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Hyperglycemia and advanced glycation end products regulate miR-126 expression in endothelial progenitor cells

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Abstract

Background/Aims: Dysfunction of endothelial progenitor cell (EPCs) contributes to diabetic vascular disease. We reported that downregulated miR-126 in diabetic patients causes EPC dysfunction. The present study was designed to investigate how high glucose (HG) and advanced glycation end products (AGEs) regulate miR-126 expression and whether miR-126 mediates the effects of HG and AGEs on EPCs.

Methods: We first tested the effects of glucose (5.5-50 mM) and AGEs (50-200 mg/L) on EPC proliferation and selected HG (50 mM) and AGEs (50 mg/L) for further experiments. EPCs were stimulated with HG and AGEs, and miR-126 expression was measured by real-time PCR. ROS was measured by immunofluorescence microscopy and flow cytometry. IL-6 and TNF- α levels in EPC supernatants were determined by ELISA. The effects of miR-126 on ROS and inflammatory markers under stimulation of HG and AGEs were also assessed. Finally, the effects of inhibitors of PI3K and Akt on AGEs-mediated miR-126 expression were examined.

Results: HG and AGEs increased IL-6, TNF- α and ROS and decreased miR-126 expression in EPCs. miR-126 negatively regulated IL-6, TNF- α and ROS. miR-126 overexpression reduced while miR-126 inhibition further increased inflammatory markers and ROS induced by HG and AGEs. Inhibitors of PI3K and Akt further decreased miR-126 expression in the presence of AGEs.

Conclusions: In conclusion, hyperglycemia and AGEs decrease miR-126 expression in EPCs. Recovering miR-126 expression may protect EPCs against dysfunction induced by HG and AGEs.

Key Words: hyperglycemia; AGEs; miR-126; EPCs

Introduction

Loss of endothelial integrity and impaired capacity for neovascularisation contribute to vascular complications of diabetes.[1] A growing body of evidence indicates that endothelial regeneration and repair do not exclusively rely on the proliferation of local endothelial cells, but also involve endothelial progenitor cells (EPCs).[2, 3] EPCs are mobilized from the bone marrow into systemic circulation and recruited at the sites of vascular repair, where they proliferate and differentiate into endothelial-like cells, thus contributing to vascular repair and angiogenesis.[4, 5] It has been found that in patients with type 2 diabetes mellitus (DM), especially with the complications of vascular disease, the number of EPCs is significantly reduced in peripheral blood, and the functions of EPCs are impaired.[6, 7] Therefore, decreased number and function of EPCs in DM patients may contribute to the pathogenesis of peripheral vascular disease and microvascular disease.[8, 9] However, the mechanisms for EPC dysfunction in DM are not fully understood.

MicroRNAs (miRNAs) are key regulators of diverse cellular processes including angiogenesis.[10] We have demonstrated that miR-126 is downregulated in EPCs derived from type 2 DM patients, and downregulation of miR-126 impairs EPCs function.[11] Since high glucose (HG) and advanced glycation end products (AGEs) are major characteristics of DM, and play important roles in vascular complications of DM, this study was aimed to investigate how HG and AGEs regulate the expression of miR-126. Previous studies have shown that HG and AGEs adversely affect EPC function, which may be related to reactive oxygen species (ROS),[12-14] so this study was also aimed to investigate whether miR-126 mediates the effects of HG and AGEs on EPCs, in relation to ROS as well as inflammatory markers. The findings of our study will help to understand the mechanism of EPC dysfunction and vascular disease in DM.

Materials and methods

Isolation and cultivation of EPCs

This study was approved by the Ethics Committee of Experimental Research, Medical College of Shanghai Jiaotong University. The use of human blood conformed to the principles outlined in the Declaration of Helsinki, and written informed consent was obtained from each patient. Blood samples were obtained from patients with type II DM at Xinhua Hospital affiliated to Shanghai Jiaotong University School of Medicine as previously described.[11] Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation with Ficoll-Isopaque Plus (Histopaque-1077, density 1.077 g/ml, Sigma) and cells (1×10^7) were plated onto culture dishes in 3 mL endothelial growth medium (EGM-2-MV Bullet Kit, LONZA), with supplements (Hydrocortisone, R3-insulin-like growth factor 1, human endothelial growth factor, vascular endothelial growth factor, human fibroblast growth factor, GA-100, ascorbic Acid, heparin and 20% fetal bovine serum) at 37°C in a 5% CO₂ incubator.

