

# Advanced glycation end products facilitate the proliferation and reduce early apoptosis of cardiac microvascular endothelial cells via PKC $\beta$ signaling pathway: Insight from diabetic cardiomyopathy

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## ABSTRACT

**Objective:** To investigate the effects of advanced glycation end products (AGEs) on the proliferation and apoptosis of cardiac microvascular endothelial cells (CMECs) in rats and their underlying signaling pathway.

**Methods:** CMECs were isolated from Sprague–Dawley rats. We first examined the effects of AGEs on the proliferation and apoptosis of CMECs and then tested whether protein kinase C (PKC)  $\beta$  blockers could counteract the effects of AGEs. The PKC agonists phorbol 12-myristate 13-acetate (PMA) and PKC $\beta$  blockers were also used to verify whether PKC could act independently on CMECs. The receptor for AGEs (RAGE)–small interfering RNA (siRNA) transfection was used to verify the effect of AGEs on PKC. Following the above steps, we explained whether AGEs regulated the CMEC proliferation and early apoptosis through the PKC $\beta$  signaling pathway. Proliferation of CMECs was detected using the Cell Counting Kit-8 (CCK-8) assay, and early apoptosis was determined using the Annexin V-Fluorescein Isothiocyanate (FITC)/propidium iodide (PI) double staining. Expression of proliferation and apoptosis-related proteins and PKC phosphorylation were determined by western blotting analysis. Cell cycle distributions were assayed using a BD FACSCalibur cell-sorting system.

**Results:** AGEs facilitated the proliferation of CMECs, upregulated phosphorylated extracellular signal regulated kinase (p-ERK), and accelerated the entry of cells from G1 phase to the S+G2/M phase, which was consistent with the upregulated cyclin D1 by AGEs. AGEs inhibited early apoptosis of CMECs by increasing the expression of survivin and decreasing the expression of cleaved-caspase3. All these effects can be reversed by PKC $\beta$ 1/2inhibitors. In addition, AGE upregulated the RAGE expression and phosphorylation of PKC $\beta$ 1/2 in CMECs, while the inhibition of RAGE reversed the phosphorylation, as well as the effects of AGEs on proliferation and apoptosis in CMECs.

**Conclusion:** The study indicated that AGEs facilitated the proliferation and reduced early apoptosis of CMECs via the PKC $\beta$  signaling pathway. (*Anatol J Cardiol* 2020; 23: 141-50)

**Keywords:** advanced glycation end products, cardiac microvascular endothelial cells, diabetic cardiomyopathy, protein kinase C

## Introduction

Diabetic cardiomyopathy (DCM) is one of the most common microvascular complications of diabetes (1-3). For patients with DCM, early clinical cardiac function can be intact, left ventricular diastolic dysfunction and cardiac hypertrophy begin to appear in the middle stage, and systolic dysfunction occurs last (4, 5). Monolayer endothelial cells, as a component of the myocardial microvascular wall, play an important role in regulating of the microvascular function. The development of many cardiovascular diseases, including diabetic heart disease, is closely

related to the damage of cardiac microvascular endothelial cells (CMECs) (6, 7).

Increasing evidence has suggested that the accumulation of advanced glycation end products (AGEs) is involved in diabetes-related diseases (5, 8). AGEs can change the structure of proteins, cross-link collagen molecules, increase the stiffness of diabetic hearts, and impair cardiac function (8, 9). Engagement of the receptor for AGEs (RAGE) with AGEs can elicit oxidative stress and subsequently evoke proliferation, inflammation, and fibrosis in a variety of cells (8). Therefore, blocking the AGEs–RAGE system is recognized as an important approach in the future treatment of

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chronic diabetic complications. Previous studies examining the AGEs–RAGE system have focused on diabetic macroangiopathy, while now the AGEs–RAGE axis has been found to have an effect on diabetic microangiopathy (10).

In addition, protein kinase C (PKC) activation can also lead to diabetic microvascular complications (11). Among various PKC isoforms,  $\beta$  isoform appears to be preferentially activated in the vascular system of diabetic animals (12). Studies have shown that PKC $\beta$  is associated with cardiac microvascular ischemia–reperfusion injury in diabetic rats (13), and PKC activation contributes to the microvascular barrier dysfunction in the heart at an early stage of diabetes (14). However, whether AGEs can regulate the CMEC function through the PKC $\beta$  signaling pathway in diabetes is still unknown.

Therefore, we explored this scientific issue based on the phenotype of CMECs and tried to provide new insights into the pathogenesis of diabetic cardiomyopathy.

## Methods

### Animal and cell culture

CMECs were isolated and cultured in Dulbecco's minimum essential medium (DMEM), supplemented with 20% fetal bovine serum (FBS) (10). Briefly, the left ventricles of male Sprague–Dawley rats (Shrek, Shanghai) (200–250 g) were harvested and minced into 1 mm<sup>3</sup> small pieces after the removal of the endocardial endothelium and epicardial coronaries. The remaining tissue was then minced in phosphate buffered saline (PBS) and incubated in 0.2% collagenase (Type II; Sigma Aldrich, St. Louis, MO, USA) for 10 min, followed by 0.2% trypsin (Sigma Aldrich, St. Louis, MO, USA) for another 6 min at 37°C in a water bath. After centrifugation, the cells were resuspended in DMEM supplemented with 20% FBS and plated on 10 cm dishes. All institutional and national guidelines for the care and use of laboratory animals were followed. The CMECs were positively identified by CD31 surface antigen expression using immunofluorescence staining and flow cytometry (Becton Dickinson, USA). Afterwards, the CMECs were cultured in different mediums including AGE albumin and bovine serum albumin (BSA) (50 mg/mL). CGP53353 (Sigma, USA) was used as a PKC $\beta$ 1 inhibitor (6.0 nmol/mL) (15) and PKC $\beta$ 2 inhibitor (0.9 nmol/mL) (16), and phorbol 12-myristate 13-acetate (PMA) (50 ng/mL) (Sigma, USA) was used as a PKC agonist.

AGE-modified albumin (AGE albumin) was synthesized under sterile conditions by incubating BSA (low endotoxin, Merck) with 0.5 M D-glucose in 100 mM PBS, as previously described (10), and then, the mixture was fully dialyzed against PBS to remove unbound glucose. BSA incubated without glucose under the same conditions was used as the negative control in all experiments.

