

Adrenomedullin₂₂₋₅₂ suppresses high-glucose-induced migration, proliferation, and tube formation of human retinal endothelial cells

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Purpose: To investigate the roles of an adrenomedullin receptor antagonist (adrenomedullin₂₂₋₅₂) on high-glucose-induced human retinal endothelial cell (HREC) in vitro cell biology.

Methods: HRECs were cultured with different concentrations of glucose and adrenomedullin₂₂₋₅₂. The proliferation of HRECs was evaluated by a cell counting kit-8 assay. Cell migration was assessed by scratch wound assay, and cell sprouting was detected by tube formation assay. The mRNA levels of adrenomedullin (ADM), vascular endothelial growth factor (VEGF), ADAMTS-1, and TSP-1 were measured by reverse-transcription polymerase chain reaction (RT-PCR). The VEGF and phosphatidylinositol 3' kinase (PI3K) pathway protein expression levels were assessed by western blot analysis.

Results: Compared with 5 mM normal glucose treatment, 30 mM glucose significantly promoted the migration of HRECs, which was attenuated by 1 µg/ml adrenomedullin₂₂₋₅₂. The proliferation of HRECs was also suppressed by 1 µg/ml adrenomedullin₂₂₋₅₂. Furthermore, compared with other groups, 5 µg/ml of adrenomedullin₂₂₋₅₂ was shown to suppress high-glucose-induced tube formation of HRECs. With adrenomedullin₂₂₋₅₂ treatment, the mRNA level of ADAMTS-1 was significantly increased. Moreover, western blot and RT-PCR analyses showed that HRECs treated with 30 mM glucose exhibited increased VEGF and PI3K pathway protein levels, while the expression levels were suppressed by 5 µg/ml of adrenomedullin₂₂₋₅₂.

Conclusions: Our study indicated that adrenomedullin₂₂₋₅₂ mediated the migration, proliferation and tube formation after HRECs were exposed to high levels of glucose, which may be related to its ability to affect the expression of VEGF through the PI3K pathway.

Diabetic retinopathy (DR) is one of the most common and severe micro-vascular complications of diabetes and can eventually lead to blindness. Early clinical signs of DR include micro aneurysms and hemorrhages, while the later signs are dilated, tortuous, irregular and narrow retinal blood vessels called retinal ischemia that ultimately result in neovascularization. At this stage, abnormal new blood vessels form at the back of the eye as a part of proliferative diabetic retinopathy (PDR) [1]. The incidence of DR is increasing every year, and it is one of the main causes of blindness. Therefore, it is necessary to develop effective measures for treating and preventing retinal neovascularization.

Adrenomedullin (ADM), a 52-amino-acid polypeptide, is a kind of expansion of blood vessels and diuretic active peptide originally isolated from human pheochromocytoma [2]. ADM belongs to a large family of peptides that includes calcitonin gene-related peptide (CGRP), calcitonin (CT), and

amylin [3]. It acts through the G protein-coupled receptor calcitonin receptor-like receptor (CRLR), with specificity for ADM being conferred by the receptor activity modifying proteins 2 (RAMP2) and 3 (RAMP3) [4].

ADM and its receptors are expressed in several tissues and organs, including the heart, lungs, kidney, blood plasma, spleen, endocrine glands, brain, and thymus [5]. Published studies have shown that vascular endothelial cells and vascular smooth muscle cells actively synthesize and secrete ADM [6,7]. Moreover, a recent study observed that each cell type in the retina could secrete ADM [8]. ADM has widely biologic functions in the vasculature, such as vasodilatation, regulation of vascular permeability, vascular protection, and promotion of angiogenesis [9]. ADM also may be involved in several ocular pathologies. The expression of ADM was increased in the vitreous humor of patients with diabetes [10,11], in patients with primary open-angle glaucoma [12], and in the aqueous humor of patients with uveitis and vitreoretinal disorders [13]; patients with retinitis pigmentosa have increased plasma ADM [14]. Adrenomedullin₂₂₋₅₂ is a truncated peptide derived from ADM and a selective ADM receptor antagonist. Several independent studies have

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suggested that ADM could significantly promote the proliferation, migration and tube formation of human umbilical vein endothelial cells [15-17], while those functions could be suppressed by adrenomedullin₂₂₋₅₂ [16,17]. A recent study reported that ADM increased the area of CNV in a laser-induced CNV model, which can be significantly blocked by both ADM antagonist and ADM antibody [18].

During the formation of new blood vessels, endothelial cells are stimulated to migrate, proliferate, and invade the surrounding tissues to form capillaries [15]. This process is closely associated with the activation of several signal pathways. Evidence suggests that ADM-induced angiogenesis is mediated by phosphatidylinositol 3' kinase (PI3K/Akt), mitogen-activated protein kinase (MAPK), focal adhesion kinase (p125FAK), and extracellular signal-regulated kinase 1/2 (ERK1/2), while these effects are counteracted by the ADM receptor antagonist (adrenomedullin₂₂₋₅₂) [19-21]. Additionally, there are several factors involved in the process of new blood vessel formation in the eyes. Vascular endothelial growth factor (VEGF) is considered to be a main stimulating factor among the various factors of pro-angiogenesis. VEGF is closely related to the ocular angiogenesis platelet protein that is an important regulatory factor in the process of blood vessel formation; it can promote vascular endothelial cell migration and proliferation, so it stimulates the generation of new blood vessels [22]. Several studies have found that the angiogenesis role of ADM is closely related with VEGF. Maki and colleagues [23] reported that ADM promoted the generation of new blood vessels via VEGF in a mice brain hypoperfusion model. In vitro, ADM can encourage the proliferation and tube formation of human umbilical vein endothelial cells and human saphenous vein endothelial cells by promoting the expression of VEGF [23,24].