After four days, medium were changed and non-adherent cells were removed. EPCs developed spindle-shaped morphology gradually at day 4 and 7 after culture. The phenotype of these cells was confirmed by the expression of CD34 (Bio Legend Inc., San Diego, CA, USA), CD133 (Bio Legend Inc., San Diego, CA, USA) and KDR (Bio Legend Inc., San Diego, CA, USA) by using flow cytometry at day 1, 4 and 7 after culture. EPCs were plated in 96-well plates with complete culture medium and stimulated with HG (5.5, 12.5, 25, and 50mM) or AGEs (50, 100, and 200mg/L) for 24, 48, and 72h.[15, 16] EPCs were visualized under a light microscope (Olympus, Japan) at a magnification of 200x.

EPC proliferation assay

The effect of HG and AGEs on EPC proliferation was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. After EPCs were treated with HG or AGEs for 24, 48, and 72 h, EPCs were supplemented with MTT (0.5 mg/mL, Sigma) and incubated for a further 6 h. The supernatant was discarded by aspiration, and dimethyl sulfoxide was then added to EPCs and shaken for 10 min.

Absorbance was measured at 490 nm. Each experiment was performed 4 times independently.

Quantification of IL-6 and TNF- α levels by ELISA

EPCs in 96-well plates were pretreated with HG (50mM) or AGEs (50mg/L) or lipopolysaccharide (LPS, 10 μ g/mL, Sigma) or PBS for 24, 48, and 72h. Cell culture supernatants were collected after the periods of cell cultivation and were analyzed for IL-6 and TNF- α with ELISA kits (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions. Experiments were repeated 4 times independently.

Measurement of ROS production

The production of ROS in EPCs was determined by flow cytometry and immunofluorescence microscopy, using 2', 7'-dichlorofluorescein diacetate (DCFH-DA, Sigma) staining. The nuclei were stained with DAPI (4', 6-diamino-2-phenylindole, dihydrochloride, Molecular Probes, US). EPCs in 96-well plates were pretreated with HG (50mM) or AGEs (50mg/L) or LPS (10 μ g/mL) for 24, 48, and 72h. After the removal of medium from the wells, cells were incubated with 20 mol/L DCFH-DA for 30 min and with 100 mg/L DAPI for 15 min at room temperature. The percentage of DCFH-DA positive cells was measured by using flow cytometry method. Mean fluorescence intensity was calculated by averaging area intensities from a number of outlined cells. The result was expressed as integrated optical density (IOD)/Area by using Image-Pro Plus 6.0 software according to standard protocol.

RNA isolation and miRNA real-time PCR

Total RNA was extracted from cells using Trizol reagent (Invitrogen) according to the manufacturer's instruction. Extracted RNA was further purified and reverse-transcribed by using the TaqMan MiRNA Reverse Transcription Kit (Applied Biosystems) along with specific primers as the following: Forward primer 5'-ACAACCTTGGTATCGTGGAAGG-3' and Reverse primer 5'-GCCATCACGCCACAGTTTC-3'.

The PCR reaction was performed as follows: stage 1, 95°C for 10 min, stage 2,

95°C for 15 s, and 60°C for 60 s. Stage 2 was repeated for 40 cycles. Real-time PCR was performed using SYBR Green PCR master mix (Applied Biosystems) with the Applied Biosystems 7900HT Fast Real-time PCR System. U6 was used as endogenous controls. All samples were normalized to internal controls, and the relative expression level was calculated through the $2^{-\Delta\Delta C_t}$ analysis method.

Lentiviral constructs, packaging, and transduction

Lentiviral constructs and packaging were performed as we described previously.[11] Expression plasmid for miR-126 was created using PCR amplification with human genomic DNA as templates with the following primers:

miR-126 F 5'- AAAGAATTCTGACAGCACATTATTACTTTTG -3',

miR-126 R 5'- AAAGGATCCTGACCACGCATTATTACTCAC -3'.