### Proliferation assay

The CMECs proliferation was detected using the Cell Counting Kit-8 (CCK-8) assay. Briefly, the CMECs were digested with trypsin and then cultured in serum-free medium in a 96-well cul-

ture plate (200  $\mu$ L/well). The four groups were identified as the control, AGEs, AGEs+PKC $\beta$ 1 inhibitor, and AGEs+PKC $\beta$ 2 inhibitor. CCK-8 solution was added to each well, and the cells were incubated for 0.5, 1, 2, or 4 hours, and the absorbance (A) at 450 nm was measured by using an automatic multi-well spectrophotometer (Bio-Rad, Richmond, CA, USA).

### Apoptosis assay

An annexin V kit (BioVision Inc. USA) was used to determine the percentage of cells undergoing apoptosis. Briefly, the CMECs were harvested after 24-hour treatments. Approximately, 15,000 cells were detected for each sample. Then, the cells were trypsinized gently and resuspended with binding buffer, and they were treated with 5  $\mu$ L annexin V–Fluorescein Isothiocyanate (FITC) and 5  $\mu$ L propidium iodide (PI). After the incubation for 5 min on ice, each sample was analyzed immediately using the FACSCalibur flow cytometer (Becton Dickinson, USA).

### Cell cycle distribution assay

DNA distributions throughout the cell cycle were assayed using a BD FACSCalibur cell-sorting system (17). The cells were placed into six-well plates and divided into several groups. After counting, 10<sup>6</sup> cells/mL were washed with PBS and fixed in 70% ethanol overnight at 4°C. The cells were centrifuged, washed again with PBS, and then stained for DNA content (1 mg PI and 25 mg ribonuclease A in 1 mL PBS) for 30 min at room temperature and promptly analyzed by flow cytometry.

### Western blotting analysis

The procedures used were similar to those described previously, with slight modifications (18). Treated CMECs were scraped in the radio immunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China). The primary antibodies anti-RAGE, anti-phosphorylated protein kinase B/extracellular signal regulated kinase (pAKT/ERK), and anti-caspase3 were purchased from Cell Signalling Technology (MA, USA). Anti-pPKC $\beta$ 1/ $\beta$ 2, anti-pPKC $\alpha$ , anti-survivin, and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) polyclonal antibodies were purchased from Santa Cruz Biotechnology (CA, USA). Anti-cyclin D1 and anti-cyclin B1 were purchased from Abcam (Cambridge, MA). The secondary antibodies conjugated to horseradish peroxidase were purchased from Cell Signalling Technology. The images were captured on an image reader LAS-4000 system (Fujifilm, Tokyo, Japan).

### Small interfering RNA (siRNA) transfection

The RAGE siRNA target duplex sequences (Sense: 5'-CAGCCAAUGUCCCUAAUTT-3', Antisense: 5'-AUUAGGGACAUUGGCUGUGTT-3') were synthesized by Thermo (CA, USA). In brief, CMECs were transferred into 24-well plates and cultured in DMEM supplemented with 20% FBS. After overnight growth, the cells were treated with 0.5 ml Opti-MEM I (Invitrogen, Canada) with each well containing 50 nmol/L siRNA duplexes and 5  $\mu$ L Lipofectamine2000.

### Statistical analysis

All determinations were performed in triplicate, and experiments were repeated at least three times. The data were presented as the means $\pm$ SEM. Analyses were conducted with SPSS 18.0, using the unpaired Student's t-test for comparisons between two groups and one-way analysis of variance for multiple comparisons. A p-value <0.05 was considered statistically significant.

## Results

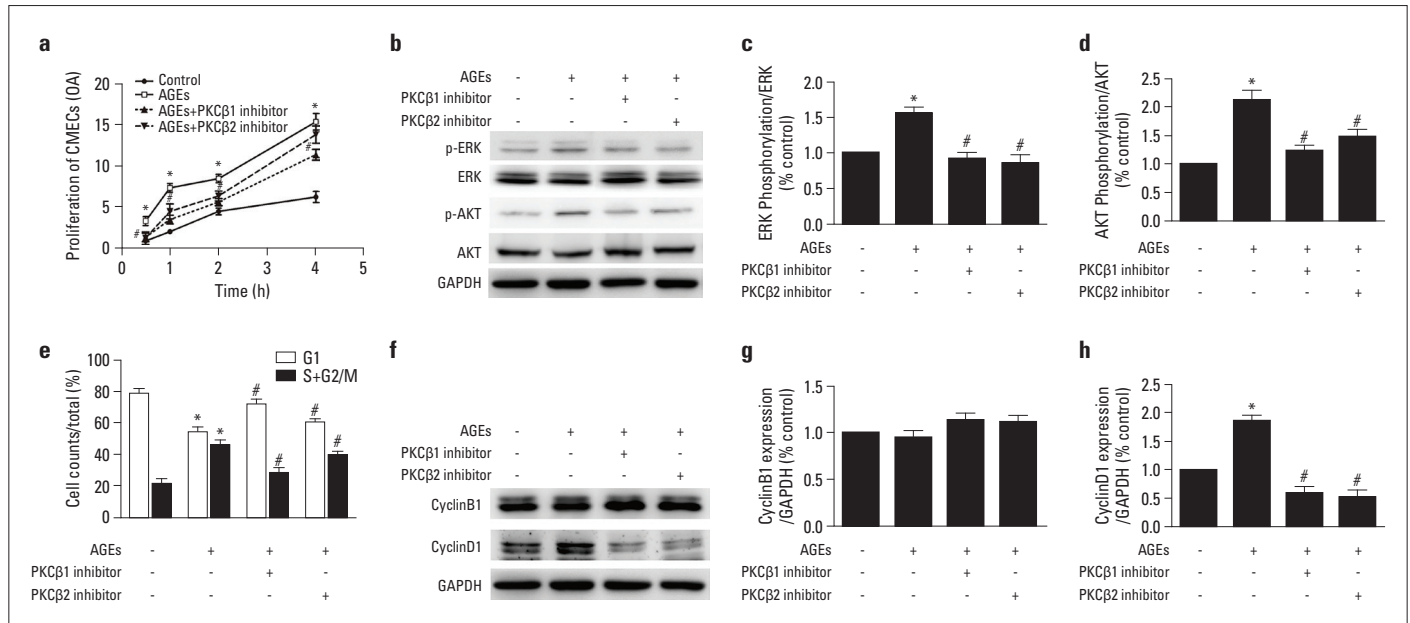
### AGEs facilitated the CMECs proliferation, and the PKC activation promoted it independently

