Effects of LY294002 on the function of retinal endothelial cell in vitro

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Received: 2018-03-18   Accepted: 2018-05-28

Abstract

AIM: To study the effects of LY294002 [phosphatidylinositol 3-kinase (PI3K) inhibitor] on the function and mechanisms of retinal endothelial cells (RECs) in vitro.

METHODS: RECs were randomly divided into control group and LY294002 treatment group. RECs in the control group were placed the incubator for hypoxic exposure in vitro. RECs in the LY294002 treatment group were pretreated with LY294002 (40 μmol/L) under hypoxic condition. The expression of matrix metalloproteinase (MMP)-2, MMP-9, vascular endothelial growth factor (VEGF), and apoptosis and proliferation of RECs were evaluated with Western blot, real-time reverse transcription-polymerase chain reaction (RT-PCR), and flow cytometric analysis, correspondently.

RESULTS: Compared with the control group, treating the RECs with LY294002 was able to remarkably inhibit cell proliferation rates (t1=2.13, t2=2.65, t3=2.36, t4=2.06, all P<0.05). Flow cytometric analysis indicated that a moderate increase in apoptosis in the LY294002 treatment group compared to the control group (t=2.51, P<0.05). The expression of MMP-2, MMP-9 and VEGF were downregulated in the LY294002 treatment group by Western blot and real-time RT-PCR (all P<0.05).

CONCLUSION: LY294002 regulates the function of RECs by reducing the expression of MMP-2, MMP-9, and VEGF in vitro. LY294002 may provide an effective method for preventing pathological angiogenesis.

KEYWORDS: LY294002; phosphatidylinositol 3-kinase; retinal endothelial cell; angiogenesis

DOI:10.18240/ijo.2018.09.03

INTRODUCTION

Ocular angiogenesis is a pathological feature of numerous pathological angiogenic diseases, including retinopathy of prematurity (ROP), proliferative diabetic retinopathy (PDR), and age-related macular degeneration (AMD)\cite{1-2}. Currently, the treatment of ocular angiogenesis includes laser surgery or inhibition of the pro-angiogenic factor vascular endothelial growth factor (VEGF) molecular therapy\cite{3-6}. However, targeting the angiogenesis pathway or binding to VEGF may lead to more effective and safer ophthalmic angiogenesis inhibitors. Recent studies have demonstrated that LY294002 [phosphatidylinositol 3-kinase (PI3K) inhibitor] is an effective and safe inhibitor for eye development and ectopic hyaloid angiogenesis\cite{7-8}.

However, the specific mechanisms, which are involved in LY294002-mediated pathological angiogenesis in retinal endothelial cells (RECs) need to be fully clarified. This study hypothesized that LY294002 could regulate the function of RECs through reducing matrix metalloproteinase (MMP)-2, MMP-9, and VEGF expression in vitro. To confirm this hypothesis, this study investigated the effect of LY294002 on the function of RECs under hypoxic condition.

MATERIALS AND METHODS

Drug Preparation

LY294002 (Sigma-Aldrich, St. Louis, MI, USA) diluted in dimethyl sulfoxide (DMSO) to create an inventory solution (the final concentration was 40 μmol/L). Subsequent experiments were carried out using the concentration of LY294002 on RECs in vitro.

Cell Culture

RECs (Cell Systems Corporation, Kirkland, WA, USA) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS) in incubator for hypoxic condition containing 5% CO₂. The cells were divided into control and LY294002 treatment group. LY294002 treatment group were pretreated with LY294002 (40 μmol/L) for 30min prior to being cultured under hypoxic condition.

Proliferation Assay

For cell counting kit-8 (CCK8) assay (Beyotime Institute of Biotechnology, Jiangsu, China), 1×10⁴ cells were inoculated at 96-well plates after different treatment and cell proliferation was measured every day for 4d after transfection. A total of 10 μL CCK8 was added and incubated for 2h. Then, the samples were vortexed for 10min and the absorbance was measured in a Sunrise™ microplate reader (Tecan Group, Ltd., Männedorf, Switzerland).
Apoptosis Detection Assay  RECs apoptosis was measured by the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. DAPI (Beyotime Institute of Biotechnology, Jiangsu Province, China) stained cellular nuclei and laser confocal microscopy (FV1000; Olympus Corp., Tokyo, Japan) were used to detect apoptosis.