The PCR products of miR-126 were cloned into the lentiviral expression plasmid pCDH-CMV-MCS-EF1-copGFP (System Biosciences). The construct of miR-126 was confirmed by sequencing. The plasmid DNA was cotransfected into HEK293 T cells with PCDH-126, psPAX2, pMD packaging construct using lipofectamine plus reagent (Invitrogen) according to the manufacturer's protocol. Medium was refreshed after 6 h, and lentiviral supernatant was collected 48 h later. EPCs were seeded into 6-well plates (5×10^5 cells/well) and transfected with different lentiviral vectors at an MOI of 10. Cells were selected by a FACS Scan (Becton Dickinson, Mountain View, CA, USA) based on the expression of GFP carried by pCDH plasmid.

Anti-miRNA transfection

To inhibit miR-126 expression, a miR-126 inhibitor or its negative control were obtained from System Biosciences and transfected into EPCs as described above. Cells were selected and cloned by culture in the presence of puromycin (2 μ g/mL) for 48 h, and miR-126 expression was analyzed by using quantitative real-time PCR.

Statistical analysis

Each experiment was performed at least three times, and all values were represented

as mean $\pm$ SD. Statistical analyses were calculated by Student's t-test or one-way ANOVA and $p < 0.05$ was considered significant. Graph Pad Prism 6.0 software (Prism 5; Graph Pad Software, San Diego, CA, USA) was used for statistical calculations.

Result

Characterization of EPCs from diabetic patients

EPCs were derived from PBMCs of type II DM patients. Attached EPCs elongated with spindle shape after changing medium at the 4th and 7th day after culture (Fig. 1A). FACS analysis showed that early EPC (at day 1, 4 and 7 after culture) expressed high levels of EPC markers such as CD34, CD133, and KDR (Fig. 1B).

HG and AGEs reduced the proliferation of EPCs

We first tested the effects of different concentration of glucose (5.5, 12.5, 25, and 50mM) and AGEs (50,100, and 200mg/L) for different time courses (24, 48, and 72h) on EPCs proliferation measured by MTT assays. At 24 and 48 h, there were no significant effects on EPCs proliferation by HG or AGE (Fig. 2) except a reduction caused by 50 mM of glucose at 48 h ($p < 0.01$, Fig. 2A, 2B). At 72 h, while glucose at 5.5 and 12.5 mM increased EPCs proliferation ($p < 0.05$, Fig. 2A), glucose at 25 and 50 mM decreased EPCs proliferation compared with PBS ($p < 0.001$, Fig. 2A, 2B). AGEs at three different concentrations (50, 100, and 200mg/L) reduced EPCs proliferation at 72 h ($p < 0.001$, Fig. 2C, 2D). We chose HG at 50mM and AGEs at 50 mg/ml for the following experiments.

HG and AGEs increased inflammatory markers and oxidative stress

We next examined the effects of HG (50 mM) and AGEs (50 mg/L) on the production of inflammation mediators (IL-6 and TNF- α) and ROS by EPCs. We included a positive control with LPS (10 μ g/mL) and a negative control with PBS. IL-6 and TNF- α released by EPCs were measured by ELISA. Both HG (Fig. 3A, C) and AGEs (Fig. 3B, D) significantly increased the production of IL-6 and TNF- α at 24, 48, and 72 h (all $p < 0.001$). ROS was measured by immunofluorescence staining (Fig. 4A)

and flow cytometry using DCFH-DA (Fig. 4B), which showed that both HG and AGEs stimulate ROS production in EPCs.

HG and AGEs inhibited miR-126 expression

In our previous study, we reported that miR-126 was down-regulated in EPCs derived from patients with diabetes, suggesting that the low level of miR-126 may be involved in functional impairment of EPCs from diabetes. In the current study, we further analyzed the expression of miR-126 in EPCs treated with HG (50 mM) or AGEs (50 mg/L) by real-time PCR. As shown in Fig. 5, gene expression of miR-126 was significantly decreased following the incubation with HG or AGEs for 24, 48, and 72h in a time-dependent manner.

