Abstract. The major ophthalmic complication in patients with diabetes is diabetic retinopathy (DR), which is one of the major eye diseases that causes blindness. It is well established that the occurrence and duration of DR is positively correlated with duration of diabetes. Advanced glycation end product (AGE) accumulation in patients with diabetes is one factor that leads to the development of DR. However, the underlying mechanisms remain unclear. In the present study, the role of phosphoinositide 3-kinase/protein kinase B (Akt) signaling in AGE-induced DR development was investigated. An in vitro experimental system was used to study the effects of AGES on human retinal capillary endothelial cells (HRCECs) and Müller cells. Flow cytometry, MTT, western blotting and BrdU incorporation assays were performed. Reverse transcription-quantitative polymerase chain reaction was used to measure the expression of angiogenesis-associated genes. Functional assays of angiogenesis, including HRCEC invasion and tube formation assays. It was demonstrated that the expression of receptor for AGES was upregulated in HRCECs and Müller cells following treatment with AGES. AGE treatment did not affect Müller cell viability, but enhanced HRCEC viability. Akt inhibition increased cell apoptosis and death in HRCECs. AGE treatment upregulated the expression of pro-angiogenic genes, which was suppressed by Akt inhibitor treatment. In addition, Akt inhibitor treatment suppressed HRCEC invasion and tube formation ability. The present study suggested that Akt-mediated signaling may serve critical roles in the development of DR due to the accumulation of AGES. Akt may be a potential therapeutic target in DR.

Introduction

Patients with diabetes mellitus and chronic hyperglycemia are at risk of developing complications, including diabetic retinopathy (DR), nephropathy, neuropathy, cardiomyopathy, rheumatoid arthritis and osteoporosis (1,2). The pathogenesis of these complications is strongly associated with the glycation of plasma proteins, which produces a large number of advanced glycation end products (AGEs) (3,4). Protein glycation interferes with their physiological function by altering molecular conformation, enzymatic activity and receptor functioning. AGEs interact with their membrane-localized receptors [receptor for advanced glycation end products (RAGE)] and alter intracellular signaling to influence various biological processes within cells (5,6). The mechanisms by which accumulation of AGEs cause DR has not yet been extensively studied.

Since its initial discovery as a proto-oncogene, protein kinase B (Akt) has become a major focus of attention, due to its critical involvement in cell apoptosis regulation, angiogenesis, autophagy, transcription, protein synthesis and glucose metabolism (7). The Akt signaling cascade is activated by multiple cell receptors, including receptor tyrosine kinases, B and T cell receptors, cytokine receptors and G-protein coupled receptors (7). Other stimuli that induce the production of phosphatidylinositol (3,4,5)-trisphosphates via phosphoinositide 3-kinase (PI3K) also induce activation of the Akt signaling cascade (7). Inhibition of the PI3K/Akt/mammalian target of rapamycin pathway has potential therapeutic function in DR pathophysiology (8). However, whether the PI3K/Akt pathway influences the function of AGEs during the development of DR remains unclear. In the present study, the mechanisms of
AGEs in DR were investigated. Specifically, the role of Akt signaling in DR pathophysiology was examined.

Materials and methods

Cell culture. Primary human retinal capillary endothelial cells (HRCECs) and primary human Müller cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Müller cells were cultured in high-glucose Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.). HRCECs were cultured in RPMI-1640 medium (Thermo Fisher Scientific, Inc.) with the same supplements. Cells were sub-cultured until 80% confluence was reached. A gradient of AGEs conjugated to bovine serum albumin (0, 25, 50 and 100 µg/ml; cat. no. 121800; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was used to treat the cells for 24 h at 37°C. LY294002 (1 µM; Sigma-Aldrich; Merck KGaA), a PI3K inhibitor (PI3Ki), was also used to treat cells for 6 h at 37°C. Akt inhibitor (Akti; 5 µM; Akt 1/2 kinase inhibitor; cat. no. sc-300173; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) was used to block Akt function in cells (cells were treated for 6 h at 37°C).

