

## Original Article

# Advanced glycation end products influence mitochondrial fusion-fission dynamics through RAGE in human aortic endothelial cells

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**Abstract:** Mitochondrial dynamics plays a critical role in maintaining healthy endothelial function, but whether the atherogenic advanced glycation end products (AGEs) can influence mitochondrial dynamics of endothelial cell remains unclear. AGE modified bovine serum albumin (AGE-BSA) was used as AGEs, primary human aortic endothelial cell line was multiplied, and divided into groups incubated with AGEs of different concentrations for different time. The expression of phosphatase and tensin homologue (PTEN)-induced putative kinase 1 (PINK1) was silenced with specific siRNA. Mitochondrial morphology of HAECs in each group was determined with transmission electron microscopy. Real time PCR method was used to detect the mRNA expression levels of mitochondrial dynamics regulatory genes mitofusin 1 (Mfn1), mitofusin 2 (Mfn2), optic atrophy 1 (Opa1), and dynamin-related protein 1 (Drp1) of HAECs, and western blot method was used to detect the protein expression levels of these regulatory genes. Specific antibody was used to block receptor for advanced glycation end products (RAGE). Treatment of different concentrations of AGEs, HAECs presented more granular mitochondrion, indicating AGEs promoted mitochondrial fission of HAECs remarkably. Silencing PINK1 induced mitochondrial fission in HAECs, and AGEs further promoted mitochondrial fragmentation in HAECs of PINK1 silenced. Different concentrations of AGEs down-regulated the mRNA and protein expression of mitochondrial pro-fusional genes Mfn1, Mfn2, Opa1, up-regulated the expression of mitochondrial pro-fissional gene Drp1, and both of the two phosphorylated Drp1 (p-ser-Drp1-616 and p-ser-Drp1-637) were increased. Time-dependent dynamic alterations of the expression levels of Mfn1, Mfn2, Opa1, and Drp1 were also found in HAECs stimulated with AGEs. Blocking RAGE with anti-RAGE inhibited AGEs induced mitochondrial fission and reversed AGEs induced expression changes of mitochondrial regulatory genes Drp1, Mfn1, Mfn2, and Opa1, indicating AGEs induced mitochondrial fission through RAGE in HAECs. In conclusion, AGEs may promote mitochondrial fission of HAECs through its receptor RAGE, silencing PINK1 induces mitochondrial fission, and AGEs further promote mitochondrial fragmentation in HAECs of PINK1 silenced. AGEs up-regulate the expression of mitochondrial pro-fissional gene Drp1 and down-regulate the expression of mitochondrial pro-fusional genes Mfn1, Mfn2, and Opa1 in HAECs.

**Keywords:** Advanced glycation end products, human aortic endothelial cells, mitochondrial dynamics, gene expression, fusion, fission, receptor for advanced glycation end products, mitophagy

## Introduction

The high prevalence of diabetes mellitus exerts increasing threats to the health of many populations all over the world. Patients with diabetes mellitus have significantly increased risk for vascular complications in comparison with individuals without diabetes mellitus [1]. Among the complications of diabetes, atherosclerotic cardiovascular disease (ASCVD) remains the principal cause of death and disability in pa-

tients with diabetes mellitus [2]. Although the mechanism of ASCVD in diabetes may be attributable to many processes, endothelial cell dysfunction plays an essential role in the pathogenesis of atherosclerosis and ASCVD [3].

Advanced glycation end products (AGEs) are the products of protein chronic non-enzymatic glycosylation, and increase in diabetes, chronic renal dysfunction, and old patients [4]. AGEs can elicit oxidative stress generation and sub-

sequently cause inflammatory and thrombotic reactions in various types of vascular cells via interaction with the receptor for AGEs (RAGE), thereby being involved in vascular complications of diabetes [5]. It has been demonstrated that AGEs play an important role in the pathogenesis of endothelial dysfunction and atherosclerosis [6]. AGEs can lead to endothelial cell apoptosis and generating more reactive oxygen species (ROS) [7]. As a result, the increment of ROS in endothelial cells is mainly attributable to the abnormality of mitochondrial electron transport chain and impairment of mitochondrial function [8].

Mitochondria is not just a energy plant, but can adapt to the change of energy requirement of cell by dynamic reconstructing and continuous fusion and fission of its framework, this dynamic changing process is called mitochondrial dynamics [9]. Mitochondrial dynamics is essential for maintaining a healthy mitochondrial network, including two opposite processes of fusion and fission which are under the fine regulation of mitochondrial fusion and fission proteins respectively. The fusion proteins include mitofusin 1 (Mfn1), mitofusin 2 (Mfn2), and optic atrophy 1 (Opa1) etc, while the fission process is mainly modulated by dynamin-related protein 1 (Drp1) and its adaptor proteins [10]. In addition, mitophagy (autophagy of mitochondria) also plays an indispensable role in maintaining mitochondrial homeostasis, mitophagy is mainly regulated by phosphatase and tensin homologue (PTEN)-induced putative kinase 1 (PINK1)/Parkin signaling pathway [11].

Recent researches demonstrated that perturbation of mitochondrial dynamics is closely related to the pathogenesis of endothelial dysfunction [12], diabetes [13], cardiovascular disease [14, 15], aging [16], neurodegenerative disease [17], and cancer [18]. However, whether the atherogenic AGEs can influence mitochondrial dynamics of endothelial cells, leading to endothelial dysfunction through this path, is not clear. Our study is to elucidate whether AGEs can influence mitochondrial dynamics in cultured human aortic endothelial cells (HAECs) in vitro, and the expression changes of the major genes involved in the regulation of mitochondrial fusion and fission processes Mfn1, Mfn2, Opa1, and Drp1 were also detected. We also detected the effect of AGEs on mitochondrial dynamics in HAECs of PINK1 the major

modulator of mitophagy is silenced. And specific antibody for receptor of AGEs (RAGE) was used to block RAGE signaling pathway to clarify the potential mechanism underlying AGEs induced changes of mitochondrial dynamics.

