·Basic Research·

Anthrax lethal toxin suppresses high glucose induced VEGF over secretion through a post-translational mechanism

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Abstract

• AIM: To prove anthrax lethal toxin (LeTx) blocks the mitogen activated protein kinases (MAPKs) activation by degrading the MAPK/ERK kinases (MEKs) to suppress vascular endothelial growth factor (VEGF) secretion.

• METHODS: Human adult retinal pigmented epithelium (ARPE) cells were cultured and treated with normal glucose, high glucose or high glucose with LeTx for additional 24, 48 or 72h for viable cell count. Total RNA from the ARPE was isolated for reverse transcription polymerase chain reaction (RT-PCR). The conditioned medium of ARPE cells treated in different group for 48h was filtered and diluted to detect the concentration of VEGF by enzyme-linked immunosorbant assays. Evaluate the role of MEK/MAPK pathway in the secretion of VEGF by immunoblotting.

• RESULTS: In this study, we proved high glucose induced activation of the MAPK extracellular signal-regulated kinase (ERK1/2) and p38 in the ARPE cell line was blocked by anthrax LeTx. LeTx also inhibited high glucose induced ARPE cell over proliferation.

• CONCLUSION: LeTx suppressed high glucose induced VEGF over secretion in the ARPE cells, mainly through a post-translational mechanism.

• KEYWORDS: angiogenesis; diabetic retinopathy; retinal pigmented epithelium; vascular endothelial growth factor; anthrax lethal toxin
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INTRODUCTION

Diabetic retinopathy (DR) is one of the major chronic complications of diabetes mellitus and is the leading cause of blindness among adults in the United States [1]. Although effective blood glucose control decreases the risk of DR, there is no threshold for blood glucose to prevent DR. The onset of DR is very early, even in the impaired glucose tolerance (IGT) population, a population whose blood glucose still lower than the standard to diagnose diabetes, the incidence rate of DR is about 10% [2]. Moreover, diabetic patients with specific genetic background have higher incidence of DR even with well controlled blood glucose. Vascular neogenesis of the retina is the major pathological change in DR. Vascular endothelial growth factor-α (here after called "VEGF") is the major growth factor promoting abnormal angiogenesis of the retina in diabetic background[3]. VEGF expression and secretion is up regulated by the mitogen activated protein kinase kinase (MAPKK, MKK or MEK)/mitogen activated protein kinasse (MAPK, including ERK1/2, p38 and JNK) pathway [4]. Activation of MAPKs depends on their phosphorylation by MEKs. MAPKs are over activated by high glucose, the condition characterized by diabetes. Thus, inhibiting the MEK/MAPK pathway may be an effective way to prevent the DR by suppressing the over secreted VEGF.

Anthrax lethal toxin (LeTx) is a binary toxin derived from the exotoxin of Bacillus anthracis [5]. LeTx is composed of lethal factor (LF) and protective antigen (PA) [5]. PA is non-toxic and serves to translocate LF to the cell cytoplasm where LF, a zinc metalloproteinase, can cleave the amino termini of MEK1-4, MEK6 and MEK7 [6]. LeTx has been shown to suppress VEGF secretion in the murine xenograft tumor model and perturb the vascular development in the murine retina [7-9]. Considering high glucose could induce MAPK activation and VEGF over secretion, we hypothesized that high glucose up-regulated expression and secretion of VEGF in retinal cells may be suppressed by LeTx-mediated inhibition of MEK/MAPKs signaling.

In the present study, we observed LeTx-mediated N-terminal cleavage of the MEKs and loss of MEK/MAPKs signaling in human adult retinal pigmented epithelial (ARPE) cells was accompanied by a substantial post-translational inhibition of VEGF releasing. Our results indicated that inhibition of MEK/MAPK signaling pathway by LeTx might be an effective strategy for treatment of DR.

MATERIALS AND METHODS

Cell Culture and Treatment The ARPE cell line was purchased from ATCC (ATCC control No.CRL-2302). Unless otherwise mentioned, ARPE cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 5.6 mmol/L glucose, 20% fetal bovine serum (FBS), 1% penicillin/streptomycin (100 units/mL of penicillin G sodium and 100 μg/mL streptomycin sulphate) at 37℃ in a humidified atmosphere of 95% air and 5% CO_2. The medium was changed every 48h. When confluency, the cells were passaged by 0.05% Trypsin (W/V, Gibco-Life Technologies, Rockville, MD, USA) in phosphate buffered saline (PBS), pH 7.4.