Based on these studies, we speculated that ADM may be involved in the development of DR. However, the role of adrenomedullin₂₂₋₅₂ on high-glucose-induced HREC in vitro cell biology has remained obscure. Therefore, we investigated the role of adrenomedullin₂₂₋₅₂ on high-glucose-induced HREC proliferation, migration, and tube formation.

METHODS

Reagents and antibodies: Human retinal endothelial cell lines (HRECs) were purchased from Ya Ji biologic technologies (Shanghai, China). Dulbecco's modified Eagle medium (DMEM) was obtained from Thermo scientific (Hyclone, UT). Fetal Bovine Serum came from PAA laboratories (Cölbe, Germany). Trypsin-EDTA, D-glucose, and adrenomedullin₂₂₋₅₂ were originated from Sigma-Aldrich (St Louis, MO). The cell counting kit-8 was purchased from Dojindo

(Kumamoto, Japan). Matrigel was bought from Becton Dickinson Labware (Bedford, MA). The primers were synthesized by the Shanghai biologic engineering technology service (Shanghai, China). Traces of RNA extraction kit (RNeasy Mini Kit) and reverse transcription Kit (Ominiscript RT Kit) came from Germany Qiagen Company (Qiagen, Hilden, Germany). Rabbit anti VEGF antibody was shipped from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-PI3K P110 alpha (Phosphatidylinositol 3 kinase catalyticalpha) was purchased from biolgend (San Diego, CA), and GAPDH antibody was obtained from the Beyotime Institute of Biotechnology (Shanghai, China).

Cell culture: HRECs were cultured in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C in DMEM medium supplemented with 10% fetal bovine serum. Cells were passaged 1:2 at confluence by release with 0.25% Trypsin-EDTA and subcultured in culture flasks. The HRECs were split at 90% confluence, and the culture media were changed every 2-3 days.

Treatment of HRECs: HRECs were divided into several groups: 5 mM normal glucose, 30 mM glucose, and 30 mM glucose plus different concentrations of adrenomedullin₂₂₋₅₂. The HRECs were split at 90% confluence and subcultured in 96-well plates or 6-well plates according to the appropriate assay conditions.

Cell migration assay: The effect of adrenomedullin₂₂₋₅₂ on high-glucose-induced migration of HRECs was evaluated by scratch wound assay. In brief, the HRECs were starved with serum-free medium for 12 h when the HRECs had grown to 90% confluence in the 6-well plates. When the HRECs had grown to over-confluence, a wound of appropriate width was made with a 200 µl tip. Then, the HRECs were washed with sterile 1×PBS three times to remove the floating cells, incubated with serum-free media containing different concentrations of glucose with or without adrenomedullin₂₂₋₅₂, and then cultured in the 6-well plate in at 37 °C in a 5% CO₂ incubator. The migration monolayer was photographed at 0 h, 12 h, 24 h, and 48 h. Five fields were photographed for each well. The migration distance was measured by using Photoshop CS2. The experiment was performed at least three times with similar results.

Cell proliferation assay: CCK-8 assay was used to evaluate the high-glucose-induced proliferation of HRECs with different concentrations of adrenomedullin₂₂₋₅₂. In brief, 3 × 10³ of HRECs were seeded into each well of a 96-well plate and allowed to adhere for 24 h. After cells were adherent to the bottom of the plate, the cells were cultured in serum-free media for starvation for 24 h. Then, the cells were treated with different concentrations of glucose and adrenomedullin₂₂₋₅₂

TABLE 1. SPECIFIC SET OF PRIMERS AND CONDITIONS OF PCR

Gene	Primmer sequences (5'-3')	Annealing temp(°C)	PCR cycles	PCR product (bp)
<i>VEGF</i>	F: TGGTCCCAGGCTGCACCCAT R: CGCATCGCATCAGGGGACACA	58	35	184
<i>ADAMTS-1</i>	F: CTCCGCCTGCACGCCTTTGA R: ATCGCCATTACGGTGCCGG	58	35	171
<i>ADM</i>	F: ATGAAGCTGGTTCCGTCG R: ATCCTAAAGAA AGTGGGGAGCACTTC	58	35	350
<i>TSP-1</i>	F: GGTCGGCCTGCACTGTCACC R: GGGGAAGCTGCTGCACTGGG	58	35	179

In the table, “F” indicates forward primer and “R” indicates reverse primer.

(10 ng/ml-1000 ng/ml) for 24 h, and the proliferative activity was determined by CCK-8 assay according to the manufacturer's instructions. In brief, 10 µl CCK-8 were added to each well followed by incubation for an additional 2–4 h. When the media changed from red to yellow, the absorbance value at a wavelength of 450 nm was detected. The experiment was performed at least three times with similar results.

Tube formation assay: The morphogenesis assay on Matrigel was conducted to evaluate the effect of adrenomedullin₂₂₋₅₂ on high-glucose induced HRECs tube formation. According to the manufacturer's instructions, the Matrigel was placed in a refrigerator at 4 °C overnight to thaw. Once it had thawed, 60 µl Matrigel was added to a pre-cooled 96-well plate and the plate was immediately placed in a humidified CO₂ incubator at 37 °C for 30 min to solidify the Matrigel. HRECs that had been cultured under different media with or without adrenomedullin₂₂₋₅₂ were seeded immediately on the solidified Matrigel at a density of 1.5×10⁴ cells per well. Then, the plates were placed in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C for 8 h to allow the formation of a capillary-like structure. The pictures were photographed, and the number of capillaries formed was qualitatively assessed using Image-Pro Plus 6.0 software. The experiment was repeated at least three times under the same conditions.