The proliferative activity of CMECs incubated in AGEs was evaluated. After treatment with AGEs for 0.5, 1, 2, and 4 hours, the proliferations of CMECs significantly increased for 240%, 249%, 89%, and 144%, compared with the control group (p<0.05) (Fig. 1a). To elucidate the relationship between the PKC signaling pathway and AGEs with regard to their effects on CMECs, we further treated cells with a PKC $\beta$  inhibitor (PKC $\beta$ 1 inhibitor 6 nmol/mL and PKC $\beta$ 2 inhibitor 0.9 nmol/mL). The proliferations of CMECs induced by AGEs were decreased (p<0.05) (Fig. 1a).

Furthermore, the phosphorylation of proliferation-related protein ERK was assessed by western blotting, and AGEs up-

regulated phosphorylation of ERK by 55.7% (p<0.05) (Fig. 1b and 1c). Both PKC $\beta$ 1 and PKC $\beta$ 2 inhibitor-treated cells downregulated the phosphorylation of ERK. In addition, AGEs had a similar impact on AKT phosphorylation (Fig. 1d).

As the cell cycle was closely related to proliferation, effects of AGEs on cell cycle distribution were examined. The percentage of AGEs-treated cells in G1 phase decreased from 78.5% to 54.1% (p<0.05), while cells in the S+G2/M phase increased from 21.5% to 45.9% (p<0.05) (Fig. 1e), which suggested AGEs accelerated the entry of cells from G1 phase to the S+G2/M phase, thereby promoting DNA synthesis and cell proliferation. The percentage of G1 phase cells pretreated with the PKC $\beta$ 1 inhibitor (6.0 nmol/mL) and PKC $\beta$ 2 inhibitor (0.9 nmol/mL) increased from 54.1% to 71.8% and 60.3% (p<0.05), respectively, while the percentage of cells in the S+G2/M phase decreased from 45.9% to 28.2% and 39.7%, respectively (p<0.05) (Fig. 1e). Thus, these results might demonstrate that the PKC $\beta$  inhibitor CGP53353 inhibited the effects of AGEs by arrest cells from G1 phase to the S+G2/M phase. We also assessed the role of cyclin B1 and cyclin D1 in the cell cycle of CMECs by western blotting and found that AGEs upregulated the expression of cyclin D1 by 88.3% (p<0.05), while no significant effect was observed on cyclin B1 (p>0.05) (Fig. 1f, 1g and 1h). Both PKC $\beta$ 1 and PKC $\beta$ 2 inhibitor-treated cells downregulated expressions of cyclin D1 significantly.



**Figure 1.** AGEs facilitate the CMECs proliferation, and PKC $\beta$  inhibitor reverses the effect (a) AGEs promote the proliferation of CMECs at 0.5, 1, 2, and 4 h (\*P<0.05 vs. control). Both PKC $\beta$ 1 and PKC $\beta$ 2 inhibitors decrease the proliferation level at 0.5, 1, 2, and 4 h (#P<0.05 vs. AGEs). (b) Effects of AGEs and PKC $\beta$  inhibitors on proliferation-associated proteins. Protein levels are assessed using the western blot. (c, d) Both the PKC $\beta$ 1 inhibitor (6 nmol/mL CGP53353) and PKC $\beta$ 2 inhibitor (0.9 nmol/mL CGP53353) downregulate the phosphorylation of ERK and AKT compared with the AGEs group (#P<0.05 vs. AGEs). (e) Effects of AGEs and PKC $\beta$ 1/2 inhibitors on cell cycle distributions. Flow shows that the percentage of AGEs-treated cells in G1 phase decreases from 78.5% to 54.1%, while the percentage in the S+G2/M phase increases from 21.5% to 45.9% (\*P<0.05). The percentage of G1 phase cells pretreated with the PKC $\beta$ 1 inhibitor (6.0 nmol/mL) and PKC $\beta$ 2 inhibitor (0.9 nmol/mL) increases from 54.1% to 71.8% and 60.3% (#P<0.05 vs. AGEs). The percentage of cells in the S+G2/M phase decreases from 45.9% to 28.2% and 39.7%, respectively (#P<0.05 vs. AGEs). (f, g, h) Effects of AGEs and PKC $\beta$ 1/2 inhibitors on the cyclin D1/B1 expression by western blotting. AGEs upregulate the expression of cyclin D1 significantly (\*P<0.05 vs. control), and no significant effect is observed on cyclin B1. PKC $\beta$ 1/2 inhibitors downregulate the expression of cyclin D1 (#P<0.05 vs. AGEs)

To demonstrate the role of PKC in the proliferation of CMECs, we used PMA to simulate the function of PKC. We treated cells with a PMA (50 ng/mL), PMA+PKC $\beta$ 1 inhibitor (CGP53353 6 nmol/mL), or PMA+PKC $\beta$ 2 inhibitor (CGP53353 0.9 nmol/mL). It was found that the PMA/PKC activation could promote the CMECs proliferation independently, while the PKC $\beta$ 1/2 inhibition reversed the effect induced by PMA/PKC (Fig. 2a).