MTT assay. Following treatment, cell media was removed by careful aspiration. Subsequently, 50 µl serum-free media and 50 µl MTT solution was added into each well (10^4 per ml). The plate was incubated at 37°C for 3 h. Following incubation, 150 µl MTT solvent (DMSO) was added into each well. The plate was then warmed in foil and shaken on an orbital shaker for 15 min. The plate was read at an absorbance of 590 nm after being maintained in the incubator for 1 h.

Western blotting. In brief, cells were collected and lysed in radioimmunoprecipitation assay lysis buffer with proteinase inhibitor (20 nM) and phosphatase inhibitor (20 nM; Thermo Fisher Scientific, Inc.). The extracted protein was measured and normalized with a bichinchoninic acid protein assay. Equivalent amounts of total proteins (30 µg per lane) were subsequently separated by electrophoresis on 4-20% gradient SDS-PAGE gels and were transferred to a polyvinylidene fluoride membrane by a half-dry transferring system. Blocking was performed by a commercial kit (cat. no. V1324; Thermo Fisher Scientific, Inc.) and the manufacturer's protocols. Cells were blocked with PBS three times and the invaded cell number on the membrane was counted using a light microscope.

Invasion assay. Transwells coated with Matrigel (Corning Incorporated, Corning, NY, USA) with an 8 µm polycarbonate filter membrane were used for the invasion assay. HRCECs (3x10^4) were added into the upper chamber with serum-free medium (cat. no. 211; Sigma-Aldrich; Merck KGaA). The lower chamber was filled with 5% serum medium (cat. no. 211; Sigma-Aldrich; Merck KGaA). HRCECs were subsequently treated with AGEs (100 µg/ml) and Akti (5 µM) for a 24 h incubation at 37°C. Cells on the top surface of the filter were removed, and the remaining cells on the underside of the filter were subsequently fixed with the fixation buffer (0.1 M sodium cacodylate buffer supplemented with 4% paraformaldehyde, 2.5% glutaraldehyde and 0.02% picric acid) at room temperature for 1 h, and stained with 5% crystal violet for 10 min at room temperature. The stained membrane was washed with PBS three times and the invaded cell number on the membrane was counted using a light microscope.

BrdU incorporation assay. BrdU incorporation assay was performed to assess the cell proliferation. A commercial BrdU cell proliferation assay kit (cat. no. 6813; Cell Signaling Technology, Inc., Danvers, MA, USA) was used and the manufacturer's protocol was carefully followed. Briefly, 5x10^4 cells were seeded in 96-well plate 24 h before the BrdU incorporation assay. Then BrdU solution was added to the plate well and incubated for 12 h at 37°C. Then, the cells were collected by centrifuging the plate at 300 x g for 10 min at 4°C. Fixing/denaturing solution was added to each well (100 µl/well) and the plate was kept at room temperature for 30 min. Following the removal of the fixing/denaturing solution, detection antibody solution from the kit was added for incubation at room temperature for 1 h. HRP-conjugated secondary antibody solution was added to incubate at room temperature for 30 min. Absorbance at 450 nm was read after the stop solution was added.

Flow cytometry. To detect cell apoptosis and necrosis, an Annexin V and propidium iodide (PI) staining method was utilized. A commercial kit (cat. no. V1324; Thermo Fisher Scientific, Inc.) was used in accordance with the manufacturer's protocols. Cells were washed twice with cold PBS and subsequently resuspended in 1X binding buffer. Then, 10^5 cells in 100 µl annexin-binding buffer were transferred to a 5 ml culture tube and 1 µl annexin V-fluorescein isothiocyanate (FITC) and 2 µl PI was added to the tube. Samples were gently mixed and incubated for 15 min at room temperature in the dark. Subsequently, 400 µl 1X binding buffer was added to each tube. Samples were analyzed by flow cytometry within 30 min following staining. A FACSCanto II machine (BD Biosciences, Franklin Lakes, NJ, USA) was used.