RNA isolation and cDNA synthesis: When the HRECs had grown to 90% confluence in the culture flasks, the cells were harvested, seeded onto 6-well culture plates, and cultured in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C in DMEM medium supplemented with 10% fetal bovine serum. When the HRECs had grown to over-confluence of the bottom, the cells were treated with different concentrations of adrenomedullin₂₂₋₅₂ for 6–8 h. Cells were harvested, and total RNA was isolated using the RNeasy Mini Kit according to the manufacturer's instructions. Reverse transcription of

RNA was performed in a 20 µl final volume using a Reverse Transcription Kit (Ominiscript RT Kit) and a cDNA Synthesis Kit according to the manufacturer's instructions. Finally, the cDNA was stored at –20 °C before its use in RT-PCR.

RT-PCR: RT-PCR was performed to detect the expression of mRNA for ADM, VEGF, ADAMTS-1, and TSP-1. These primers used are summarized in Table 1. A 2 µl aliquot of cDNA was amplified by PCR in a total volume of 25 µl containing 17.575 µl ddH₂O, 2.5 µl 10 ×PCR buffer, 2 µl dNTP, 0.125 µl rTaq, and 2 µl of each primer. The PCR was performed under the following conditions: an initial denaturation step at 94 °C for 2 min followed by 35 or 27 cycles of standard PCR (30 s denaturation at 94 °C, 30 s annealing at 58 °C, 30 s extension at 72 °C), and re-extension at 72 °C for 10 min. Levels of VEGF, ADAMTS-1, TSP-1, and ADM mRNA were normalized to those of GAPDH mRNA.

Western blot analysis: HRECs were split at 90% confluence and cultured in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C with different concentrations of adrenomedullin₂₂₋₅₂ for 24 h. Cells were harvested and lysed in 100 µl of lysis buffer with protease inhibitor cocktail for total protein extraction. The lysates were then centrifuged from the samples at 13,800 ×g at 4 °C for 10 min. The supernatant was removed, placed in a fresh tube, mixed with 6×SDS sample buffer and heated for 10 min at 100 °C. The protein samples were separated with 10% SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was blocked for 1 h in PBS containing 0.05% Tween-20 (PBST) and 5% nonfat dry milk powder at room temperature. The membrane was then incubated with the primary antibody at 4 °C overnight, washed three times with PBST containing 0.05% Tween-20 for 10 min, and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The blot was visualized using chemiluminescence

detection reagents (Cell Signing Technology, Danvers, MA), and the pictures were photographed using the ChemiDoc™ MP Imaging System (BIO-RAD, Hercules, CA). The data were analyzed by Image J software. GAPDH was used as a loading control. The experiment was performed three times with similar results.

Statistical analysis: All data are expressed as mean ±SD. Data were analyzed statistically by one way ANOVA (ANOVA) or two-tailed Student *t* test with the Statistical Package for the Social Sciences (SPSS) 13.0 software. A *p* value <0.05 was accepted as statistically significant.

RESULTS

Effect of high glucose on ADM mRNA expression in HRECs: Published results have shown that ADM is increased in the plasma of DR patients [25]. RT-PCR was performed to observe whether the high glucose level would affect the HRECs' ADM secretion. As shown in Figure 1, when compared with 5 mM glucose conditions, ADM expression was upregulated in HRECs under 30 mM glucose conditions. Therefore, the high glucose condition appeared to upregulate the expression of ADM in HRECs.

Adrenomedullin₂₂₋₅₂ inhibited HREC proliferation induced by high glucose: To assess the effect of adrenomedullin₂₂₋₅₂ on the proliferation of HRECs, in vitro CCK-8 assay was performed to measure the proliferation ability of HRECs at different concentrations of glucose and adrenomedullin₂₂₋₅₂

