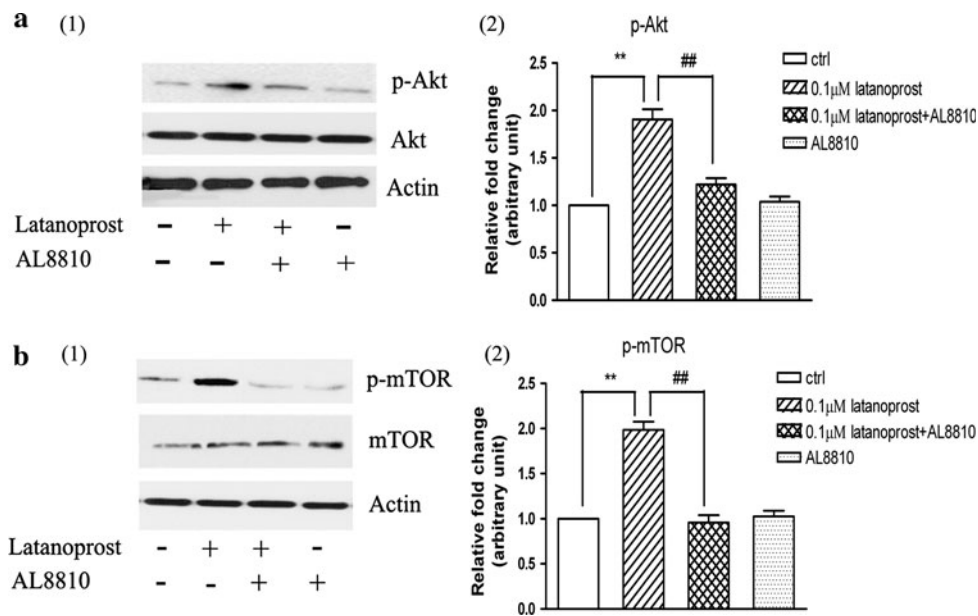


Fig. 4 Effects of latanoprost and FP receptor inhibitor AL8810 on the protein levels of p-Akt and p-mTOR in differentiated RGC-5 cells. **a.1** After pretreatment with 10 μM of the FP receptor inhibitor AL8810 for 1 h, RGC-5 cell cultures were exposed to 0.1 μM latanoprost for 24 h. Immunoreactive bands of p-Akt, Akt, and actin. **a.2** The densitometric quantification ratio of p-Akt was normalized to

total Akt. **b.1** After pretreatment with 10 μM of the FP receptor inhibitor AL8810 for 1 h, RGC-5 cell cultures were exposed to 0.1 μM latanoprost for 24 h. Immunoreactive bands of p-mTOR, mTOR, and actin. **b.2** The densitometric quantification ratio of p-mTOR was normalized to total mTOR. ** $P < 0.01$ compared with the control. ## $P < 0.01$ compared with the latanoprost group

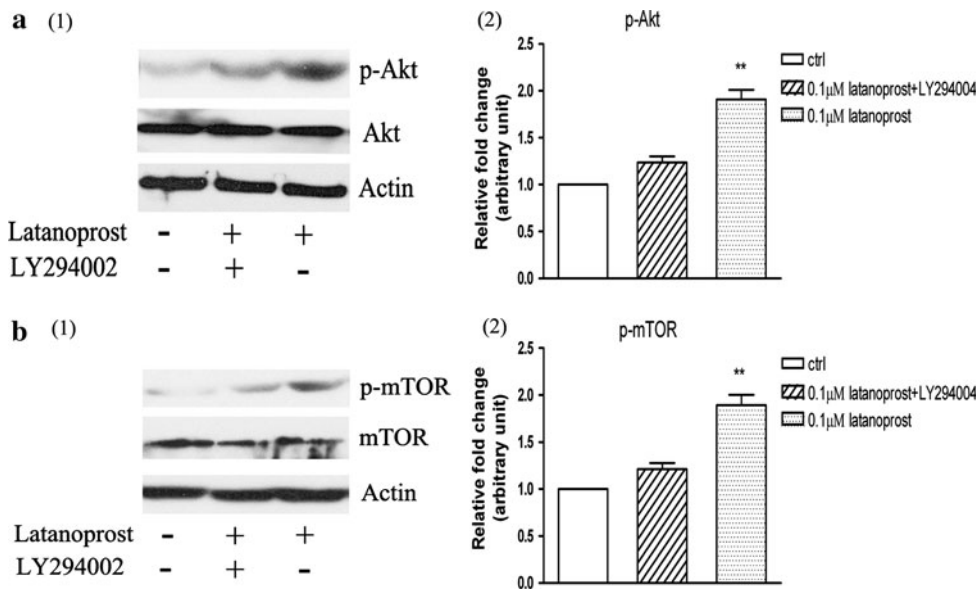


Fig. 5 Effects of latanoprost and PI3K inhibitor LY294004 on the protein levels of p-Akt and p-mTOR in differentiated RGC-5 cells. **a.1** After pretreatment with 10 μM of the PI3K inhibitor LY294004 for 1 h, RGC-5 cell cultures were exposed to 0.1 μM latanoprost for 24 h. Immunoreactive bands of p-Akt, Akt, and actin. **a.2** The densitometric quantification ratio of p-Akt was normalized to total

Akt. **b.1** After pretreatment with 10 μM of the PI3K inhibitor LY294004 for 1 h, RGC-5 cell cultures were exposed to 0.1 μM latanoprost for 24 h. Immunoreactive bands of p-mTOR, mTOR, and actin. **b.2** The densitometric quantification ratio of p-mTOR was normalized to total mTOR. ** $P < 0.01$ compared with the control

latanoprost-promoted neurite outgrowth, our results confirmed that the inhibition of mTOR by rapamycin blocked latanoprost-induced neurite outgrowth.

In conclusion, the above results demonstrate that latanoprost at a clinically therapeutic concentration may promote neurite outgrowth through an FP receptor-mediated

modulation of PI3K-Akt-mTOR signaling pathway. This finding may provide insight for a better understanding of a new mechanism of latanoprost for glaucoma therapy and into the physiological-modulating activities of prostaglandins. This signaling pathway study may provide further clues concerning how neurons lose the capacity to extend axons following neurodegenerative diseases and injury.

Acknowledgments This study was supported by grants from the National Natural Science Foundation of China (No. 30700280), the National Basic Research Program of China (No. 2010CB529806), the Shanghai Municipal Science and Technology Commission (No. 09ZR1416000), and the Shanghai Leading Academic Discipline Project (No. S30205).

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