The anthrax LF and PA were obtained from Dr. Nicholas Duesbery of Van Andel Research Institute in Grand Rapids, MI. Before each *in vitro* assay, ARPE cells were starved in serum free medium. Then the cells were treated with 30 mmol/L glucose (high glucose group, indicated as H), 5.6 mmol/L glucose (normal glucose group, indicated as N) or high glucose containing 100 ng/mL LF and 1 μg/mL PA (LeTx group, indicated as L) in DMEM with 2% FBS for either 24, 48 or 72h.

Viable Cell Count Assay ARPE cells in an exponential phase of growth were seeded in 96-well plates at the density of 6000 cells per well. After starvation, the cells were treated with normal glucose, high glucose or high glucose with LeTx for additional 24, 48 or 72h. Then the conditioned medium was removed and collected for further analysis. The plates were washed by sterile PBS twice. To measure the viable cell number, 100 μL of pre-warmed phenol free medium with 20 μL of 0.5% 3- (4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was added into each well. Plates were incubated at 37℃ for additional 4h. Then the medium was removed and the plates were washed by sterile PBS twice. Cells containing the trapped MTT crystals were then solubilized in 150 μL dimethyl sulfoxide (DMSO) at room temperature for 10min with slightly agitation. Absorbance was determined at 490 nm.

Reverse Transcript–Polymerase Chain Reaction Total RNA from the ARPE cells growing in 6-well plates in each group for 48h was isolated by the RNApure rapid RNA isolation kit (Nanjing Bioteke Corporation, RP1201) according to the manufactures' instructions. The first strand cDNA was synthesized by EasyScript reverse transcriptase (Beijing Transgen Biotech, AE101-01) at 42℃ for 60min. The fragments of VEGF and GAPDH were amplified with 40 cycles for 15s at 95℃, 30s at 55℃, 40s at 72℃ by TransTaq DNA polymerase (Beijing Transgen Biotech, AP121). The primers for the amplification of VEGF were: forward primer: 5'-CTTTCTGCTGTCTTGGGTGCATTG-3'; reverse primer: 5'-TAATCTGCATGGTGATGTTGG-3'. The primers for amplifying the GAPDH were: forward primer: 5'-CAAGGTC ATCCATGACAACTTTG-3'; reverse primer: 5'-GTCCACC ACCCTGTTGCTGTAG-3'. The 314 bp VEGF polymerase chain reaction (PCR) product and the 496 bp GAPDH PCR products were electrophoresed on the 1% agarose gel and visualized under UV light. Semi-quantitive analysis of VEGF and GAPDH mRNA expression was evaluated using Bio-rad Quantity One software, version 4.6.8.

Enzyme–linked Immunosorbant Assays VEGF enzyme-linked immunosorbant assays (ELISA) kit was ordered from Shanghai Genetimes Technology (No.EH015). The conditioned medium of ARPE cells treated in different group for 48h was filtered and diluted in sample dilution buffer in the kit. Supplied VEGF in the kit was serially diluted to set up a standard curve. The ELISA was conducted according to the kit's manufactures' instruction. The absorbance at 450 nm was read to reflect the concentration of VEGF at the end of the performance. The VEGF concentration was calculated by the standard curve. Samples were from four independent experiments and each was tested triplicate.

Immunoblotting Cells were cultured in 10-cm plates to about 80% confluent and treated with normal glucose, high glucose or high glucose with LeTx in DMEM containing 2% FBS for 48h after starvation. Following three rinses with PBS, cells in each plate were lysed by 300 μL 1×SDS buffer (62.5 mmol/L Tris-HCl, pH 6.8 containing 2% w/v SDS, 10% glycerol, 50 mmol/L DTT, 0.01% w/v bromophenol blue) on ice for 30min. Equal amount of cell lysate of each group was separated by sodium dodecyl sulphate poly-acrylamide gel electrophoresis (SDS-PAGE) and transferred to the nitrocellulose membrane. After blocking with 5% non-fat milk in 0.1% TWEEN-PBS at 37℃ for 60min, membranes were incubated with primary antibodies (1:100-1:1000 diluted) at 4℃ overnight with slightly shaking. The individual blots were probed with antibodies against human ERK (cell signaling, No.9107), phosphorylated ERK (cell signaling, No.4370), p38 (cell signaling, No.9212), phosphorylated p38 (cell signaling, No.9216), N-termini of MEK-1 (upstate, 07-641), VEGF (gifted by Dr. Brian Cao of the Van Andel Institute in Grand Rapids, MI) or β-actin (Boster biotech, BA-1404). Then the primary antibodies were removed and membranes were washed three times by 0.1% TWEEN-PBS. Membranes were incubated with 1:3000 diluted horseradish peroxidase-conjugated anti mouse (1:3000, Boster biotech, BA-1050) or anti rabbit (1:3000, Boster biotech, BA-1054) secondary antibodies at room temperature