### Materials and methods

#### *Cell culture*

Primary human aortic endothelial cell (HAECs) line bought from Wuhan Pricells Biomedical Technology company (Wuhan, Hubei Province, China) were seeded in 1% gelatin-coated plastic plate containing special culture solution for HAECs and incubated at 37°C under a humidified 95% air and 5% CO<sub>2</sub> atmosphere. HAECs were multiplied and transferred to generations, and divided into groups for different purposes.

#### *siRNA transfection*

Silencing PINK1 expression was achieved by using specific siRNA bought from Genepharma Company (Shanghai, China). Transfection of HAECs was performed using LipofectAMINETM 2000 (Invitrogen, Grand Island, NY) according to the manufacturer's instruction. The interfering efficiency was verified with real-time PCR and Western blot methods.

#### *Blocking RAGE with specific antibody*

To block RAGE, a specific blocking anti-RAGE antibody bought from R&D Systems (Abingdon, UK) was used. HAECs were incubated with 20 µg/ml anti-RAGE antibody for 1 hour before treatment with AGEs in serum free media to block AGE-RAGE interaction.

#### *Treatment with AGEs*

AGE modified bovine serum albumin (AGE-BSA) bought from Millipore company (Billerica, MA, USA) was used as AGEs in our study, which was prepared by reacting BSA with glycoaldehyde under sterile conditions. For concentration dependent experiment, HAECs were divided into four groups treated with 0 mg/L (Control), 50 mg/L, 100 mg/L and 200 mg/L AGEs respectively. When HUVECs grew to about 80% confluence, all cell culture solutions of the four groups were discharged and changed, and control group was cultured with the special culture solution continuously, while in the left three groups, 50 mg/L, 100 mg/L and 200 mg/L

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AGEs was added in the cell culture solution respectively. Continued to culture for 24 hours, the cell culture solution in each group was discharged, and HAECs was collected for next experiment.

For time dependent experiment, HAECs were divided into five groups, including control group and other four groups which were incubated with 100 mg/L AGEs for 6 hours, 12 hours, 24 hours, and 48 hours respectively. When HAECs grew to about 80% confluence, all cell culture solutions of the five groups were discharged, cells of control group were immediately collected for next experiment, the left four intervention groups were then added 100 mg/L AGEs into cell culture solution to continue culturing for 6, 12, 24, and 48 hours respectively, cells then collected for next experiment.

### *Transmission electron microscopy imaging*

Each group of HAECs was fixed in 2.5% glutaraldehyde overnight. Subsequently, cells were dehydrated, infiltrated, and embedded in Epon 812 at 60°C for 48 hours. 120 nm ultrathin sections were stained with 4% uranyl acetate for 20 min, then with lead citrate for 5 min. Stained ultrathin sections were mounted on copper grids, and then examined using a TECNAI 10 Transmission electron microscopy (Philips, Amsterdam, Netherlands) to observe mitochondrial shape of HAECs and electron micrographs were recorded.

### *Real-time PCR detection*

High purity total RNA rapid extraction kit was bought from Generey Company (Shanghai, China). Total RNA was isolated from each group of HAECs using a standard protocol, and the synthesis of cDNA was performed using RevertAid First Strand cDNA synthesis Kit bought from Thermo Fisher Scientific Company (Waltham, MA, USA). Real time PCR reaction system was IQ SYBR Green Supermix (Tiangen, Beijing, China) 10 µL system, containing 10 µM/L forward primer 1 µL, 10 µM/L reverse primer 1 µL, cDNA 8 µL. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control. The sequences of primers were as follows: Mfn1, 5'-AGACTGAGCTGGACACCCATG-3' (forward) and 5'-TTAGGATTCTTCATTGCTTGAAGGTAGA-3' (reverse); Mfn2, 5'-GCATCTTCTTTGTGTCTGCT-3' (forward) and 5'-

CTCCTCAAATCTCCTCTCAA-3' (reverse); Opa1, 5'-TCTTTAGTCAAACACAGCTC-3' (forward) and 5'-TGGGGTCGTTGAAGCTTTAA-3' (reverse); Drp1, 5'-CAAGACAGTGTGCCAAAGGCAGT-3' (forward) and 5'-TCACCAAAGATGA GTCTCCCGG AT-3' (reverse); PINK1, 5'-CAT CTAAGCCTCTGGGGTG-3' (forward) and 5'-TCACAGGGCTGCCCTCCAT-3' (reverse); GAPDH, 5'-CGGG AACTGTGGCGTG-ATGG-3' (forward) and 5'-CCTCTTCAAGGGGTC-TACATGG-3' (reverse).

### *Western blot detection*

Total protein concentrations of the cytosolic extracts were determined using a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Proteins were separated in SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked in 5% nonfat dry milk and probed with primary antibodies 1:1000 dilution overnight. GAPDH was used for the cytosolic loading control. Primary antibodies included anti-GAPDH from Bioworld (St. Louis PARK, MN, USA), anti-Mfn1, anti-Mfn2, anti-Drp1, anti-p-Drp1-616 and anti-p-Drp1-637 from CST (Danvers, MA, USA), anti-Opa1 and anti-PINK1 from Abcam (Cambridge, UK).