miR-126 negatively regulated the production of IL-6, TNF- α and ROS

To investigate the role of miR-126 in the regulation of inflammatory factors and ROS, EPCs were infected with lentivirus overexpressing miR-126 or a miR-126 inhibitor, and the production of IL-6, TNF- α and ROS was measured. Overexpression of miR-126 significantly inhibited while inhibition of miR-126 significantly promoted the production of IL-6 and TNF- α , compared with their respective control group (Fig. 6A, 6B all $p < 0.001$). Similarly, overexpression of miR-126 decreased ROS production ($p < 0.001$), while down-regulation of miR-126 increased ROS production ($p < 0.01$) (Fig. 6C, 6F). We confirmed that the expression of miR-126 was increased by lenti-miR-126, but decreased by anti-miR-126, compared with their respective control group (Fig. 6E, both $p < 0.001$).

miR-126 regulated the production of inflammatory factors and ROS in the presence of HG or AGEs

We then examined how manipulation of miR-126 affected the production of inflammatory factors (IL-6 and TNF- α) and ROS in EPCs treated with HG or AGEs. anti-miR-126 significantly increased the levels of IL-6, TNF- α and ROS, while mimic-miR-126 decreased the levels of IL-6, TNF- α (Fig. 7A and 7B) and ROS (Fig. 7C) in EPCs treated with either HG or AGEs. Noticeably, an untreated control group (PBS) was comparable to anti-vector group or lenti-vector group indicating that adding a virus

did not contribute to the production of these factors.

miR-126 expression in EPCs was mediated via PI3K/Akt pathway by a positive feedback loop

We later examined the roles of signaling pathways in regulating miR-126 in EPCs by AGEs. We blocked PI3K/Akt signaling pathway which is known related to AGEs with specific inhibitors: anti-phosphatidylinositol-3-Kinase (LY294002) and anti-Akt (PX-316) antibodies. Inhibitors of PI3K and Akt further decreased miR-126 expression (Fig. 8A) and p-Akt expression (Fig. 8B) in AGEs-stimulated EPCs, suggesting that the regulation of miR-126 by AGEs in EPCs may be related to PI3K/Akt-dependent signaling pathway. Since miR-126 regulates PI3K/Akt/eNOs signaling pathway, [11] miR-126 expression is likely to be mediated via PI3K/Akt by a positive feedback loop (Fig. 8C).

Discussion

In this study we found that HG and AGEs inhibited EPC proliferation and decreased miR-126 expression in EPCs, accompanied by increased production of inflammatory markers (IL-6 and TNF- α) and ROS. miR-126 negatively regulated inflammatory markers and ROS. Furthermore, overexpression of miR-126 reduced while inhibition of miR-126 promoted inflammatory markers and ROS induced by HG and AGEs, suggesting that miR-126 mediates the effects of HG and AGEs on inflammation and ROS in EPCs. Therefore, miR-126 may counteract EPC dysfunction induced by HG and AGEs. In addition, inhibitors of PI3K and Akt further decreased miR-126 expression in AGEs-stimulated EPCs. (The main findings of the study are summarized in Fig. 8C)

Decreased EPCs levels have been described in diabetic patients and EPCs recruitment at re-endothelization sites following arterial injury is impaired in DM,[6, 7] likely contributing to diabetic vasculopathy. Our previous work has demonstrated that miR-126 is down-regulated in EPCs derived from type 2 DM patients, and the down-regulation of miR-126 impairs EPCs function.[11] Thus miR-126 deficiency in diabetic EPCs might be associated with the EPCs functional disorders in DM. In the present study, we aimed to investigate whether HG and AGEs, two main features of DM, cause miR-126 deficiency in DM.

We first tested the effects of different doses of glucose (5.5-50 mM) and AGEs (50-200 mg/L) on EPC proliferation to select the suitable dose of HG and AGEs for investigation of their effects on miR-126 and inflammatory markers and ROS. Previous studies showed that different doses of HG impaired EPCs number and function. Previous studies demonstrated that HG (12 to 33 mM) impaired EPC function.[17-19] In the present study, we tested the effects of different doses of glucose (5.5-50mM) on EPC proliferation. 5.5 mM D-glucose is the normal concentration in DMEM culture medium and equivalent to the serum glucose concentration in normal individuals (99 mg/dl). D-glucose (12.5, 25, and 50mM) reflect hyperglycemia in DM individuals (equivalent to 225, 450, and 900mg/dl, respectively). It is known that AGEs, which accumulate in DM,[20, 21] also contribute to impaired EPC function in DM. Previous studies showed that AGEs induced EPC dysfunction including promoting EPC apoptosis, inhibiting

EPC proliferation, migration, adhesion and tube-forming ability [12, 13, 22], thus promoting diabetes-accelerated atherosclerosis.[23] We tested the effect of AGEs (50-200 mg/L) on EPC proliferation. We selected 50 mM of glucose and 50 mg/L of AGEs for investigation of their effects on miR-126 and inflammatory markers and ROS in EPCs.