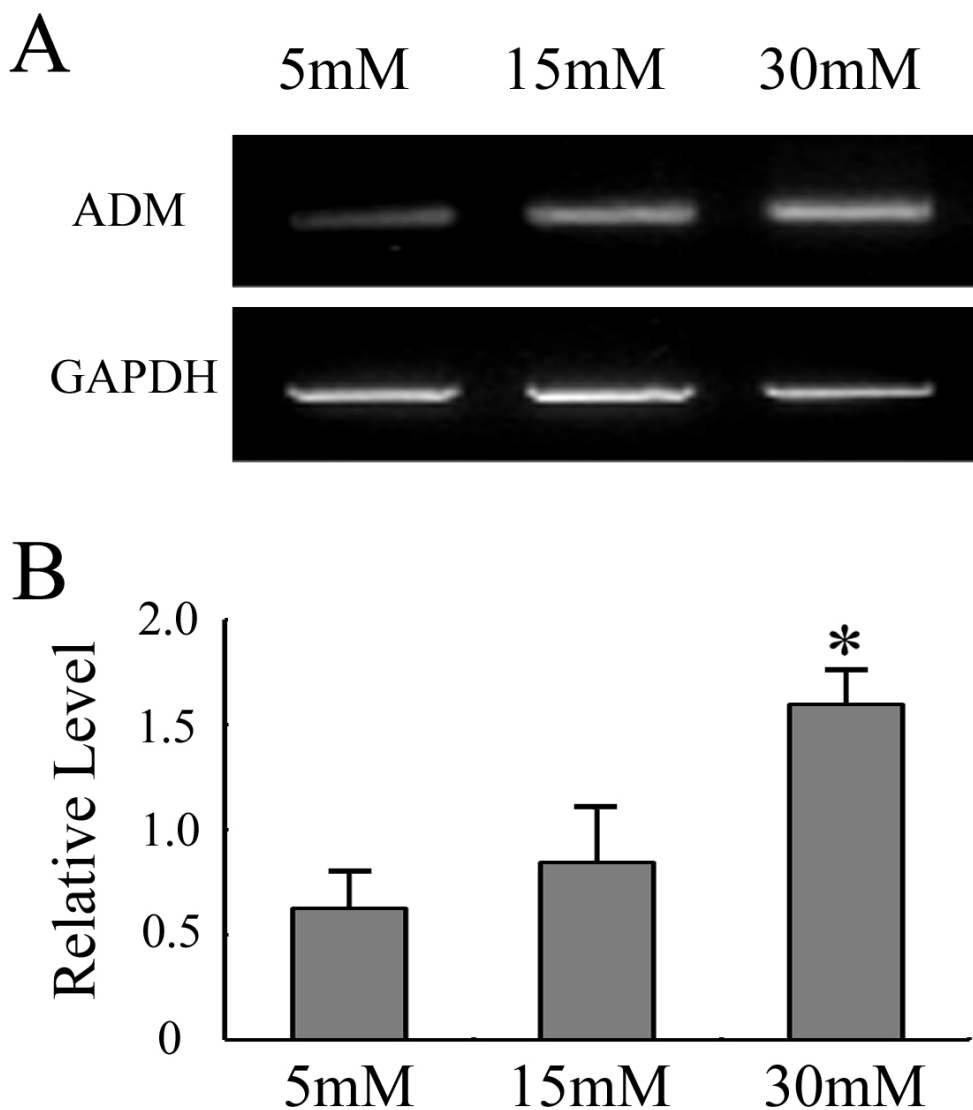


Figure 1. High glucose promoted the expression of ADM. After HRECs were treated with different concentrations of glucose (5 mM, 15 mM, and 30 mM) for 48 h, RT-PCR was performed to assess the mRNA expression of ADM. A: Representative results from three independent trials of RT-PCR. B: Ratios of ADM to GAPDH were determined by RT-PCR. The quantitative data of the ratio were from three independent experiments. All values represent Mean±SD (n=3). * *p*<0.05 versus the 5 mM group.

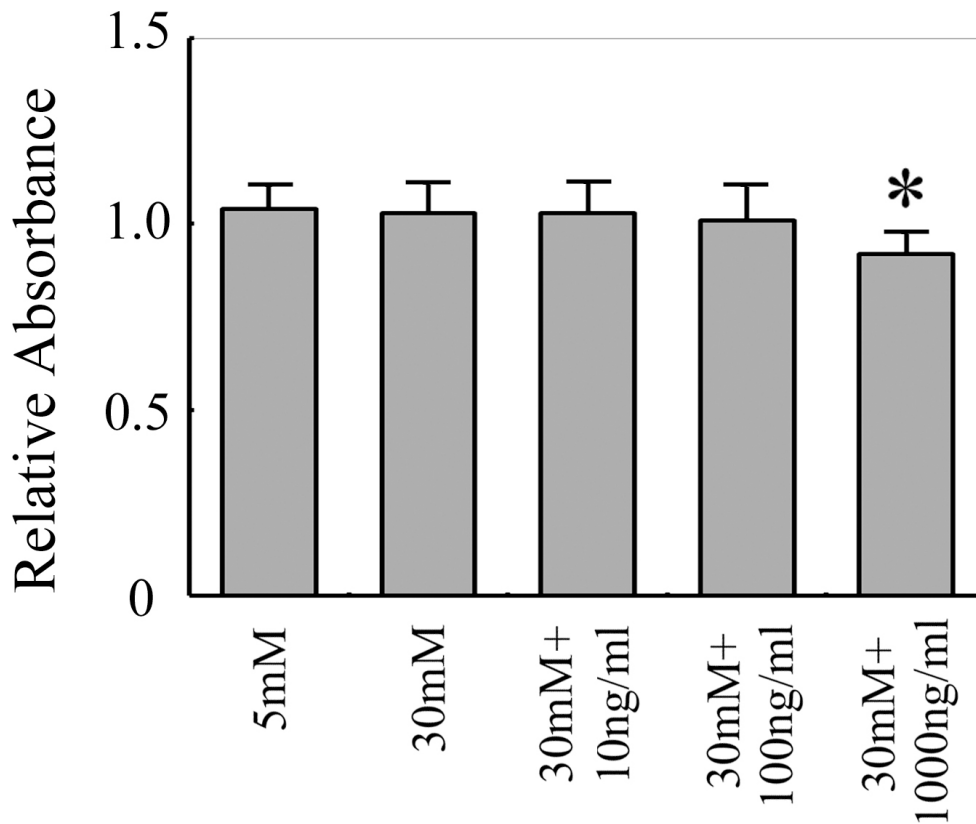


Figure 2. Adrenomedullin₂₂₋₅₂ inhibited HREC proliferation induced by high glucose. After the HRECs were treated with high glucose or high glucose plus different concentrations of adrenomedullin₂₂₋₅₂ for 24 h, cell proliferation was measured by CCK-8 assay. The absorbance value was detected using an enzyme-linked immunity instrument at a wavelength of 450 nm. All values represent Mean±SD (n=3). * p<0.05 versus the 30 mM group.

(10 ng/ml-1000 ng/ml). The results shown in Figure 2 indicate that compared with the 5 mM glucose treatment, the proliferation ability of HRECs had no significant effect when treated with 30 mM glucose. The proliferation ability of HRECs was inhibited by the treatment with 30 mM glucose and 1000 ng/ml of adrenomedullin₂₂₋₅₂ compared to 30 mM glucose.

Adrenomedullin₂₂₋₅₂ inhibited HREC migration induced by high glucose: To evaluate whether adrenomedullin₂₂₋₅₂ had an effect on the migration of HRECs, we performed an in vitro scratch wound test to measure the migration ability of HRECs at different concentrations of glucose and adrenomedullin₂₂₋₅₂. As shown in Figure 3, a significantly accelerated wound closure was observed in the group treated with 30 mM glucose compared with the 5 mM glucose treatment group. However, the wound area was still wide in the HRECs with high glucose co-treatment with 1 µg/ml adrenomedullin₂₂₋₅₂ at 24 h. These results indicated that adrenomedullin₂₂₋₅₂ could effectively inhibit the migration ability of HRECs cultured in 30 mM glucose.