### *Statistical analysis*

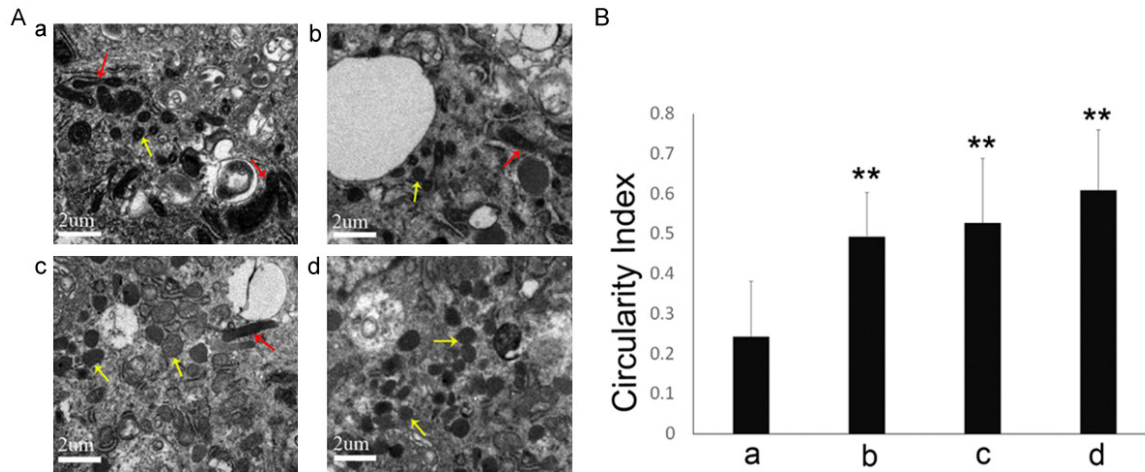
The gray scale of Western blot images was analyzed semi-quantitatively with image analysis software Image J 1.43. Circularity index detected and analyzed with Image J 1.43 was used as index to evaluate mitochondrial fragmentation. For multiple comparisons, data were first analyzed by one-way ANOVA on SPSS17.0, followed by pairwise comparisons with unpaired two-tailed Student's *t*-test on SPSS17.0. Graphs were produced with Excel or GraphPad Prism 6.02. Results are expressed as mean ± standard deviation of at least three independent experiments. Statistical significance is indicated by *P*<0.05.

## Results

### *AGEs promote mitochondrial fission in HAECs*

Transmission electron microscopy imaging showed that HAECs incubated with different concentrations of AGEs present more granular mitochondrion and less tubular mitochondrion (**Figure 1Ab-Ad**) in comparison to HAECs of control group (**Figure 1Aa**). Analysis with multi-

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**Figure 1.** Transmission electron microscopy (TEM) images of mitochondrion from control and AGEs-treated HAECs. The left part of the picture is representative TEM images of mitochondrion of HAECs, the right part is the corresponding bar chart of circularity index for each group. a: Control group; b: 50 mg/L AGEs intervention group; c: 100 mg/L AGEs intervention group; d: 200 mg/L AGEs intervention group. The magnification is  $\times 8300$ . The Scale bar presents 2  $\mu\text{m}$ . Red arrows indicate tubular mitochondrion, yellow arrows indicate granular mitochondrion. Data are from at least 30 mitochondrion for each control and AGEs-treated cells examined from three separate experiments. Data are expressed as Mean  $\pm$  SD, N=3; \*\* $P < 0.01$  versus control group.

measure ROI tool of Image J 1.43 demonstrated that mitochondrial circularity indexes of HAECs incubated with 50 mg/L, 100 mg/L and 200 mg/L AGEs are all much higher than HAECs in control group ( $P < 0.01$  versus control group) (Figure 1B). These changes indicate that different concentrations of AGEs promote mitochondrial fission significantly in HAECs.