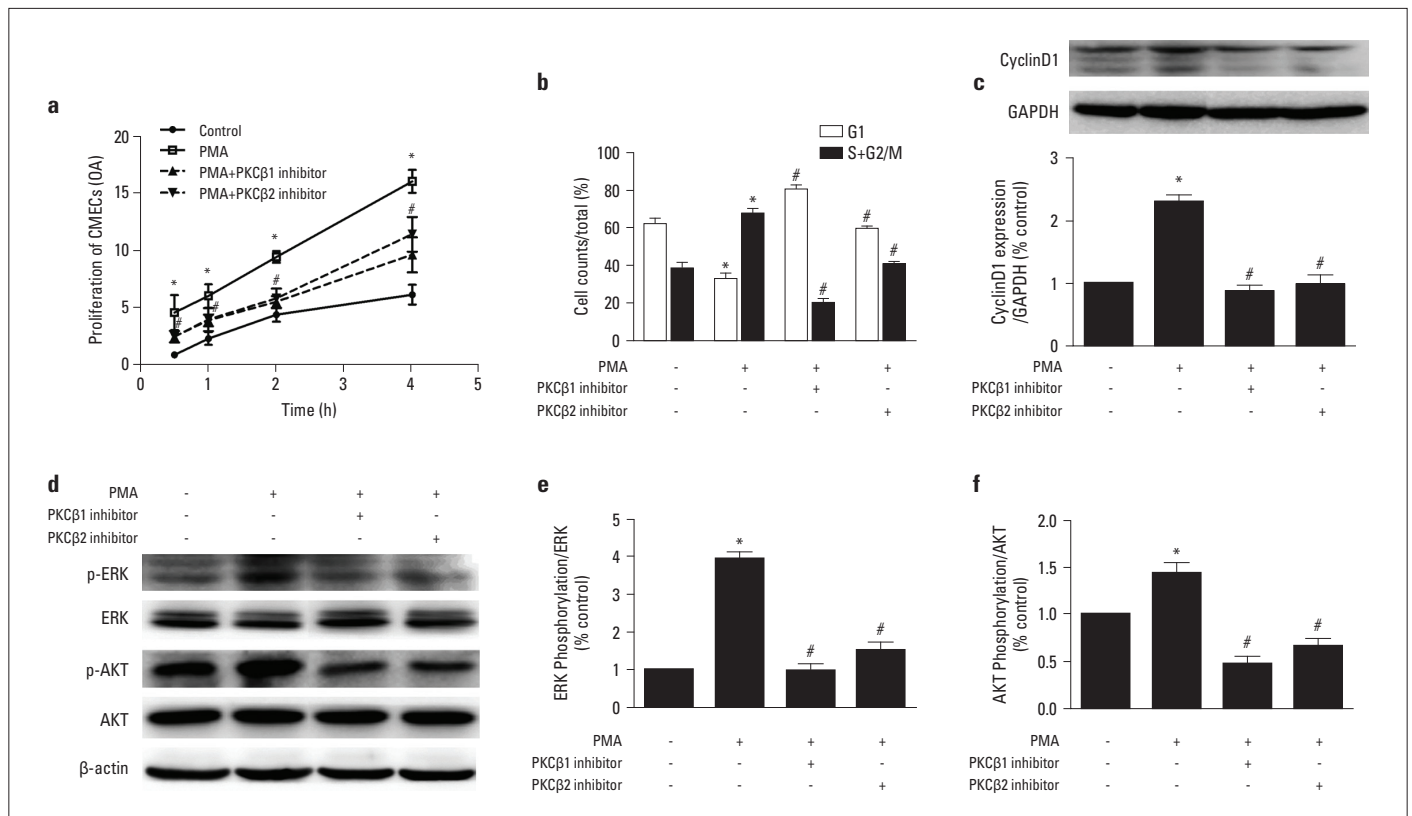
The percentage of PMA-treated cells in G1 phase decreased from 61.8% to 32.6% ( $p < 0.05$ ), while cells in the S+G2/M phase increased from 38.2% to 67.4% ( $p < 0.05$ ). We treated cells with PMA+PKC $\beta$ 1/2 inhibitors and observed that the percentage of cells in G1 phase significantly increased from 32.6% to 80.3% and 59.4% ( $p < 0.05$ ), and cells in the S+G2/M phase decreased from 67.4% to 19.7% and 40.6% ( $p < 0.05$ ) (Fig. 2b). The results were consistent with those observed for AGEs treatments. Simultaneously, western blotting analysis indicated that PMA/PKC upregulated the cyclin D1 expression significantly by 140%, while PKC $\beta$ 1/2 inhibitors reversed the effect induced by PMA/PKC ( $p < 0.05$ ) (Fig. 2c). The western blotting analysis indicated that PMA/PKC upregulated phosphorylation of ERK ( $p < 0.05$ ), and

PKC $\beta$ 1/ $\beta$ 2 inhibitor-treated cells downregulated phosphorylation of ERK (Fig. 2d, 2e).

### AGEs and PKC activation both reduced early apoptosis of CMECs

Flow cytometry revealed that early apoptosis reduced from 5.01% to 1.83% in AGEs-treated cells when compared with the control group ( $p < 0.05$ ) (Fig. 3a). However, both the numbers of early apoptotic cells evidently increased after treatment with the PKC $\beta$ 1/2 inhibition when compared with the AGEs group ( $p < 0.05$ ) (Fig. 3a), which implied that PKC inhibitors could reverse the early apoptosis reduction induced by AGEs.

Furthermore, the expressions of apoptosis-related protein, including survivin, cleaved-caspase3, and B cell lymphoma-2 (Bcl-2)/Bcl-2 associated X protein (Bax) were assessed using the western blotting analysis. The expression of survivin was increased by 75.7%, and the expression of cleaved-caspase3 was decreased by 57.6% after treatment with AGEs ( $p < 0.05$ ), although the expression of Bcl-2 and Bax were not affected by AGEs (Fig. 3b, 3c, 3d, and 3e). Both PKC $\beta$ 1/2 inhibitors were able



**Figure 2.** PKC activation promotes CMECs proliferation (a) Effects of PMA on proliferation in CMECs. Compared with control, PMA increases the proliferation of CMECs, and PKC $\beta$ 1/2 inhibitors reverse these effects induced by PMA ( $*P < 0.05$  vs. control;  $#P < 0.05$  vs. PMA). (b) Effects of PMA and PKC $\beta$ 1/2 inhibitors on cell cycle distribution. The percentage of PMA-treated cells in G1 phase decreases from 61.8% to 32.6% and cells in the S+G2/M phase increase from 38.2% to 67.4%. The percentage of cells in G1 phase significantly increases from 32.6% to 80.3% and 59.4% after PMA+PKC $\beta$ 1/2 inhibitors, and cells in the S+G2/M phase decrease from 67.4% to 19.7% and 40.6% ( $*P < 0.05$  vs. control;  $#P < 0.05$  vs. PMA). (c) Effects of PMA and PKC $\beta$ 1/2 inhibitors on the cyclin D1 expression. PMA upregulates the cyclin D1 expression by approximately 140%. PKC $\beta$ 1/2 inhibitors reverse the effect induced by PMA/PKC ( $*P < 0.05$  vs. control;  $#P < 0.05$  vs. PMA). (d, e, f) Effects of PMA and PKC $\beta$  inhibitors on the expression of ERK and AKT. PMA upregulates the phosphorylation of ERK and AKT, and PKC $\beta$ 1/2 inhibitors decrease the phosphorylation of ERK and AKT compared with the PMA group ( $*P < 0.05$  vs. control;  $#P < 0.05$  vs. PMA)

to downregulate the expression of survivin and upregulate the expression of cleaved-caspase3 (Fig. 3c, 3d, and 3e), which might indicate that AGEs could reduce early apoptosis of CMECs, while PKC inhibitors CGP53353 could reverse the effect of AGEs in cell apoptosis.