In addition, the expression of glial fibrillary acidic protein (GFAP) on Müller cells and cluster of differentiation (CD)34 protein on HRCECs was measured by flow cytometry. Cell pellets of Müller cells and HRCECs were harvested following centrifugation at 500 x g and 5 min at 4°C. Then an equal number (10^7) of these cells were collected for subsequent steps. They were blocked with PBS with 5% bovine serum albumin (BSA; cat. no. A2058; Sigma-Aldrich; Merck KGaA) for 10 min at room temperature. Then mouse antibodies of
GFAP (1:200; cat. no. G3893, Sigma-Aldrich; Merck KGaA) and CD34 (1:200; cat. no. SAB4700160, Sigma-Aldrich; Merck KGaA) were incubated with Müller cells and HRCECs for 10 min at room temperature, respectively. To measure the expression of cleaved caspase-3, the 10^5 harvested HRCECs and Müller cells were washed in cold PBS once, and 2 ml cold 4% paraformaldehyde (Sigma-aldrich; Merck KGaA). Following washing with cold PBS for 5 min, permeabilization buffer (cat. no. 22016, Biotium, Inc.) was added to incubate at 4˚C for 30 min. Cells were washed in PBS prior to the addition of cleaved caspase-3 (1:100; cat. no. ab2302, Abcam) antibodies to the cells. Following incubation with GFAP, CD34 or cleaved caspase-3 antibodies for 30 min at 4˚C, the cells were washed by PBS. Subsequently, FITC-labeled anti-mouse secondary antibodies (1:5,000, cat. no. SAB3701081; Sigma-Aldrich; Merck KGaA) and APC-preadsorbed anti-rabbit secondary antibodies (1:5,000, cat. no. ab130805, Abcam) were added to the cells to incubate for 20 min at room temperature. Following three washes in PBS, three cells were analyzed using the FACScanto II machine (BD Biosciences) and FlowJo software (version 10.0.7, FlowJo LLC, Ashland, OR, USA) was used to analyze the data.

Tube formation assay. Prior to the tube formation assay, HRCECs (1x10^5 cells/well) were starved in medium containing 1% FBS for 12 h at 37˚C. Cells were incubated with different concentrations of AGEs for another 12 h at 37˚C. Cells were subsequently seeded into 24 well plates pre-coated with Matrigel (BD Biosciences) and treated with AGEs (50 µg/ml) or Akti (5 µM) for 8 h at 37˚C. Images were captured under an inverted microscope at 200x magnification and 5 fields were randomly selected for assessment.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). PCR was performed to detect expression of vascular endothelial growth factor (VEGF), VEGF receptor (VEGFR), pigment epithelium-derived factor (PEDF), angiopoietin (Ang) 1, Ang 2, fibroblast growth factor (FGF), platelet-derived growth factor (PDGF) and 18S rRNA. The primers used for these genes are listed in Table I. RNA was isolated from the cell lines according to the manufacturer's protocol using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.). Total RNA (1 µg) was reverse transcribed using High Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, Inc.). PCR was performed on a LightCycler II 480 (Roche Diagnostics, Basel, Switzerland) with SYBR Green I dye (Roche Diagnostics). The results were normalized to the housekeeping 18S rRNA gene.

Statistical analysis. Statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Software, Inc., CA, USA). All experiments were repeated 3 times. Data were expressed as the mean ± standard error of the mean. Two-tailed Student's t-test was used to evaluate the significance of differences between two groups. One-way analysis of variance was used to compare results with more than three groups. Tukey's post-hoc test was performed for multiple comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

RAGE expression is upregulated in HRCECs and Müller cells in response to AGE treatment. To investigate the effects of AGEs on DR, primary Müller cells (Fig. 1A) and HRCECs (Fig. 1B) were cultured in vitro, and the expression of RAGE was measured. Biomarkers of human retinal capillary endothelial cells (CD34) and Müller cells (GFAP) were assessed by flow cytometry. These two biomarkers were highly expressed in the two kinds of cells, respectively (Fig. IC and D). The results revealed that there was a basal level of RAGE expression in Müller cells (Fig. 1E) and HRCECs (Fig. 1F). Following AGE treatment at 100 µg/ml, RAGE expression was significantly upregulated in the two cell cultures by more than five-fold, compared with the untreated cells (P<0.0001; Fig. 1G and H). The increase in RAGE expression was proportional to the concentration of AGE treatment. These data provided initial verification that Müller cells and HRCECs were responsive to AGE treatment.