### AGEs down-regulate the expression of mitochondrial pro-fusional genes *Mfn1*, *Mfn2* and *Opa1*

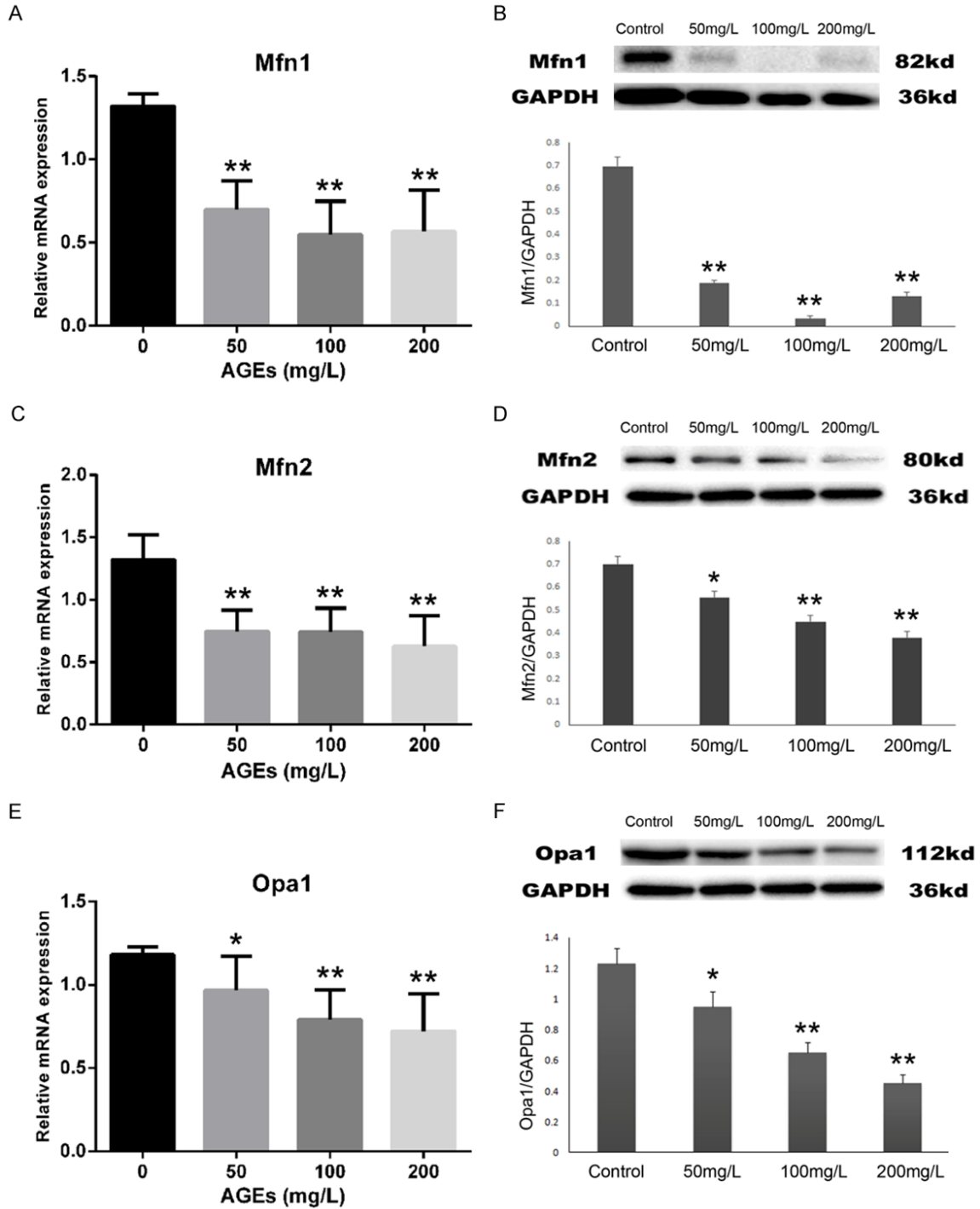
The fusion process of mitochondrial dynamics is mainly controlled by the pro-fusional *Mfn1*, *Mfn2*, and *Opa1*, so we determined whether AGEs can influence the expression of *Mfn1*, *Mfn2* and *Opa1* genes in HAECs. Both the mRNA and protein expression of these genes of HAECs treated with different concentrations of AGEs for different time were determined. We found that incubation with AGEs of 50 mg/L, 100 mg/L and 200 mg/L concentrations for 24 hours significantly down-regulated both the mRNA and protein expression levels of *Mfn1*, *Mfn2*, and *Opa1* ( $P < 0.05$  or  $P < 0.01$  versus control group, Figure 2). We also found that the mRNA and protein expression level of *Mfn1*, *Mfn2*, and *Opa1* down-regulated when incubation with 100 mg/L AGEs for 12 hours to 48 hours, but the expression level did not down-

regulated significantly when incubation for 6 hours (Figure 3).

### AGEs up-regulate the expression of mitochondrial pro-fissional gene *Drp1*

The fission process of mitochondrial dynamics is mainly modulated by *Drp1*, which is a GTPase mediating mitochondrial membrane fission through oligomerization into ring-like structures around the scission site to constrict and sever the mitochondrial membrane through a GTP hydrolysis-dependent mechanism. However, the pro-fissional role of *Drp1* depends on its translocation from cytoplasm to mitochondria, and it has been demonstrated that phosphorylation at 616 and 637 serine residues of *Drp1* controlling this translocation process. Phosphorylation at 616 serine residue of *Drp1* (p-Ser-616-*Drp1*) promotes *Drp1* translocation to mitochondrial membrane to play its pro-fissional role, while phosphorylation at 637 serine residue (p-Ser-637-*Drp1*) blocks *Drp1* translocation to mitochondrial membrane. In this study we observed whether AGEs can influence the expression of *Drp1* of HAECs, both the mRNA and protein expression changes of *Drp1* were detected in HAECs incubated with different concentrations of *Drp1* for different time. We also detected the protein concentrations of p-ser-616-*Drp1* and p-ser-637-*Drp1* in HAECs treated with AGEs.