To demonstrate the role of PKC in the apoptosis of CMECs, we also used PMA to simulate the function of PKC. The number of apoptotic cells after pretreatment with PMA was detected. It was found that PMA/PKC significantly decreased the early apoptosis cell counts ( $p < 0.05$ ), which could be reversed by the PKC $\beta$ /2 inhibition ( $p < 0.05$ ) (Fig. 4a). The western blot analysis demonstrated that PMA upregulated the expression of survivin, but downregulated the expression of cleaved-caspase3 ( $p < 0.05$ ). Both PKC $\beta$ /2 inhibitors were able to reverse these effects, compared with the PMA group ( $p < 0.05$ ) (Fig. 4b, 4c, and 4d), which might imply that PMA/PKC could reduce early apoptosis of CMECs.

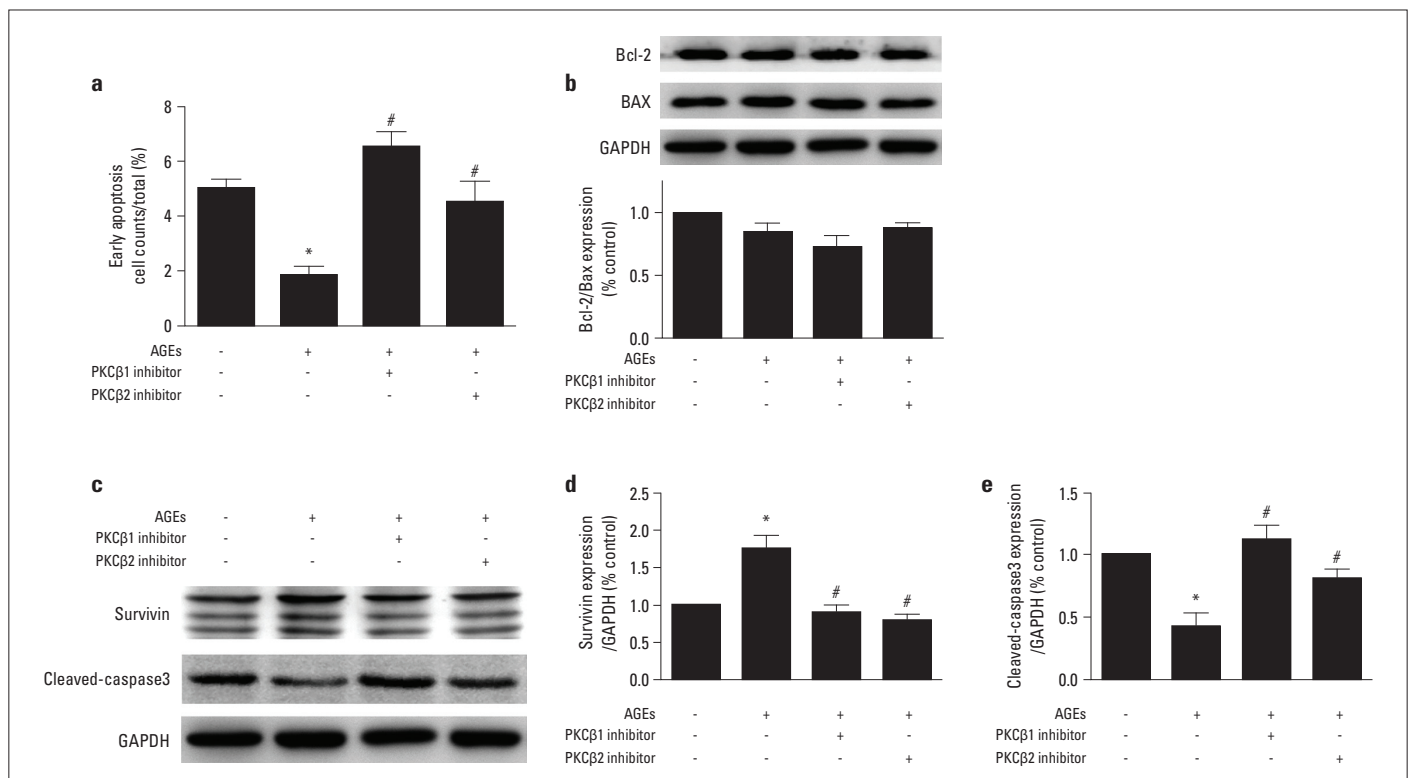
### AGEs upregulated the phosphorylation of PKC $\beta$ 1 and PKC $\beta$ 2

The RAGE expression was detected using the western blot, and it significantly increased ( $p < 0.05$ ) with the treatment of AGEs (Fig. 5a). AGEs also significantly promoted the phosphorylation of PKC $\beta$ 1 by 121% ( $p < 0.05$ ) and phosphorylation of PKC $\beta$ 2 by 197%