Western Blot Analysis  The protein from each sample was extracted with lysis buffer. Proteins were isolated and transferred to polyvinylidene fluoride (PVDF) membranes (Merck Millipore, Billerica, MA, USA). Membranes were incubated for the night at 4°C with MMP-2 (sc-13595), MMP-9 (sc-21733), VEGF (sc-57496) and β-actin (1:2000, Santa Cruz Biotechnology Inc.). Then the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:2000, Zhongshan Jinqiao Biotechnology Co., Ltd., China) for 1h at 37°C and visualized with an enhanced-chemiluminescence kit (Thermo Fisher Scientific).

Real-time Reverse Transcription-polymerase Chain Reaction  Total RNA was extracted from each sample using TRizol reagent (Invitrogen, Carlsbad, CA, USA). cDNA synthesis was performed according to the RNA polymerase chain reaction protocol (PrimeScript™ RT Reagent Kit-Perfect Real Time, Takara Bio, Otsu, Japan). The primer sequences were MMP-2: 5’-GGCTTGGACCGAATGCT-3’ (forward) and 5’-TTGTTGAAGGCTGTGGC -3’ (reverse); MMP-9: 5’-AGCAAACAGGCTCACAGGT T-3’ (forward) and 5’-TAAGTCCTCCCCATCTCCCT-3’ (reverse); VEGF: 5’-CCCGACAGGGAA GACAAT-3’ (forward) and 5’-TCTGGAAGTGAGCCAACG -3’ (reverse); β-actin: 5’-GAGAGGGAAATCGTGCGTGA-3’ (forward) and 5’-GCCTAGAAGCAT TTGCGGTG-3’ (reverse). Real-time reverse transcription-polymerase chain reaction (RT-PCR) reaction was performed using SYBR green RT-PCR Master Mix (Premix Ex TaqTM-Perfect Real Time, Takara Bio, Otsu, Japan) on the 7300 real-time RT-PCR system (Applied Biosystems, Foster City, CA, USA).

Statistical Analysis  The data were represented by the mean±standard deviation (SD). Statistical analysis of cell proliferation, cell apoptosis, protein and mRNA were carried out using independent-sample t-test. P<0.05 was significant significance.

RESULTS

Inhibition of Cell Proliferation and Promotion of Cell Apoptosis by LY294002  RECs proliferation was measured using a CCK8 assay. The cell growth curves of 1, 2, 3 and 4d showed that the growth rate was slower in the LY294002 treatment group than in the control group at the same time point (t1d=2.13, t2d=2.65, t3d=2.36, t4d=2.06, all P<0.05; Figure 1, Table 1). In order to detect the effect of LY294002 on RECs apoptosis, TUNEL staining was performed. The experimental result indicated that there were significant differences in apoptosis between the two groups (t=2.51, P<0.05; Figure 2). These results demonstrated that LY294002 treatment cells had a more significant anti-proliferation and pro-apoptotic effects on RECs.

Inhibitory Effect of LY294002 on MMP-2, MMP-9 and Vascular Endothelial Growth Factor Protein Expression  To verify the mechanism of LY294002 on RECs function, the expression of MMP-2, MMP-9 and VEGF were detected by Western blot. Our findings indicated that under hypoxic environment, MMP-2, MMP-9 and VEGF protein levels in the LY294002 treatment group were reduced by 45.01%, 36.91% and 38.84%, respectively, contrasted with the control group (all P<0.05). After LY294002 treatment, MMP-2, MMP-9 and VEGF expressions were inhibited significantly (Figure 3). These results suggested that LY294002 could regulate the function of RECs by reducing the protein expression of MMP-2, MMP-9 and VEGF in vitro.