Effect of high glucose and adrenomedullin₂₂₋₅₂ on capillary tube formation of HRECs: To explore the effect of high glucose and adrenomedullin₂₂₋₅₂ on angiogenesis, we measured HREC tube formation by Matrigel assay. As

shown in Figure 4, the number of capillary-like structures was significantly enhanced under the high glucose condition compared with the 5 mM glucose group. However, the treatment with 5 µg/ml of adrenomedullin₂₂₋₅₂ caused significantly fewer capillary-like structures than those produced by high glucose. These results provide further proof that adrenomedullin₂₂₋₅₂ can inhibit the ability of high glucose-induced capillary-like formation.

Effect of high glucose and adrenomedullin₂₂₋₅₂ on VEGF, ADAMTS-1, and TSP-1 mRNA expression in HRECs: The effect of adrenomedullin₂₂₋₅₂ on the mRNA expression of VEGF, ADAMTS-1, and TSP-1 in high-glucose-induced HRECs was determined next. As shown in Figure 5, the expression of VEGF was significantly increased in 30 mM glucose conditions compared with 5 mM glucose, while the level was significantly suppressed by 5 µg/ml adrenomedullin₂₂₋₅₂. Following treatment with 5 µg/ml adrenomedullin₂₂₋₅₂, the mRNA level of ADAMTS-1 was significantly increased.

Effect of high glucose and adrenomedullin₂₂₋₅₂ on VEGF and PI3K pathway protein expression in HRECs: Consistent with our RT-PCR data, the western blot shown in Figure 6 indicated that compared with 5 mM glucose, the 30 mM

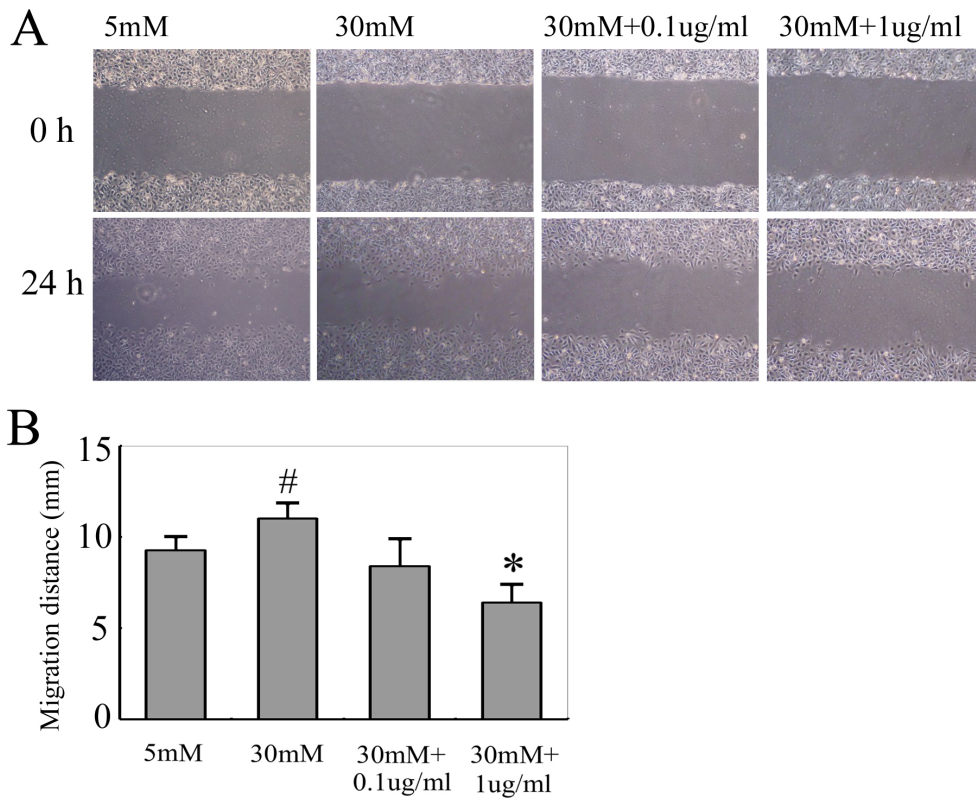


Figure 3. Adrenomedullin₂₂₋₅₂ inhibited HREC migration induced by high glucose. HRECs were analyzed for the ability to migrate into a wound area using a scratch wound assay. **A:** After HRECs were wounded using a 200 μ l yellow micropipette tip, they were incubated with serum-free media containing high glucose with or without adrenomedullin₂₂₋₅₂ for 24 h. The re-endothelialized monolayer area was photographed. A magnification of $\times 200$ was used to image the re-endothelialized monolayer area. **B:** The quantitative data of the migration distance were from three independent experiments. All values represent Mean \pm SD (n=3). * p<0.05 versus the 30 mM group. # p<0.05 versus the 5 mM group.

glucose treatment markedly increased the VEGF protein expression, while this increase was significantly blocked by 5 μ g/ml adrenomedullin₂₂₋₅₂. This result suggested that 5 μ g/ml adrenomedullin₂₂₋₅₂ could inhibit VEGF secretion and

significantly inhibited the levels of VEGF in the media of HRECs under the high glucose condition. The western blot results regarding the PI3K pathway shown in Figure 7 further supported the VEGF result.