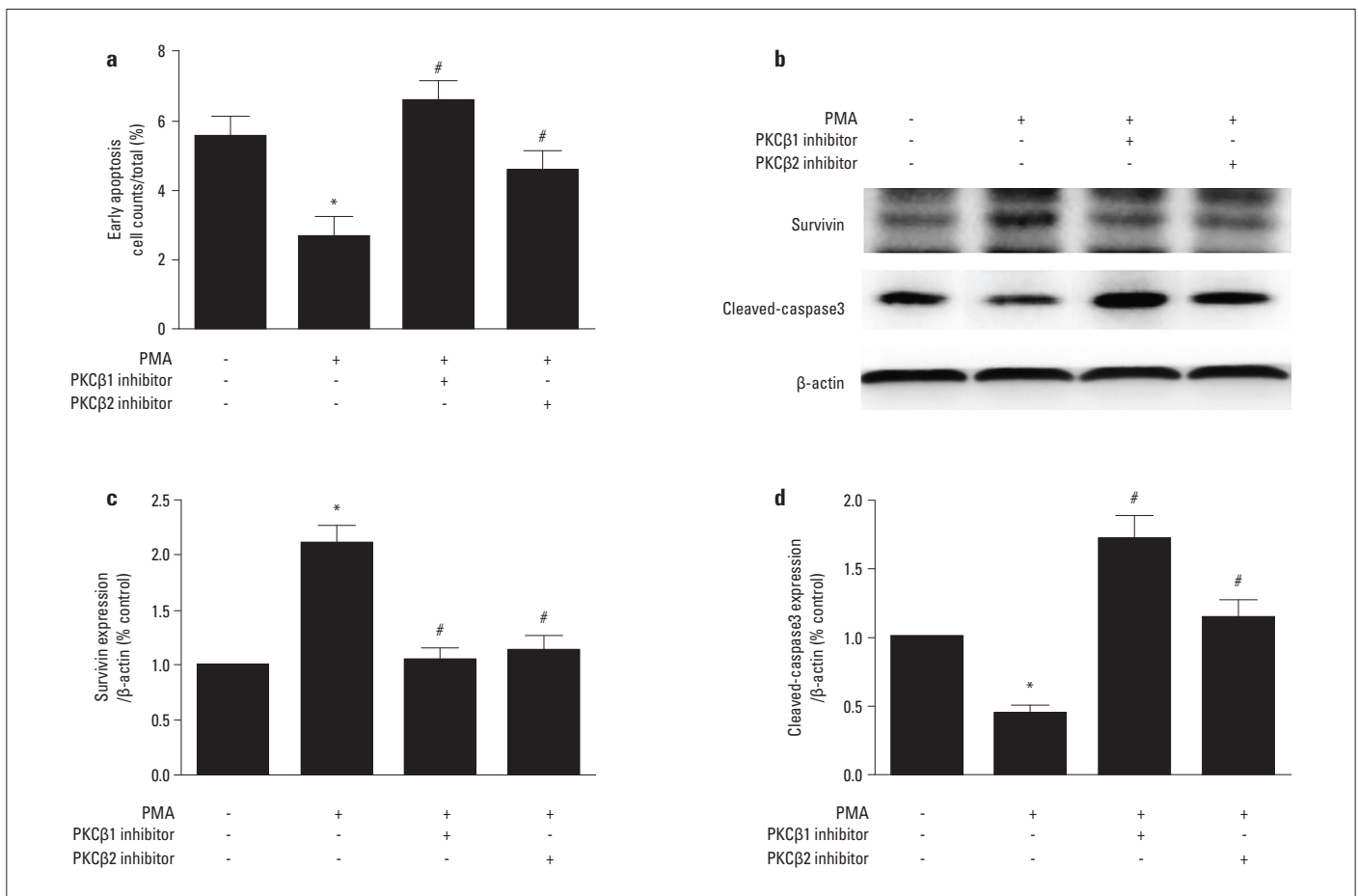
when compared with the control group ( $p < 0.05$ ) (Fig. 5d, 5e, and 5f). These results implied that RAGE and PKC $\beta$ 1/2 were activated in the AGEs-treated CMECs.

### RAGE-siRNA transfection reduced phosphorylation of PKC $\beta$ 1/2 and obstructed the effects of AGEs on the CMECs proliferation and apoptosis

AGEs promoted the proliferation and reduced the apoptosis of CMECs. To further elucidate whether the PKC pathway participates in the regulation of AGEs-induced proliferation and apoptosis, we used RAGE-siRNA to block the RAGE expression. First, CMECs were cultured in AGEs with 50 nmol/mL RAGE-siRNA for 24 hours. Then the phosphorylations of PKC $\beta$ 1/2 were detected. The results indicated that phosphorylations of PKC $\beta$ 1 and PKC $\beta$ 2 were reduced by 33.6% and 35.4%, respectively, after the siRNA treatment ( $p < 0.05$ ) (Fig. 5d, 5e, and 5f), whereas no changes were observed in the phosphorylation of PKC $\alpha$  (Fig. 5g, 5h). Furthermore, we found that after the siRNA transfection, the proliferations of CMECs significantly reduced by 58.1%, 54.5%, 26.9%, and 28% for 0.5, 1, 2, and 4 hours, respectively, compared with AGEs-treated cells ( $p < 0.05$ ) (Fig. 5b), and the number of early apoptotic cells increased ( $p < 0.05$ ) (Fig. 5c).



**Figure 3.** AGEs reduce early apoptosis of CMECs, and the PKC $\beta$  inhibitor reverses the effect (a) Apoptosis is determined using the Annexin V-FITC/PI double staining. Early apoptosis of CMECs is reduced from 5.01% to 1.83% in AGEs-treated cells when compared with the control group ( $*P < 0.05$  vs. control). The numbers of the early apoptotic cells increase after treatment with the PKC $\beta$ 1/2 inhibitor when compared with the AGEs group ( $#P < 0.05$  vs. AGEs). (b, c, d, e) Effects of AGEs and PKC $\beta$  inhibitors on apoptosis-associated proteins. The western blotting analysis showed that the survivin expression is upregulated by 75.7%, and the expression of cleaved-caspase3 is decreased by 57.6% after treatment with AGEs ( $*P < 0.05$  vs. control). The expression of Bcl-2 and Bax are not affected by AGEs. Both the PKC $\beta$ 1/2 inhibitors are able to reverse the effect of AGEs in cell apoptosis ( $#P < 0.05$  vs. AGEs)



**Figure 4.** PKC activation reduces early apoptosis of CMECs (a) The number of apoptotic cells after pretreatment with the PKC agonist PMA is detected. PMA/PKC significantly decreases the early apoptosis cell counts from 5.58% to 2.64% ( $*P < 0.05$  vs. control), which could be reversed by the PKC $\beta$ 1/2 inhibition ( $\#P < 0.05$  vs. PMA). (b, c, d) The western blot analysis demonstrated that PMA upregulates the expression of survivin, but downregulates the expression of cleaved-caspase3 ( $*P < 0.05$  vs. control). Both PKC $\beta$ 1/2 inhibitors are able to reverse these effects ( $\#P < 0.05$  vs. PMA).