Inhibitory Effect of LY294002 on MMP-2, MMP-9 and Vascular Endothelial Growth Factor mRNA Expression  In order to detect the interference effect of LY294002, we performed RT-PCR for quantitative detection. Real-time RT-PCR revealed similar results in cell samples. MMP-2, MMP-9 and VEGF mRNA levels in the LY294002 treatment group (+56.23%, +59.45% and +46.45%) were reduced by 43.77%,...
40.55%, and 53.55%, respectively, contrasted with the control group (all $P<0.05$; Figure 4). These results suggested that LY294002 could regulate the function of RECs by reducing the mRNA expression of MMP-2, MMP-9 and VEGF in vitro.

**DISCUSSION**

Pathological angiogenic diseases is characterized by progressive changes in retinal microvessels, including endothelial dysfunction, breakdown of the blood-retina barrier, retinal rupture, ischemia, and retinal neovascularization (RNV)\[^5-6,9\]. The biological behavior of RECs plays a dominant role in the formation of RNV.

The best inhibitor for PI3K is LY294002. Previous studies have directly\[^10-12\] and indirectly\[^13-14\] demonstrated that LY294002 inhibited the growth of various tumor cells by inhibiting the functions of PI3K and downstream components\[^15-16\]. LY294002 may inhibit RECs proliferation and induce RECs apoptosis by inhibiting PI3K. But, the downstream molecules that LY294002 inhibit the apoptosis of RECs by inhibition of PI3K/Akt still to be identified.

In the present study, we used RECs under hypoxic condition to simulate pathological angiogenic diseases in vitro model. Examination of the proliferation of RECs following LY294002 treatment under hypoxic conditions demonstrated that LY294002 significantly inhibited cell proliferation. Furthermore, it was demonstrated that LY294002 promoted apoptosis of the cells, thus interfering with angiogenesis. RNV is a complex series of processes in which many of the gene products are involved in the regulation of MMPs, which play a major role in destroying the extracellular matrix, are overexpressed in pathological angiogenic diseases and are considered to contribute to angiogenesis\[^17-18\]. MMP-2 and MMP-9 are related very closely to RNV, and have been considered as important factors in promoting RNV\[^19-20\]. VEGF is one of the agents to promote angiogenesis and plays a central role in RNV\[^21-22\]. In this study, MMP-2, MMP-9 and VEGF were significantly different between the RECs under

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**Figure 2** LY294002 induces RECs apoptosis under hypoxia A: Cell apoptosis was determined by TUNEL staining. The red arrows show the nucleus of the apoptotic cells. B: Cell viability in each groups. LY294002 remarkably induced cell apoptosis under hypoxic conditions. $^{a}P<0.05$ vs control group.

**Figure 3** LY294002 regulated the function of RECs through inhibition of the expression of MMP-2, MMP-9 and VEGF in vitro A: Western blot assay for protein expression; B: Statistical analysis. $^{a}P<0.05$ vs control group.

**Figure 4** LY294002 regulated the function of RECs through inhibition of MMP-2, MMP-9 and VEGF mRNA expression in vitro mRNA expressions of MMP-2, MMP-9 and VEGF were determined by real-time RT-PCR. $^{a}P<0.05$ vs control group.
hypooxic condition treated by LY294002 and that of the control group \( (P<0.05) \). It is suggested that LY294002 can inhibit the gene expression of MMP-2, MMP-9, and VEGF. Furthermore, we found that LY294002 induced apoptosis in the RECs and had the potential to inhibit pathological angiogenesis. Taken together, this study showed that LY294002 inhibited the proliferation of RECs, induced the apoptosis of RECs, and downregulated cytokines expression, including MMP-2, MMP-9 and VEGF, which acted through inhibiting PI3K/Akt activation. Inhibition of the PI3K pathway is an effective strategy for the treatment of pathological angiogenesis.

**ACKNOWLEDGEMENTS**

**Foundations:** Supported by National Natural Science Foundation of China (No.81600747); Startup Foundation for Doctors of Liaoning Province (No.201501020).

**Conflicts of Interest:** Di Y, Chen XL, None; Chao Y, None; Doctors of Liaoning Province (No.201501020).

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