Akt inhibition reduces HRCEC viability. As the expression of RAGE was upregulated by the AGE treatment in Müller cells and HRCECs, the biological effects of AGE treatment (100 µg/ml) were examined. The cell viability assay results demonstrated that the AGE treatment did not exert...
a significant effect on Müller cell viability (Fig. 2A), but enhanced HRCEC viability (Fig. 2B). The PI3K/Akt pathway has a profound impact on cell proliferation and survival (8). Müller cells and HRCECs were treated with PI3Ki and Akti, and it was noticed that Akti significantly reduced the effects induced by AGE treatment (P<0.0001; Fig. 2B), although...
PI2Ki did not notably influence the effect of AGE treatment. Western blotting analysis confirmed that AGE treatment upregulated Akt phosphorylation, but not PI3K phosphorylation (Fig. 2C and D).

**Akt inhibition suppresses HRCEC proliferation and induces apoptosis.** To understand the mechanisms underlying the alterations in cell viability as a result of AGE and Akt treatment in HRCECs, cell proliferation and apoptosis was measured via a BrdU incorporation assay and flow cytometry, respectively. Flow cytometry demonstrated that Akt treatment markedly increased the cell death rate in HRCECs (Fig. 3A). The expression of full length and cleaved caspase-3 was also quantified in Müller cells and HRCECs following treatment. As presented in Fig. 3B, neither the AGE treatment nor the Akt treatment stimulated cell apoptosis in Müller cells. However, in HRCECs, the Akt treatment induced cleaved caspase-3 expression (Fig. 3B). The BrdU incorporation assay detected cell proliferation. In HRCECs, AGE treatment significantly increased cell proliferation, and Akt treatment suppressed cell proliferation (Fig. 3C). These results indicated that AGE treatment enhanced HRCEC viability by increasing cell proliferation, and that Akt treatment reduced HRCEC viability via suppressing cell proliferation, as well as via induction of apoptosis.

**Akt inhibition reduces angiogenesis-associated gene expression.** The angiogenic function of HRCECs was further investigated by quantifying the relative expression of angiogenesis-associated genes using RT-qPCR, including the pro-angiogenic genes Ang1, Ang2, VEGF, VEGFR, PDGF and FGF, as well as the anti-angiogenic gene PEDF. The expression level was normalized by calculating the z scores. Expression levels of these pro-angiogenic genes were upregulated by AGEs treatment, whereas PEDF expression was downregulated (Fig. 4). Furthermore, Akti treatment suppressed pro-angiogenic gene expression induced AGE treatment.

**Akt inhibition suppresses the tube formation ability of HRCECs.** In addition to measuring the expression of angiogenesis-associated genes, functional assays were performed to further assess the HRCEC angiogenesis. In the in vitro invasion assay, HRCECs treated with AGEs (100 µg/ml) had the highest invasive ability (Fig. 5A), whereas the AGEs and Akti-treated HRCECs exhibited a comparable invasive ability to control cells (Fig. 5A). A similar trend was observed in the tube formation assay (Fig. 5B). Quantitative analysis revealed that these differences were significant (P<0.01; Fig. 5C and D). These data, together with the angiogenic gene expression data, suggested that AGEs had a pro-angiogenic effect in HRCECs, and Akt inhibition had an anti-angiogenic effect.

**Discussion**

Patients with a long history of diabetes mellitus frequently have comorbidities, including DR and cardiomyopathy, which are highly associated with a chronic hyperglycemic state (1,2,9). Abnormal, newly formed blood vessels grow from the retina and result in subsequent tractional retinal detachment and hemorrhage, which is the major concern of DR (9-11). Although the underlying mechanisms of aberrant angiogenesis in DR remains unclear, evidence has demonstrated AGE
accumulation is a major factor that contributes to angiogenesis (12). AGEs interact with RAGEs, therefore altering intracellular signaling and influencing several essential biological processes within cells (3-6). The exact pathological mechanisms of AGE-induced angiogenesis in DR remain to be elucidated.
Müller cells are a type of retinal glial cell that have been demonstrated to be critical to retinal development, by serving as promoters of retinal growth and histogenesis (13). Hrcecs are the major cell type that causes vascularization in dr (14). In the present study; the effects of a Ges on these two cell types were examined. Initially, the expression of RAGEs in Müller cells and Hrcecs following aGe treatment was investigated. These two cell types expressed a basal level of RAGEs without any treatment. When treated with AGES, they expressed a much higher level of RAGEs, compared with