for 90min. The specific proteins were visualized using X-ray films after incubation with the SuperSignal west pico chemiluminescent substrate (pierce, No.34077).

Statistical Analysis All the above experiments were performed at least three times. The quantitative data was presented as mean±standard deviation (SD) and was analyzed by Graphpad Prism for one-way ANOVA. The statistical significance was defined as the P value less than 0.05.

RESULTS

High Glucose Activated ERK1/2 and p38 is Blocked by Anthrax Lethal Toxin RPE cells secrete more VEGF in the presence of high glucose and under hypoxic conditions [10]. Therefore we chose the ARPE cell line as the model to evaluate the role of MEK/MAPK pathway in the secretion of VEGF upon high glucose challenge. However, to date, no report has demonstrated that LeTx can bind and enter ARPE cells. Thus, to determine whether LF enters RPE cells and inactivates MEKs, we immunoblotted lysates with antibodies against the amino terminus of MEK1 and phosphorylated (active) substrates ERK1/2 and p38 MAPK. We observed decreased levels of MEK1 in cells treated with LeTx. Moreover, we observed diminished levels of phosphorylated ERK1/2 and p38 MAPK only in cells treated with LeTx (Figure 1). These results indicated that LF was able to enter ARPE cells and on entry it effectively cleaved MEK1 and blocked downstream substrate activation.

High Glucose Induced ARPE Cells Over Proliferation is Inhibited by Anthrax Lethal Toxin Whereas LeTx is acutely toxic for some macrophage-derived and tumor cells lines, it is non-toxic for other cells [9]. To test whether ARPE cells are sensitive to LeTx we evaluated cell viability in medium containing normal and high levels of glucose as well as in medium containing high glucose plus LeTx (1 μg/mL PA with 100 ng/mL LF). As shown in Figure 2, LeTx did not significantly alter cell viability over the course of 48h incubation though we observed a modest decrease of cell number (approx. 15%) after 72h incubation. These results indicated that ARPE cells were not acutely sensitive to LeTx but instead they appeared to undergo modest changes following extended toxin treatment. Since the 48h incubation with LeTx in high glucose condition rescued the ARPE cells number near to that in the normal glucose condition, other experiments were performed after 48h treatment (Figure 2).

Anthrax Lethal Toxin Prevents VEGF Release From ARPE Cells by a Post -transcriptional Mechanism MAPKs regulate VEGF expression at transcriptional level through SP-1 and AP-2 binding site in the upstream of the VEGF transcription initiation site [11]. We further asked whether LeTx could decrease VEGF expression or secretion in ARPE cells. We designed PCR primers to specifically amplify a fragment of VEGF and observed that in the absence of elevated glucose levels very little VEGF was