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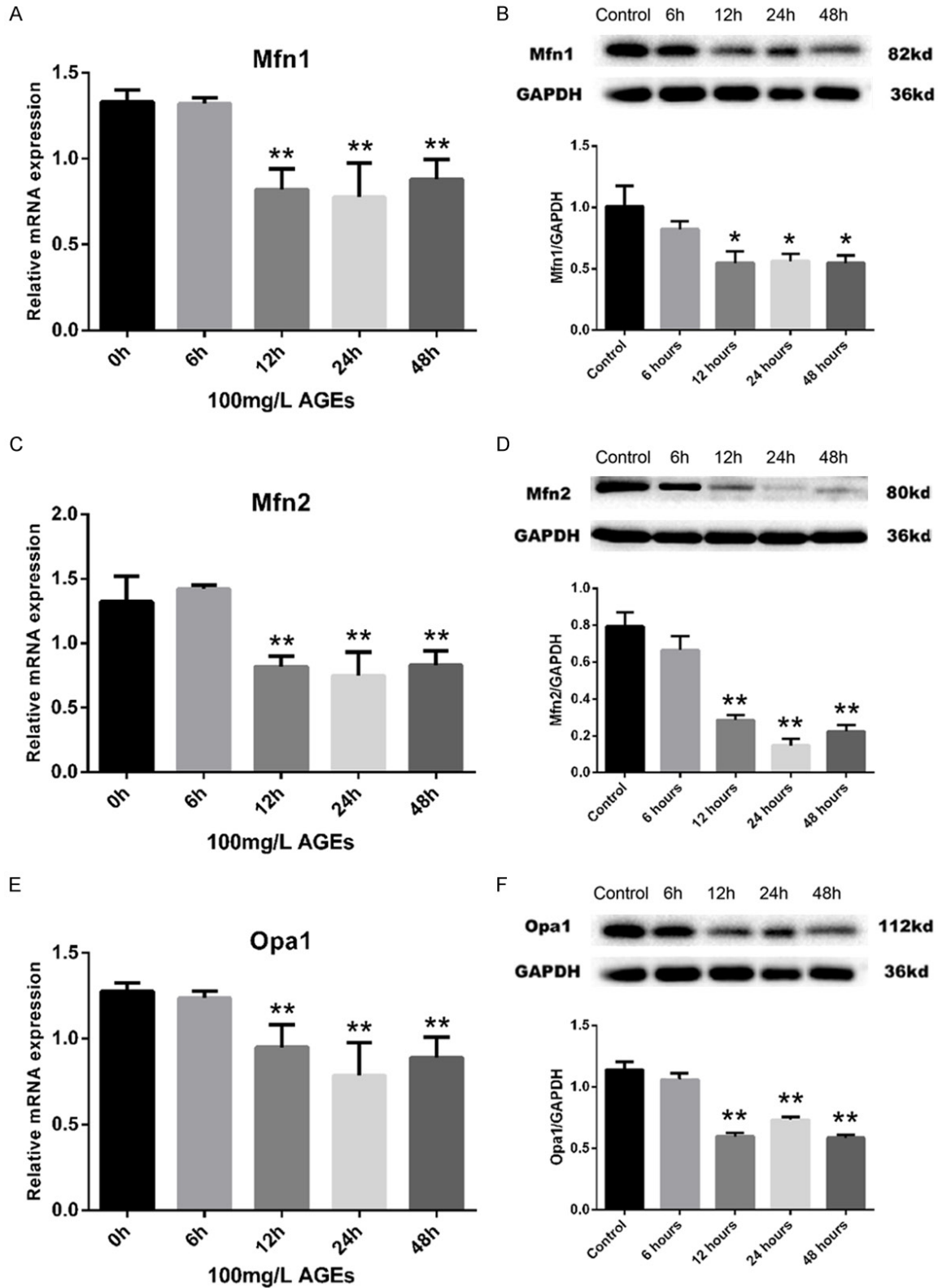


**Figure 2.** Different concentrations of AGEs down-regulated the expression of Mfn1, Mfn2, and Opa1. Different concentrations of AGEs down-regulated the mRNA and protein expression levels of Mfn1, Mfn2, and Opa1 genes. The amount of target mRNAs was normalized to respective internal standard GAPDH mRNA, relative fold was calculated based on the ratio of the normalized values of the cells incubated with AGEs to that without AGEs ( $2^{-\Delta\Delta Ct}$ ). GAPDH: glyceraldehyde-3-phosphate dehydrogenase. Data are expressed as Mean  $\pm$  SD, N=3; \* $P$ <0.05 versus control group, \*\* $P$ <0.01 versus control group.

We found that incubation with different concentrations of AGEs for different time all up-regu-

lated the mRNA and protein expression of Drp1 significantly ( $P$ <0.05 or  $P$ <0.01 versus control

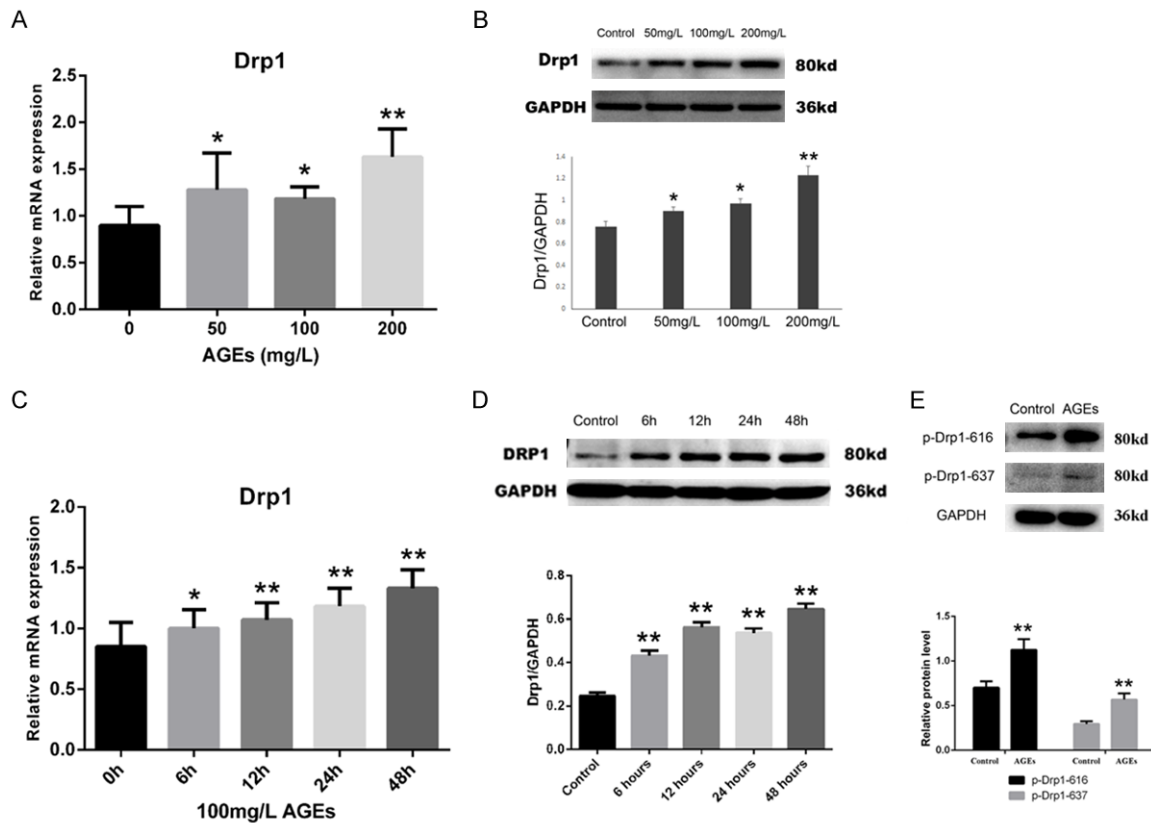
## AGEs influence mitochondrial dynamics