We found that both HG (50 mM) and AGEs (50 mg/L) decreased miR-126 expression and also induced the production of IL-6 and TNF- α and ROS in EPCs. Moreover, miR-126 exerted an inhibitory effect on the production of inflammatory markers and ROS in EPCs. Overexpression of miR-126 reduced while inhibition of miR-126 exacerbated the production of inflammatory markers and ROS induced by HG and AGEs, suggesting that down-regulation of miR-126 mediates the effects of HG and AGEs on inflammation and ROS, thus mediating EPC dysfunction. It has been known that low baseline levels of ROS produced in response to tissue ischemia serve as intracellular signaling molecules to prevent tissue injury and promote angiogenesis,[24, 25] but excess amounts of ROS impair EPC function and induce vascular injury.[26] Previous studies also showed that HG and AGEs induced ROS production and overpressed oxidant stress in EPCs and other cell types.[12-14, 27, 28] Inflammatory markers such as TNF- α and CRP also caused vascular dysfunction [29] and the reduction and dysfunction of EPCs,[30-32] and HG and AGEs induced IL-6 and TNF- α expression.[33, 34] Taken together, overexpression of miR-126 may rescue EPC dysfunction caused by HG and AGEs, likely via the inhibitory effect of miR-126 on inflammation and ROS production.

Previous studies showed that HG and AGEs induced EPCs dysfunction through down-regulation of PI3K/Akt/eNOS pathways.[15, 22, 35] Our previous work demonstrated that PI3K, Akt, and eNOS were decreased in EPCs derived from diabetic patients, which were increased by overexpression of miR-126.[11] In the present study, we found that inhibitors of PI3K and Akt further decreased miR-126 expression in AGEs-stimulated EPCs, suggesting a positive feedback loop between miR-126 expression and PI3K/Akt pathway (Fig. 8C). It has been known the PI3K/Akt/eNOS pathway plays a pivotal role in the regulation of EPCs.[15, 36] So, PI3K/Akt/eNOS

pathway is likely an important mediator of the effects of HG and AGEs on EPCs, through regulating miR-126 expression. Our previous work demonstrated that Spred-1 was an important target gene of miR-126 in regulating EPCs colony formation, proliferation, migration, and apoptosis.[11] Whether Spred-1 also acts as the targets of miR-126 in the process of inflammation in EPCs warrants further investigation.

Conclusion

In summary, we have found that hyperglycemia and AGEs decrease miR-126 expression in EPCs through inhibiting PI3K/Akt pathway. miR-126 negatively mediates the effects of HG and AGEs on the production of inflammatory markers and ROS. Recovering miR-126 expression may partly protect EPCs against dysfunction induced by HG and AGEs.

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Author Contributions

ShuMeng focused on the study design, Yeting Li is responsible for the experiments, Yeting Li, Qing Zhou, Chongzhe Pei, Bo Liu and MaoQuan Li are responsible for data analysis and writing, Lu Fang, YingGang Sun and YiGang Li are responsible for modifying the study design and writing. There are no conflicts of interest to declare.

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Figure Legend

Fig. 1 The morphology of EPCs isolated from PBMCs at day 1, 4 and 7 after culture (A).

Flow cytometry analysis showed that EPCs expressed CD34, CD133 and KDR at day 1, 4 and 7 after culture (B). Magnification $\times 200$. Scale bars=20 μ m.

Fig. 2 The proliferation of EPCs in response to different concentrations of glucose and advanced glycation end products (AGEs). EPC were treated with various concentrations of glucose (5.5, 12.5, 25, and 50mM) (A) or AGEs (50,100, and 200mg/L) (C) for 24, 48, and 72h. Cell proliferation was assessed by MTT assay. The time-dependent effects of 50mM of glucose (B) or 50mg/L of AGEs (D) were also shown. Data represent means $\pm$ SD (n=4/group). *p< 0.05, **p< 0.01, ***p < 0.001 vs. the control group (PBS).