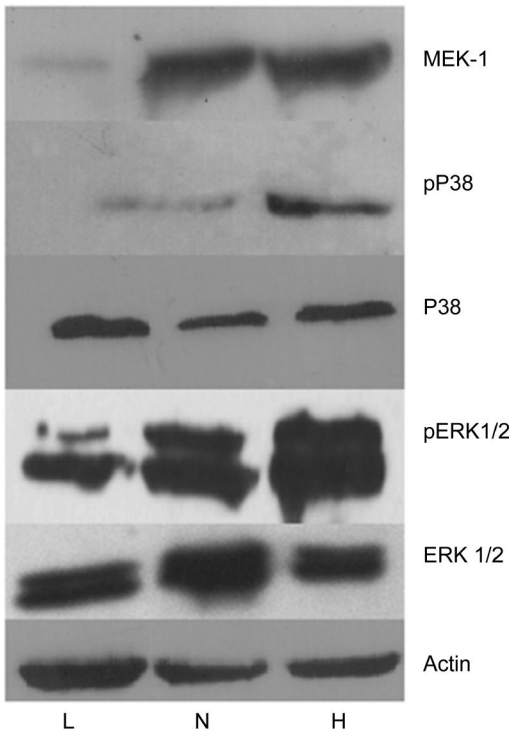

Figure 1 Over activated MKK/MAPK pathway in the ARPE cultured in high glucose was blocked by LeTx Immunoblotting of lysate of ARPE cells from different groups shows high glucose induced MAPK ERK1/2 and p38 over activation, this effect was blocked by LeTx by cleaving the MKK. N: 5.6 mmol/L glucose (normal glucose, N); H: 30 mmol/L glucose (high glucose, H); L: 100 ng/mL LF with 1 μg/mL PA in 30 mmol/L glucose.

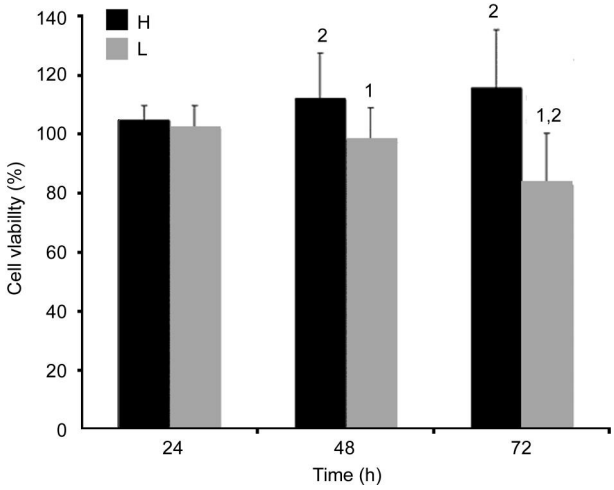

Figure 2 LeTx prevented high glucose induced ARPE cell over proliferation Results are expressed as a percentage of the proliferation of control cells. The high glucose promoted the ARPE cells proliferation after 48 and 72h' incubation, whereas the 100 ng/mL LF with 1 μg/mL PA (L) slightly inhibited the over proliferation of ARPE cells. 1: *vs* high glucose, P <0.05; 2: *vs* nomral glucose, P <0.05.

expressed. In contrast, high glucose induced elevated VEGF expression. The addition of LeTx caused only a modest and statistically insignificant decrease in VEGF expression (Figure 3). However, LeTx suppressed level of soluble VEGF in conditioned medium from ARPE cells under high glucose

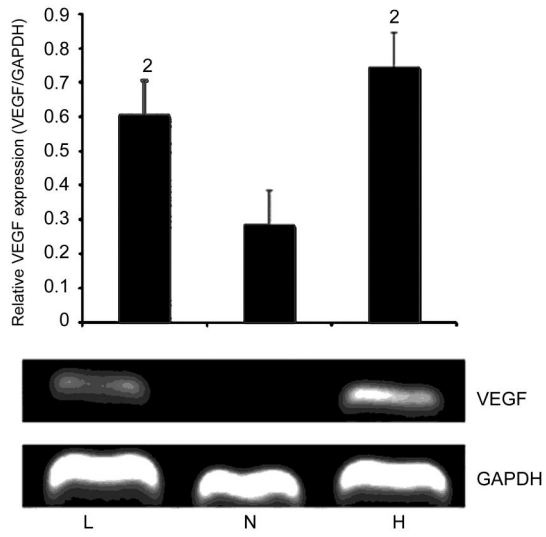

Figure 3 LeTx slightly decreased high glucose enhanced VEGF expression in ARPE cells without statistical significance VEGF mRNA over expression under high glucose was slightly inhibited by 100 ng/mL LF with 1 μg/mL PA without significance; 2: *vs* normal glucose, $P<0.05$.