**Figure 3.** Time-dependent effects of AGEs on the expression of Mfn1, Mfn2, and Opa1. Incubation with 100 mg/L AGEs for 12 to 48 hours significantly down-regulated the mRNA and protein expression levels of Mfn1, Mfn2, and Opa1 genes, while incubation for 6 hours did not alter the expression of Mfn1, Mfn2, and Opa1 significantly. The amount of target mRNAs was normalized to respective internal standard GAPDH mRNA, relative fold was calculated

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based on the ratio of the normalized values of the cells incubated with AGEs to that without AGEs ( $2^{-\Delta\Delta Ct}$ ). GAPDH: glyceraldehyde-3-phosphate dehydrogenase. Data are expressed as Mean  $\pm$  SD, N=3; \* $P$ <0.05 versus control group, \*\* $P$ <0.01 versus control group.



**Figure 4.** AGEs up-regulate the expression of Drp1 in HAECs. Incubation with different concentrations of AGEs up-regulated the mRNA and protein expression levels of Drp1 gene significantly, both the two phosphorylated Drp1 p-Drp1-616 and p-Drp1-637 were up-regulated. The amount of target mRNAs was normalized to respective internal standard GAPDH mRNA, relative fold was calculated based on the ratio of the normalized values of the cells incubated with AGEs to that without AGEs ( $2^{-\Delta\Delta Ct}$ ). GAPDH: glyceraldehyde-3-phosphate dehydrogenase. Data are expressed as Mean  $\pm$  SD, N=3; \* $P$ <0.05 versus control group, \*\* $P$ <0.01 versus control group.

group, **Figure 4A-D**). We also found that both p-ser-616-Drp1 and p-ser-637-Drp1 protein concentrations of HAECs were increased when incubated with 100 mg/L AGEs for 24 hours (**Figure 4E**).

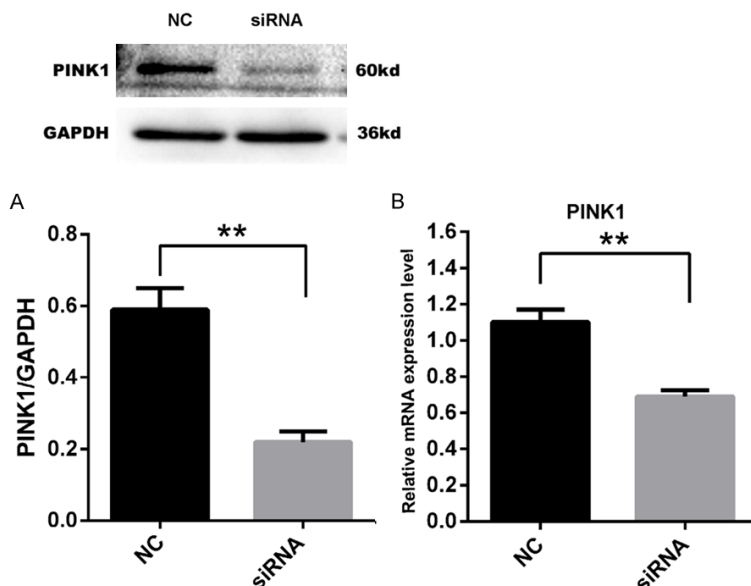
### *AGEs further promote mitochondrial fragmentation in HAECs of PINK1 knocked down*

PINK1-Parkin pathway is one of the major regulatory pathways for mitophagy (autophagy of mitochondria), while mitophagy plays a critical role in maintaining mitochondrial homeostasis. In our study, we observed how AGEs can influence mitochondrial dynamics of HAECs in a condition of mitophagy was blocked. We successfully silenced the expression of PINK1 in HAECs with specific siRNA for PINK1 which was

verified with real-time PCR (**Figure 5B**) and western blot (**Figure 5A**) methods. We found that silencing PINK1 promoted mitochondrial fission, and AGEs further promoted mitochondrial fission in HAECs of PINK1 gene silenced (**Figure 6**). These results further demonstrated the pro-fission role of AGEs for mitochondrial dynamics in HAECs.

### *Inhibition of RAGE prevents AGEs induced mitochondrial fission and reverses the expression changes of mitochondrial dynamics regulatory genes*

In order to clarify whether AGEs inducing mitochondrial fission of HAECs via its receptor RAGE, we inhibited RAGE activity with specific antibody for RAGE. We found that pre-incuba-



**Figure 5.** Silenced the expression of PINK1 in HAECs with specific siRNA. Transfection of specific siRNA for PINK1 down-regulated the mRNA and protein expression of PINK1 in HAECs remarkably, indicating PINK1 was successfully silenced. The amount of target mRNAs was normalized to respective internal standard GAPDH mRNA, relative fold was calculated based on the ratio of the normalized values of the cells in siRNA transfected group to NC group ( $2^{-\Delta\Delta Ct}$ ). GAPDH: glyceraldehyde-3-phosphate dehydrogenase. NC: negative control. Data are expressed as Mean  $\pm$  SD, N=3; \*\* $P < 0.01$  versus NC group.

tion with antibody of RAGE clearly blocked AGEs induced mitochondrial fission in HAECs (**Figure 7**). We then detected the protein expression level of Mfn1, Mfn2, Opa1, and Drp1 in HAECs treated with both anti-RAGE antibody and AGEs, and compared with that of HAECs treated only with AGEs and control group. We observed that pre-incubation of anti-RAGE significantly reversed AGEs-induced up-regulation of Drp1 (**Figure 8A**) and down-regulation of Mfn1, Mfn2, and Opa1 (**Figure 8B-D**). These results indicate that AGEs induce mitochondrial fission of HAECs through RAGE signaling pathway.