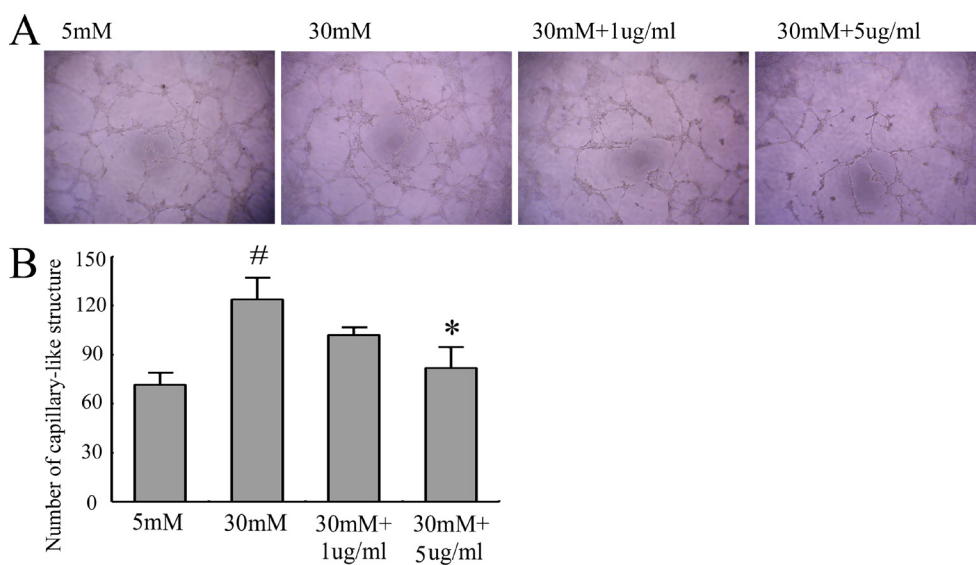


Figure 4. Adrenomedullin₂₂₋₅₂ inhibited high-glucose-induced tube formation. The tube formation was determined by Matrigel assay. **A:** After culture under appropriate glucose conditions for 24 h, HRECs (1.5×10^4 cells/well) were added to 96-well culture plates and incubated for 8 h to allow the formation of capillary-like structures. A magnification of $\times 200$ was used to image the cells. **B:** The quantitative data of the capillary-like structures

were obtained from three independent experiments. All values represent Mean \pm SD (n=3). * p<0.05 versus the 30 mM group. # p<0.05 versus the 5 mM group.

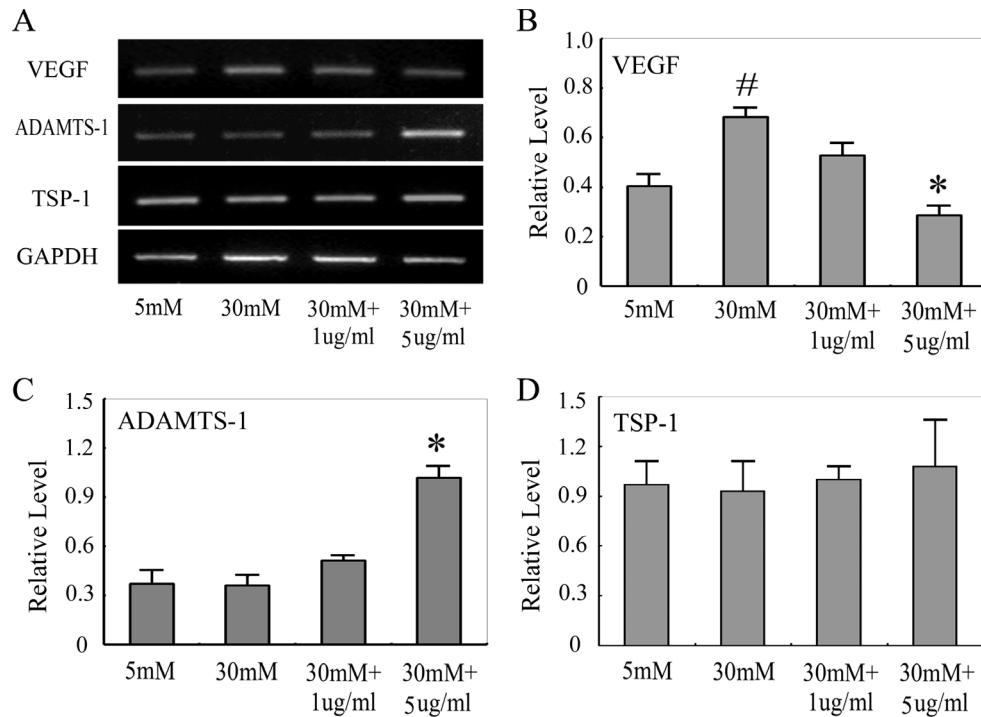


Figure 5. Angiogenic factors expression. After the HRECs were treated with high glucose or high glucose plus different concentrations of adrenomedullin₂₂₋₅₂ for 8 h, RT-PCR analysis was performed to detect pro-angiogenic and anti-angiogenic factor gene expression in the HRECs. **A:** Representative results from three independent runs of RT-PCR. **B:** Ratios of target gene expression to GAPDH were determined by RT-PCR. The quantitative data of the ratios were from three independent experiments. All values represent Mean±SD (n=3). * p<0.05 versus the 30 mM group. # p<0.05 versus the 5 mM group.

DISCUSSION

High glucose is considered to be an important risk factor in the development of DR. It could activate the polyol pathway of glucose metabolism. It generates cellular stress, injury of vascular pericytes and endothelial cells, and the development of abnormal capillaries [26,27]. Endothelial cells play a crucial role in regulating vascular tone, and injury of these cells is the initial step that leads to irreversible structural abnormalities [28]. In our current study, HRECs were used to imitate the pathogenesis of DR under the condition of high glucose. Similar to previous reports, HRECs in the current study were cultured in either physiologic (5 mM) or high glucose (30 mM) media [28,29]. In line with other studies, our results suggested that high glucose could promote the migration and tube formation of HRECs. However, there have been conflicting reports of high glucose on the proliferation of HRECs. Yuan et al. found the growth of HRECs in 30 mM glucose after 48 h was significantly higher than in 5 mM glucose [28]. Premanad et al. observed that although HREC treated with 30 mM glucose for 72 h showed no significant increase in proliferation when compared with cells treated with 5 mM glucose, there was an increasing trend in the high-glucose condition [29]. In our study, compared to the normal glucose condition, treatment with high glucose for 24 h failed to accelerate the proliferation of HRECs. One reason for this discrepancy may be that endothelial cells used in our study are the cell line while in the previous study, they used the

primary cultured endothelial cells from the eyes of donors. Another explanation could be the difference of the culture time in the 30 mM glucose condition.