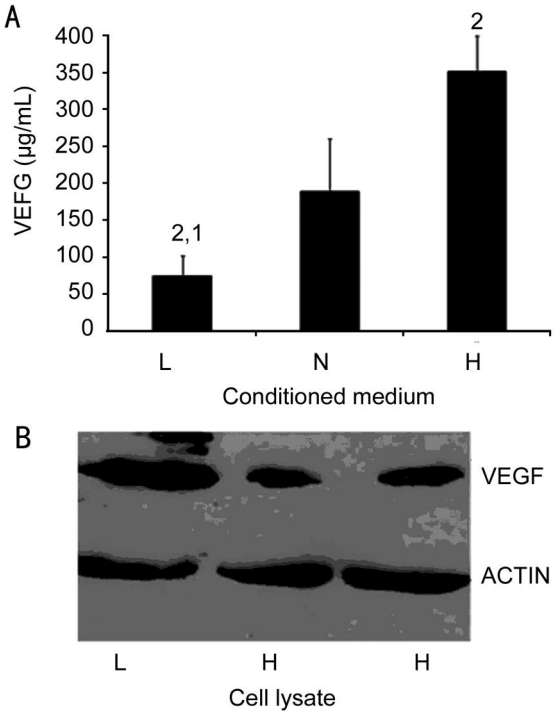

Figure 4 LeTx decreased VEGF concentration in the conditioned medium of ARPE cells upon high glucose stimulation through a post-translational mechanism A: LeTx decreased VEGF concentration in the conditioned medium of ARPE cells under high glucose. 1: *vs* high glucose, $P<0.05$; 2: *vs* normal glucose, $P<0.05$. B: LeTx sequestered over expressed VEGF inside ARPE cells under high glucose condition. N: 5.6 mmol/L glucose (normal glucose, N); H: 30 mmol/L glucose (high glucose, H); L: 100 ng/mL LF with 1 μg/mL PA in 30 mmol/L glucose.

(>3-fold, $P<0.01$, Figure 4), even lower than normal glucose incubation. Combined these results, we hypothesize LeTx prevented the VEGF secretion by a post-translational way where LeTx sequestered the VEGF inside the cells. Immunoblotting of the intracellular VEGF disclosed LeTx interferred VEGF releasing by sequestering it inside the ARPE cells (Figure 4). We thus concluded LeTx inhibited high glucose induced VEGF over secretion in ARPE cells by blocking its releasing.

DISCUSSION

In this study, we observed inhibition of high glucose induced VEGF over secretion by anthrax LeTx. VEGF is over secreted in diabetic background and mediates diabetic chronic complications including diabetic nephropathy, diabetic neuropathy and DR[12-14]. In early DR, VEGF induces the vascular hyperpermeability, leading to the macular edema and protein exudate from retinal vessels [15] while in the proliferative DR stage, VEGF enhances collateral vessel formation by promoting vascular endothelial over proliferation[10]. VEGF could also induce EPC transforming to endothelial cells [16]. To date, the published therapy targeting VEGF to treat DR in clinic is its neutralizing antibody bevacizumab which blocks the down stream signal transduction and biological effects of VEGF [17,18]. RPE over proliferation is a major morphological change in DR, either inducing the death of the RPE or blocking the proliferation is a strategy to prevent such pathology. Therefore, we use MTT to measure the total number of the RPE cells to show the potential of LeTx could inhibit the proliferation of RPE induced by high glucose .Secretion of VEGF is regulated by the MEK/MAPK pathway, therefore, in this study, we targeted MEK/MAPK pathway to suppress high glucose induced VEGF over secretion.

Our findings suggested that high glucose did not increase the amount of total ERK1/2 and p38 but promote their phosporylations. Actually, MEK/MAPK pathway is over activated throughout the natural course of diabetes. In the pre-diabetic stage, over activation of MEK/MAPK pathway leads to either the impaired insulin secretion [19] or the decreased insulin sensitivity, the most important factors promoting the onset of diabetes [20,21]. As a vicious return, hyperinsulinemia, the result of insulin resistance, also leads to the highly activated MEK/MAPK pathway, a phenomenon called "selective insulin resistance" [22]. After the onset of diabetes, multiple disorders subjected to long term hyperglycemia exposure lead to MAPK activation including the oxidative stress [23], imbalanced growth factor secretion[23], the inflammation status [24], the advanced glycated end products [25], local hypoxia and the cross activation of cell membrane molecules including epidermal growth factor receptor (EGFR) [26]. The over activation of MEK/MAPK pathway contributes to further diabetic complications through of angiogenesis, endothelial dysfunction [27], renal fibrosis[28], and epithelium to mesenchyme transition (EMT) [29].