## Discussion

Atherosclerosis as one of the major cardiovascular complications of diabetes threatens people's health seriously, its pathogenesis is related to many factors, of which endothelial dysfunction plays a critical role, especially in diabetes patients [3]. Improving endothelial function can prevent the progress of atherosclerosis, and correspondingly ameliorate symptoms of atherosclerosis related diseases. So improv-

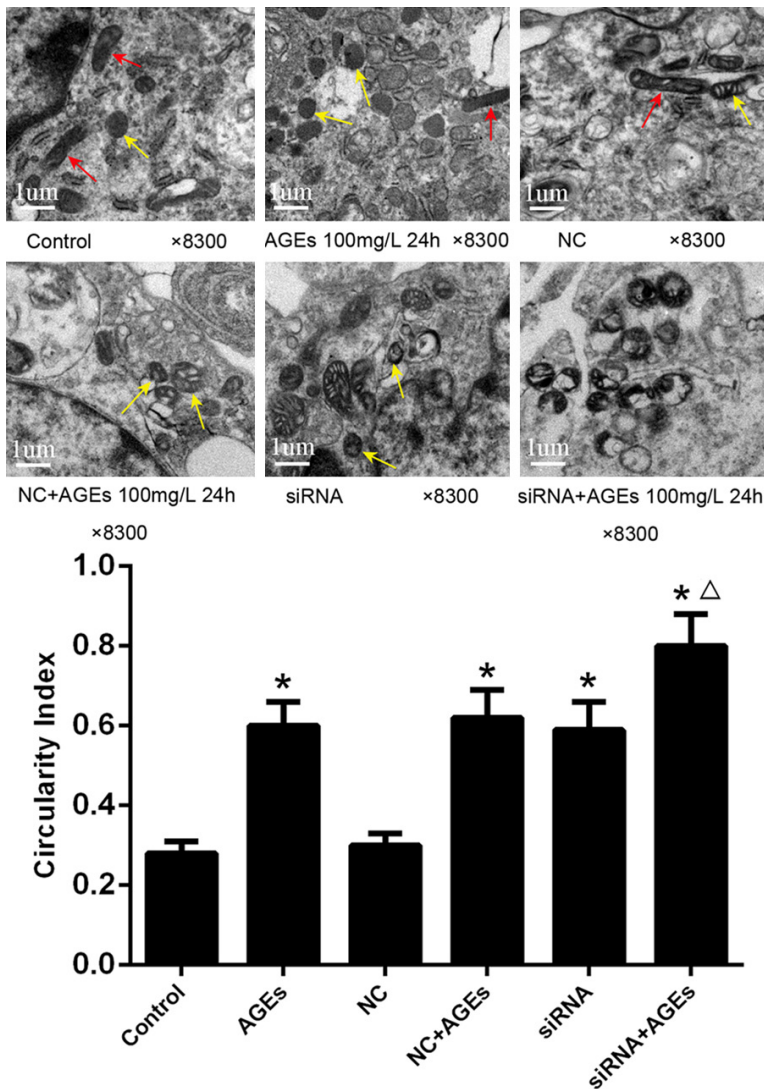
ing endothelial function is one of the major targets to prevent and cure atherosclerosis [19]. Recent studies found that healthy endothelial cell function is closely related to a balanced mitochondrial dynamics, abnormality of mitochondrial dynamics leads to endothelial dysfunction, and then further promotes the initiation and progression of atherosclerosis [12, 20]. In fact, the important role of mitochondrial dynamics in the pathogenesis of human diseases is increasingly arousing people's attention [21].

On the other hand, it has been demonstrated that AGEs has a tight link with the mechanism of atherosclerosis, it's a key causative agent for cardiovascular complication of diabetes, causing endothelial dysfunction of blood vessel [22]. There is accumulating evidences that AGEs elicit oxidative stress generation and subsequently evoke inflammatory and thrombogenic reactions in a variety of cells through the interaction with the receptor of AGEs (RAGE), thereby being involved in vascular complications of diabetes [5, 23]. In addition, one of our previous studies [24] indicated that AGEs can promote autophagy of myocardium cells, further demonstrating the importance of AGEs in the pathogenesis of diabetic cardiovascular complications.

However, whether AGEs inducing endothelial dysfunction is through influencing mitochondrial dynamics of endothelial cells remained elusive. Considering the essential role of balanced mitochondrial dynamics in maintaining normal cell function, undoubtedly this is a target worthy of studying. Mitochondrial dynamics is a dynamically balanced process of fusion and fission, in normal condition fusion and fission maintain a balance, mitochondria presents a shape between tubular and granular shape. However, in pathological situation when fusion increased, mitochondria presents a long tubular shape, whereas fission increased, mitochondria presents a fragmented granular shape.



## AGEs influence mitochondrial dynamics



**Figure 6.** AGEs further promote mitochondrial fission in HAECs of PINK1 silenced. Silencing PINK1 induced mitochondrial fission in HAECs, and AGEs further promoted mitochondrial fission in HAECs of PINK1 silenced. The upper part of the picture is representative TEM images of mitochondrion of HAECs, the lower part is the corresponding bar chart of circularity index for each group. The magnification is  $\times 8300$ . Red arrows indicate tubular mitochondrion, yellow arrows indicate granular mitochondrion. The Scale bar presents 1  $\mu\text{m}$ . NC: negative control. Data are from at least 30 mitochondrion for each group of cells examined from three separate experiments. Data are expressed as Mean  $\pm$  SD, N=3; \* $P < 0.01$  versus control group; \* $P < 0.01$  versus siRNA transfection group.