ADM, a 52-amino acid polypeptide, is well known as an expander of blood vessels and a diuretic active peptide [2]. Accumulating evidence suggests that ADM is a pro-angiogenesis factor that promotes the proliferation, migration, and tube formation of endothelial cells [15-17,19]. Additionally, several lines of proof have suggested that inhibiting the ADM pathway with antibodies or antagonists directed against ADM or ADM receptors can reduce angiogenesis and tumor cell proliferation in mouse cancer models [30]. Zakareia and colleagues [25] reported that ADM was specifically enhanced in the plasma and vitreous humor of DR patients. Recently, it has been shown that ADM promoted the size of laser-induced CNV and ADM antagonist significantly inhibited the CNV [18]. Based on these studies, we assumed that ADM is involved in the process of ocular angiogenesis and adrenomedullin₂₂₋₅₂ might be a useful target for ocular angiogenesis intervention.

The angiogenic cascade is a complex and multistep process. Vascular endothelial cell migration and proliferation make up the initial step in angiogenesis, and then endothelial cells differentiate into a capillary-like network [31]. This process is tightly regulated by a series of pro- and anti-angiogenic molecules in normal physiology; when this

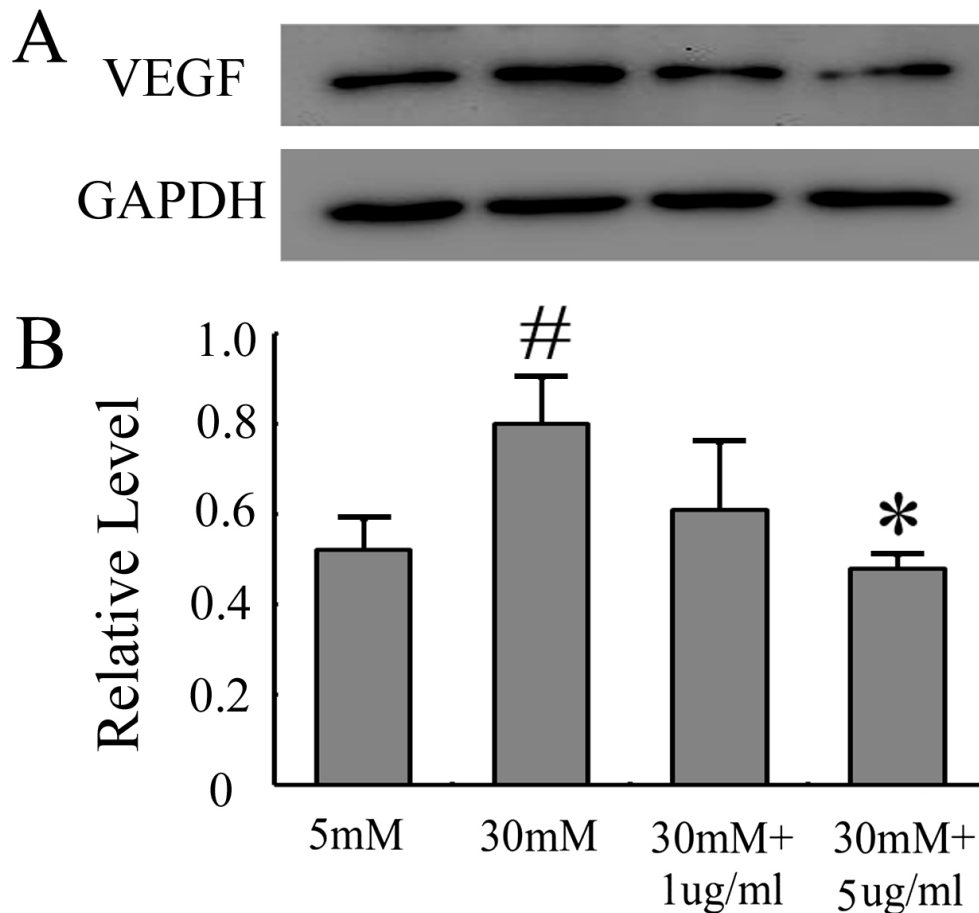


Figure 6. Adrenomedullin₂₂₋₅₂ inhibited high-glucose-induced VEGF expression. After HRECs were treated with high glucose or high glucose plus different concentrations of adrenomedullin₂₂₋₅₂ for 24 h, the protein level of VEGF was determined by western blot assay. **A**: Representative results from three independent trials of western blot. **B**: Ratios of VEGF to GAPDH were determined by western blot. The quantitative data of the ratios were from three independent experiments. All values represent Mean±SD (n=3). * p<0.05 versus the 30 mM group. # p<0.05 versus the 5 mM group.

equilibrium is broken, serious consequences may arise [22]. VEGF is considered to be a major factor of the abnormal angiogenesis that occurs in response to high glucose. At present, most researchers believe that high glucose could stimulate VEGF secretion, which is similar to our findings. However, the relationship between ADM and VEGF is still unclear. Some researchers found adrenomedullin could enhance VEGF-induced EC capillary formation and Akt pathway activation [32]. Other studies have indicated that blocking antibodies to VEGF cannot significantly inhibit AM-induced human umbilical vein endothelial cell (HUVEC) capillary tube formation [15]. Maki and colleagues [23] reported adrenomedullin induced capillary tube formation, which can be suppressed by VEGF neutralization. Our results demonstrated 5 µg/ml adrenomedullin₂₂₋₅₂ could inhibit high-glucose-induced HRECs VEGF expression. These seemingly contradictory findings may be due to the differences of cell types, culture media, treatment times, dose of agents, and so on.