## Discussion

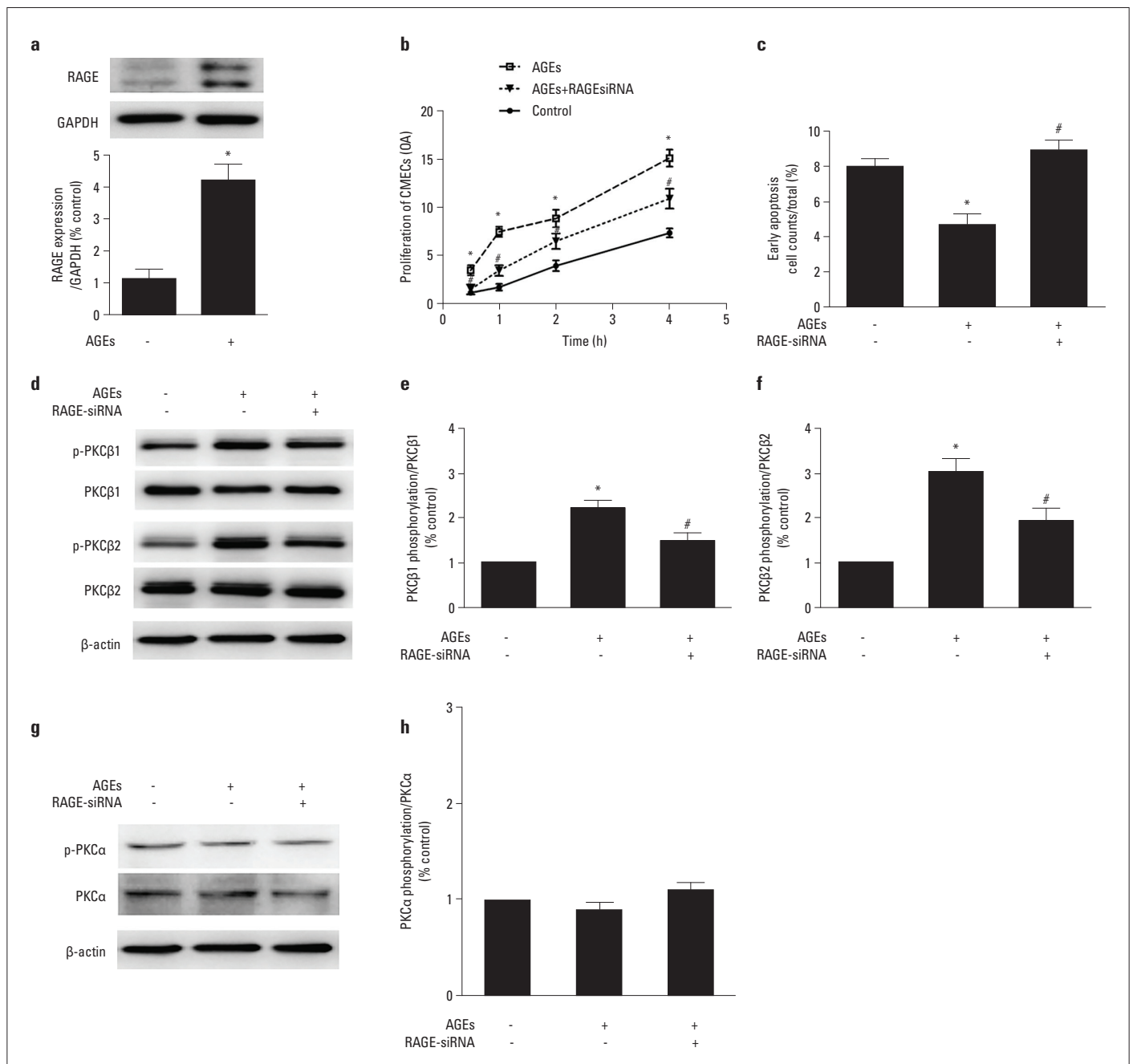
There are two types of vascular diseases in diabetes. One is microangiopathy, which mainly affects capillaries and arterioles and is unique to diabetes, and the other is macroangiopathy, which is similar to non-diabetic atherosclerosis (3). Microvessels are the most widely distributed blood vessel connecting the arterioles and venules. Although their largest diameter is only 100  $\mu$ m, their wall is highly permeable, facilitating a sufficient material exchange between blood and tissues (19). Myocardial tissue has an extremely rich capillary network. The microcirculation of the heart is composed of blood circulation between its arterioles and venules, which can affect myocardial perfusion (20).

At present, coronary angiography and percutaneous coronary intervention are preferable in the clinical treatment of macrovascular diseases. However, completely effective treatments for microvascular diseases have not been found, which leads to clinical presence of cardiac patients with persisting and progressing symptoms, although they have received stan-

dard care. Therefore, more researches should focus on microangiopathy.

CMECs contribute to the pathogenesis of cardiac microvascular diseases and have different specific functions compared with other vascular endothelial cells. Researches have supported the involvement of AGEs in the pathogenesis of diabetic vascular complications (10, 21). However, the effects of AGEs on CMECs, especially the relationship with the PKC $\beta$  signaling pathway, remain unclear.