Fig. 3 High glucose and advanced glycation end products (AGEs) promote the secretion of inflammatory markers by EPCs. IL-6 (A, B) and TNF- α (C, D) in supernatants of EPCs stimulated with high glucose (50mM, A, C) or AGEs (50mg/L, B, D) or LPS (10 μ g/mL) for 24, 48, and 72h were measured by Elisa assay. Data represent mean $\pm$ SD (n=4/group). ***p< 0.001 vs. the control group (PBS). P>0.05 vs. the positive control group (LPS).

Fig. 4 The effect of high glucose and advanced glycation end products (AGEs) on ROS production by EPCs. EPCs were treated with HG (50 mM) and AGEs (50 mg/L) or LPS (10 μ g/mL) for 24 h. Cells were stained with DCFH-DA and DAPI, and production of ROS was detected by immunofluorescence microscopy (A) and flow cytometry (B). Data are shown expressed as mean $\pm$ SD (n=3/group). *p< 0.05, **p< 0.01 vs. the control

group (PBS). Magnification $\times 200$. Scale bars=10 μ m.

Fig. 5 miR-126 is down-regulated by high glucose and advanced glycation end products (AGEs). After the treatment with HG (50mM) or AGEs (50mg/L) for 24, 48, and 72h, miRNA-126 expression in EPCs was measured by real-time PCR, normalized to U6 expression and depicted as fold changes relative to the corresponding PBS group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. PBS group. Values represent mean $\pm$ SD (n=3/group).

Fig. 6 miR-126 inhibits the production of IL-6, TNF- α and ROS. EPCs were transfected with lentiviral miR-126, miR-126 empty vector (EV), miR-126 inhibitor, and its negative control. (A and B) The levels of IL-6 and TNF- α were measured by ELISA. Production of ROS was detected by immunofluorescence microscopy (C) and flow cytometry (F). Fluorescence intensity was calculated as IOD/Area (D). miR-126 was increased after treatment with lenti-miR-126, while miR-126 was decreased after treatment with anti-miR-126, compared with their respective control group (Fig. 6E all $p < 0.001$). ** $p < 0.01$, *** $p < 0.001$ vs. their respective control groups. Data are expressed as mean $\pm$ SD (n=3/group). Magnification $\times 200$. Scale bars=10 μ m.

Fig. 7 miR-126 inhibits the production of IL-6, TNF- α and ROS of EPCs in the presence of high glucose or advanced glycation end products (AGEs). EPCs were transfected with lentiviral miR-126, miR-126 empty vector (EV), anti-miR-126 inhibitor, and anti-miR-

negative control. After transfection, cells were treated with high glucose (50mM) or AGEs (50mg/L) for 24 h. (A) IL-6 and (B) TNF- α levels were measured by ELISA. Production of ROS was detected by immunofluorescence microscopy (C). Fluorescence intensity was calculated as IOD/Area (D and E). Data are expressed as means $\pm$ SD (n=3/group). ***p<0.001. Magnification $\times$ 200. Scale bars=10 μ m.

Fig. 8 The regulation of miR-126 by advanced glycation end products (AGEs) is PI3K/Akt signaling dependent. miR-126 (Fig. 8A) and p-AKT (Fig. 8B) was analyzed using real-time PCR after the incubation of AGEs for 48 h with the presence and absence of LY294002(PI3K inhibitor) and PX-316 (Akt inhibitor). Fig. 8C summarizes the main findings of the present study. HG and AGEs decrease miR-126 expression in EPCs. miR-126 negatively regulates inflammatory markers and ROS, and also mediates the effects of HG and AGEs on inflammation and ROS in EPCs. In addition, PI3K/Akt promotes miR-126 expression since inhibitors of PI3K and Akt further decreased miR-126 expression in AGEs-stimulated EPCs. Our previous study has shown miR-126 regulates PI3K/Akt/eNOs signaling pathway, so miR-126 expression is likely to be mediated via PI3K/Akt by a positive feedback loop. Data are shown as means $\pm$ SD (n=3/group). **p<0.01, ***p<0.001.