As antiangiogenic molecules, TSP-1 and ADAMTS-1, which are also constitutively present in Ecs, can preclude

vasculature development [33,34]. TSP-1 levels in Bruch's membrane and choroidal vessels were decreased in age-related macular degeneration (AMD), and a reduction in TSP-1 may permit the formation of choroidal neovascularization [35]. TSP-1 is processed to release antiangiogenic polypeptides by the action of ADAMTS-1 [36]. At high concentration, ADAMTS-1 can also inhibit endothelial migration induced by the combined treatment with VEGF and basic fibroblast growth factor [37]. Macrophages are the source of TSP-1, ADAMTS-1, and VEGF [38-40]. It was published that adrenomedullin₂₂₋₅₂ could suppress the proliferation of Ecs, which was potentiated by TNF-α-treated macrophage cell lines [18]. Thus, macrophages may be associated with the anti-angiogenic effect of adrenomedullin₂₂₋₅₂.

Several signaling pathways are involved in the process of neovascularization. The PI3K/Akt signaling pathway can stimulate EC migration, proliferation, and tube formation [41,42], and PI3K is a downstream mediator of VEGF [43]. Maki and colleagues [23] found that ADM could upregulate the expression of VEGF via the PI3K pathway. Furthermore, pretreatment with 10⁻¹⁰ mol/l adrenomedullin₂₂₋₅₂ slightly

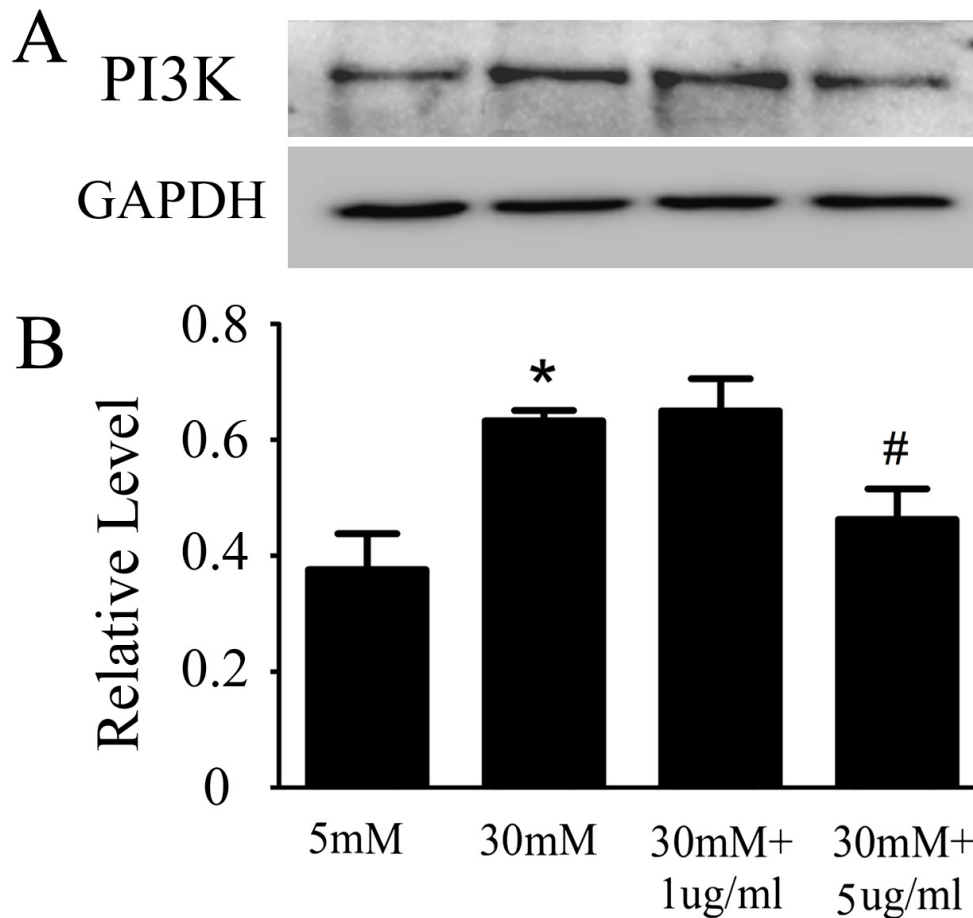


Figure 7. Adrenomedullin₂₂₋₅₂ inhibited the activation of PI3K by high glucose. After HRECs were treated with high glucose or high glucose plus different concentrations of adrenomedullin₂₂₋₅₂ for 24 h, the protein level of PI3K was determined by western blot assay. **A**: Representative results from three independent runs of western blot. **B**: Ratios of PI3K to GAPDH were determined by western blot. The quantitative data of the ratios were collected from three independent experiments. All values represent Mean±SD (n=3). * p<0.05 versus the 30 mM group. # p<0.05 versus the 5 mM group.

decreased the phosphorylation of Akt, and treatment with 10^{-6} mol/l partially suppressed the phosphorylation of Akt [19]. We found that high glucose could stimulate the phosphorylation of PI3K, which can be suppressed by 5 μ g/ml adrenomedullin₂₂₋₅₂. These results indicate that adrenomedullin₂₂₋₅₂ can inhibit the phosphorylation of PI3K.

In conclusion, all of our results suggest that adrenomedullin₂₂₋₅₂ could inhibit HREC migration, proliferation, and tube formation induced by high glucose by decreasing the expression of VEGF and the phosphorylation of the PI3K pathway. Although our findings provide evidence that ADM may be a potential drug to control the incidence and progression of DR in the future, further in vivo studies are still needed to evaluate its safety and efficacy.

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