MEK/MAPK pathway also up-regulates other angiogenic factors secretion such as basic fibroblast growth factor (bFGF), interlukin-8(IL-8), TNF-alpha[11].

It is therefore reasonable to reduce VEGF production by blocking the MEK/MAPK pathway. Indeed, sorafenib, an inhibitor of B-raf and C-raf (kinases that activate MEK1/2), improves age related macular degeneration-a disease similar to DR [30]. Treating diabetic rats with MEK1/2 inhibitor U0126[31] or p38 inhibitor PHA666859[32] has been shown to prevent the DR. Anthrax LeTx is another MEK/MAPK pathway inhibitor which broadly cleaves the MEKs and blocks the activation of ERK1/2, p38 and JNK. In tumor cells, anthrax LeTx was reported to suppress VEGF secretion by inactivation of MAPKs. More recently, Bromberg-White *et al* [9] found LeTx perturbed retinal vascular development. However, it is not clear whether anthrax LeTx could inhibit high glucose induced VEGF expression and secretion in human ARPE cell line, thus in this study, we investigated whether anthrax LeTx could inhibit high glucose induced VEGF secretion by blocking MEK/MAPK pathway.

MEK/MAPK pathway, especially the ERK1/2 pathway, is important in some cell proliferation. In the retina, murine retinal capillary endothelial cells are slightly sensitive to anthrax LeTx, no study has been reported whether the human ARPE cells are sensitive to LeTx. In our system, we investigated high glucose increased the viable ARPE cell number and ARPE cells under high glucose condition are sensitive to anthrax. LeTx could normalize the viable cell number to the number of cell cultured under 5.6 mmol/L glucose after 48h incubation but moderately decrease the viable cell number to less than that of the normal glucose after 72h incubation. So 48h incubation with LeTx was chosen in other experiments of this study.

LeTx cleaves the N-terminus of MEK1-4 and MEK6-7 to block the activation of MAPKs in tumor cells [7,8]. MAPK activity is essential for the survival of sarcoma. We therefore tested whether prevention of high glucose induced ARPE cell over proliferation by anthrax LeTx was based on the blocking of MEK/MAPK pathway. Indeed, high glucose over activated ERK1/2 and p38 after 48h incubation in ARPE cells while anthrax LeTx blocked this effect by degrading the MEKs.

VEGF mRNA expression is under the control of transcription factor AP-2 and SP1, both of which are the downstream molecules of ERK1/2 and p38 [31,32]. This could explain high glucose increased the VEGF mRNA production as it activated MAPKs in our study. Alternative splicing of VEGF mRNA results the 5 forms of VEGF-α, VEGF121 and VEGF165 are secreted into the conditioned medium while VEGF189 partly binds to the cell membranes to be the cell associated form [33]. The secreted VEGF in free form has stronger potential to manipulate its angiogenic and the mitogenic effect while the cell associated form of VEGF is usually immature[33-36]. Therefore, releasing from the cell is an important regulation for VEGF maturation and function. Urokinase, matrix metalproteinase (MMP) and heparanase[33-36] have been observed to cleave the cell associated VEGF for its secretion. Expression of these molecules is also regulated by MEK/MAPK pathway, indicating an indirect way to promote VEGF secretion. Our findings suggest, high glucose induced VEGF over secretion by promoting its mRNA transcription. Anthrax LeTx decreased VEGF concentration in the ARPE conditioned medium under high glucose to even lower than that under normal glucose, but mainly through a post translational mechanism of sequestering VEGF inside the ARPE cells. The VEGF leading to DR is the secreted form, so it is very important to decrease VEGF secretion. Although LeTx targets MEK1-4, MEK6-7, MEK5 is not the target of LeTx, indicating MEK5 promotes VEGF expressing under high glucose condition, but not regulates its translation and secretion.

In conclusion, our findings suggest inhibition of MEK/MAPK pathway by LeTx is effective to block high glucose induced VEGF secretion in ARPE cells, mainly at the post-translational level; this effect is independent of its anti-proliferation effect during high glucose background. Based on these findings, further experiments will be designed to test whether the anthrax LeTx could prevent the DR *in vivo*.

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