Some studies showed that AGEs inhibited proliferation and promoted apoptosis in endothelial progenitor cells (22), mesangial cells (23), and acute myeloid leukemia cells (24), and others found that the activation of RAGE facilitated proliferation and reduced apoptosis in pulmonary artery smooth muscle cells (25). Those controversial outcomes suggested that AGEs might selectively act on several types of cells under different pathophysiological conditions. In the present study, we found that AGEs facilitated proliferation and reduced early apoptosis of CMECs, and all these effects could be reversed by PKC $\beta$ 1/2 inhibitors. We subsequently confirmed that PKC could also in-



**Figure 5.** Effect of AGEs on PKC (a) Effects of AGEs on RAGE protein expression. Protein levels were assessed using the western blot. AGEs upregulates the RAGE expression. The significance of differences is as indicated ( $*P<0.05$ ). (b) After siRNA transfections, the proliferation of CMECs is reduced by 58.1%, 54.5%, 26.9%, and 28% at 0.5, 1, 2, and 4 h respectively, compared with AGEs-treated cells ( $\#P<0.05$  vs. AGEs). (c) After siRNA transfections, the number of early apoptosis cells significantly increases versus AGEs-treated group ( $\#P<0.05$  vs. AGEs). (d, e, f) Phosphorylation of PKC $\beta$ 1, PKC $\beta$ 2 induced by AGEs in CMECs and relative PKC $\beta$ 1/2 phosphorylation levels after RAGE-siRNA transfections. AGEs enhance phosphorylation of PKC $\beta$ 1/2 ( $*P<0.05$  vs. control), and phosphorylation of PKC $\beta$ 1/2 is significantly downregulated by RAGE-siRNA transfection ( $\#P<0.05$  vs. AGEs). (g, h) Phosphorylation of PKC $\alpha$  in AGEs-treated CMECs and relative PKC $\alpha$  phosphorylation levels after RAGE-siRNA transfections. There is no significant difference in phosphorylation of PKC $\alpha$  ( $P>0.05$  vs. control)

independently regulate the phenotype of CMECs, indicating the AGEs may work on CMECs through PKC $\beta$ . Hence, we explored further and verified that AGEs indeed upregulated phosphorylation of PKC $\beta$ 1 and PKC $\beta$ 2, and RAGE-siRNA transfection reduced phosphorylation of PKC $\beta$ 1/2 and obstructed the effects of AGEs on CMECs.

PKC is a family of protein kinase enzymes involved in controlling the function of other proteins through the phosphorylation of hydroxyl groups of serine and threonine amino acid residues on these proteins. PKC enzymes are activated by signals such as increases in the diacylglycerol (DAG) concentration or calcium ions. Previous studies indicated that diabetes led to vas-

cular DAG accumulation and ensuing PKC activation, causing a variety of cardiovascular complications (26). It was reported that the PKC activation by hyperglycemia was likely to be responsible for specific vascular pathologies such as endothelial cells and smooth muscle cells dysfunction, extracellular matrix synthesis and fibrosis, monocyte activation, and vascular insulin dysfunction (11). Moreover, the PKC $\beta$  inhibition might represent a novel therapy in the prevention of diabetic cardiovascular complications through attenuating diastolic dysfunction, myocyte hypertrophy, collagen deposition, and preserving cardiac contractility (27, 28). It was also reported that the PKC $\beta$  inhibition reduced the cardiac microvascular barrier impairment (14). All the above findings supported the importance of PKC $\beta$  in myocardial cells and endotheliocyte.

As the cell cycle was related to proliferation, the effects of AGEs on cell cycle distribution were also examined. Results showed that AGEs accelerated the CMECs from G1 phase to the S+G2/M phase, which resulted in elevated DNA synthesis (29). At the same time, the expression of cyclin D1, which was the crucial checkpoint protein from G1 to S phase, was upregulated by AGEs. The result was reversed by PKC $\beta$ 1/2 inhibitors, which was in agreement with the effects of PMA/PKC. Thus, we inferred that AGEs promote the proliferation of CMECs through the activation of the PKC $\beta$  pathway and subsequent transcriptional activation of cyclin D1.

RAGE can be found on many cells, including endothelial cells, smooth muscle, and cells of the immune system. When binding with AGEs, it contributes to diabetes-related chronic inflammatory diseases and is associated with the progression of cardiovascular diseases and diabetes (30). The AGE/RAGE interaction observed in diabetes stimulates endothelial pro-inflammatory gene expression and reactive oxygen species (ROS) production in a nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase dependent manner (31), which might contribute to endothelial dysfunction. AGE/RAGE also plays a critical role in the chronic activation of the immune inflammatory processes that accelerate atherosclerosis in diabetes (32). In our study, we demonstrated that AGE/RAGE played a critical role in the CMECs proliferation and apoptosis in diabetic cardiomyopathy. By specific RAGE-siRNA transfection in CMECs, we successfully reduced the phosphorylation of PKC $\beta$ 1/2 and obstructed the process of CMECs' proliferation and apoptosis. These findings suggested that the AGEs-RAGE-PKC $\beta$  was one of the mechanisms in the pathophysiological process of CMECs' proliferation and apoptosis and provided a novel insight into the pathogenesis of diabetic cardiomyopathy.

PKC signaling pathways are activated in diabetic cardiomyopathy. To date,  $\approx$ 15 isoforms of PKC have been described in humans. PKC $\alpha$ ,  $\beta$ ,  $\epsilon$ ,  $\theta$ , and  $\delta$  isoforms have been proposed to be involved in the development of diabetic cardiac hypertrophy (5, 33, 34). PKC $\alpha$  is the predominant member among the different PKC isozymes expressed in the cardiac tissue (35-38). PKC $\alpha$  is increased in human cardiac pathology (36, 39) and during the

transition to heart failure (40). It plays crucial roles in cardiomyocyte hypertrophy, and it is an important regulator of muscle contractility (41, 42).

To exclude the possible contribution of other isoforms for the signaling mechanism, we also investigated PKC $\alpha$  in addition to PKC $\beta$ . It showed that AGEs upregulated the expression of RAGE and phosphorylated PKC $\beta$ 1 and PKC $\beta$ 2, but not phosphorylated PKC $\alpha$ . Although PKC $\alpha$  is involved in the development of diabetic cardiomyopathy, our results implied that AGEs facilitate proliferation and reduce early apoptosis of cardiac microvascular endothelial cell via PKC $\beta$ , but not the PKC $\alpha$  signaling pathway. Although AGEs do not act through PKC $\alpha$ , this does not affect other PKC $\alpha$  physiological effects in cardiomyopathy mentioned and discussed above.

### Study limitations

Although this research did not provide evidence in vivo to support the hypotheses, it is still pioneering, and we can clarify that AGEs can regulate CMEC function through the PKC $\beta$  signaling pathway in diabetes.

### Conclusion

Our findings demonstrated that AGEs facilitated proliferation and reduced early apoptosis of CMECs through the activation of the PKC $\beta$  signaling pathway, which might be related to the pathogenesis of diabetic cardiomyopathy.

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