Figure 4. Expression of angiogenesis-associated genes in human retinal capillary endothelial cells. mRNA expression of was plotted as a heatmap. Red indicates relatively high expression level, and green indicates a relatively low expression level. AGE, advanced glycation end products; Ctrl, control; VEGF, vascular endothelial growth factor; VEGFRA, vascular endothelial growth factor receptor; PEDF, pigment epithelium-derived factor; Ang1, angiopoietin 1; Ang2, angiopoietin 2; FGF, fibroblast growth factor; PDGF, platelet-derived growth factor; PI3K, phosphoinositide 3-kinase; Akt, protein kinase B; i, inhibitor; Ctrl, control.

Figure 5. Invasive and tube forming ability of Hrcecs. (A) Representative results of HRCEC invasion in control group following AGE and Akti treatment. AGE treatment significantly increased HRCECs invasion, and Akt inhibitor reduced this effect. (B) The tube formation ability of HRCECs was measured in control HRCECs, AGES treated HRCECs, and Akt inhibitor-treated HRCECs. Magnification, x200. (C) Differences in invasive and (D) tube formation ability were statistically significant. **P<0.01, ***P<0.001, ****P<0.0001 vs. the AGES treated group. Hrcecs, human retinal capillary endothelial cells; AGE, advanced glycation end products; PI3K, phosphoinositide 3-kinase; Akt, protein kinase B; i, inhibitor.
the control group. The effects of AGEs on Müller cells and HRCEC viability were subsequently examined. AGE treatment significantly enhanced HRCEC viability. The PI3K/Akt pathway has critical roles in regulating cell proliferation and death, and has been demonstrated to be involved in a number of AGE-associated biological processes, including autophagy and cell migration (15,16). When cells were treated with PI3Ki, however, no obvious alterations in cell viability were observed in Müller cells or HRCECs. When cells were treated with Akti, the viability of HRCECs was dramatically suppressed, whereas Müller cell viability was not influenced. The following cell proliferation and apoptosis assays confirmed the findings of the cell viability assay, and suggested that AGE facilitated the survival of HRCECs in an Akt-dependent way. These initial results suggested that the AGEs primarily influenced HRCECs, rather than Müller cells.

Retinal vascularization is a coordinated collaboration involving several cell types, including endothelial cells, pericytes and astrocytes, and a dynamic balance of positive and negative regulatory factors (17-19). In angiogenesis-associated DR, this delicate balance is disturbed (10). Since AGEs regulate HRCEC proliferation via Akt, whether or not AGEs regulate the vascularization of the retina was investigated. By measuring the expression of genes associated with angiogenesis, it was demonstrated that the AGE treatment induced a pro-angiogenic gene expression, including VEGF, VEGFR, FGF, Ang1, Ang2 and PDGF. In addition, PEDF expression was decreased. Treatment with Akti inhibited these effects. Furthermore, the HRCEC invasion and tube formation assays also indicated the Akt-dependent pro-angiogenic effects of AGEs.

Studies of HRCECs have shed light on the earliest stages of DR and other diseases of the retinal microvasculature (20-22). However, whether or not the PI3K/Akt pathway influences AGE-mediated DR development remains unclear. The present study reported that AGEs induced HRCEC proliferation and angiogenesis via an Akt-dependent mechanism. Inhibiting the Akt pathway prevented the effects of AGEs on HRCEC proliferation and vascularization. Therefore, targeted therapies that suppress Akt function may be a promising treatment for retinal vascularization-associated diseases, including DR.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

DT was responsible for study design, major experiments, data analysis and manuscript preparation. NN was responsible for experiments, data analysis and manuscript preparation. TZ conducted some experiments, and performed data analysis and manuscript preparation. CL was responsible for literature review, data analysis, and manuscript preparation and revision. QS was responsible for data interpretation, manuscript revision, and data collection. LW performed some experiments and was responsible for manuscript preparation and revision. YM was responsible for funding collection, study design and manuscript revision.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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