Mitochondrial dynamics is a adaptable process which is coordinated by pro-fusional and pro-fissional modulators, the former include the three large GTPases of the dynamin superfamily Mfn1, Mfn2, and Opa1, the latter include Drp1 and its' several adaptor proteins [25, 26].

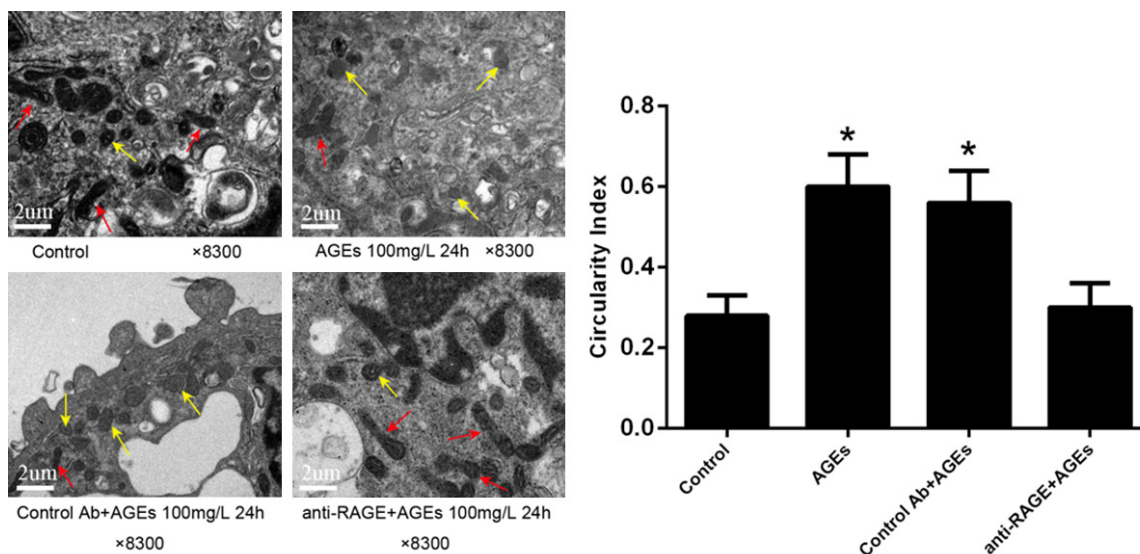
Our study showed that incubation with different concentrations of AGEs, mitochondrion of HA-

ECs presented a much more granular shape as demonstrated by increased circularity index of mitochondrion under transmission electron microscopy, indicating mitochondrial dynamics of HAECs was perturbed, with a increased fission process happened. Apart from mitochondrial dynamics, mitophagy also plays a critical role in maintaining normal mitochondrial homeostasis. PINK1-Parkin pathway is one of the major regulatory pathways for mitophagy, we found that silencing PINK1 induced mitochondrial fission, and AGEs further promoted mitochondrial fragmentation in HAECs of PINK1 silenced.

As mentioned above, mitochondrial dynamics is a dynamically balanced process of fusion and fission, and many proteins regulate this process precisely. Thus, in our study the mRNA and protein expression changes of these mitochondrial dynamics regulatory genes were detected. We found that incubation with AGEs of different concentrations down-regulated the mRNA and protein expression of the pro-fusional genes Mfn1, Mfn2 and Opa1 in cultured HAECs, whereas up-regulated the pro-fissional gene Drp1. And both the two phosphorylated Drp1 forms p-Drp1-616 and p-Drp1-637 were increased in HAECs incubated with AGEs. Meanwhile, time-dependent dynamic alterations of the mRNA and

protein expression of these genes were also found in HAECs incubated with AGEs. As mentioned above, mitochondrial dynamics is modulated by the pro-fusional Mfn1, Mfn2, Opa1 and the pro-fissional Drp1, so it is reasonable that the decreased expression of Mfn1, Mfn2, Opa1 and increased expression of Drp1 are combined to induce mitochondrial fission. Mitophagy is to maintain mitochondrial homeostasis

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**Figure 7.** Pre-incubation with anti-RAGE antibody blocked AGEs induced mitochondrial fission in HAECs. Pre-incubation with specific anti-RAGE antibody prevented AGEs induced mitochondrial fission in HAECs. The left part of the picture is representative TEM images of mitochondrion of HAECs, the right part is the corresponding bar chart of circularity index for each group. The magnification is  $\times 8300$ . Red arrows indicate tubular mitochondrion, yellow arrows indicate granular mitochondrion. The Scale bar presents 2  $\mu\text{m}$ . Data are from at least 30 mitochondrion for each group of cells examined from three separate experiments. Data are expressed as Mean  $\pm$  SD, N=3; \* $P < 0.01$  versus control group.

by clearing fragmented and impaired mitochondrion, so it is reasonable to found that blocking mitophagy by silencing PINK1 induced mitochondrial fission and AGEs further increased mitochondrial fragmentation in HAECs.

In order to clarify the underlying mechanism of AGEs-induced mitochondrial fission, RAGE the receptor of AGEs was blocked with specific anti-RAGE antibody, and we found that blocking RAGE prevented AGEs induced mitochondrial fission and reversed up-regulation of Drp1 expression and down-regulation of Mfn1, Mfn2, and Opa1 expression, indicating AGEs promote mitochondrial fission through its receptor RAGE.

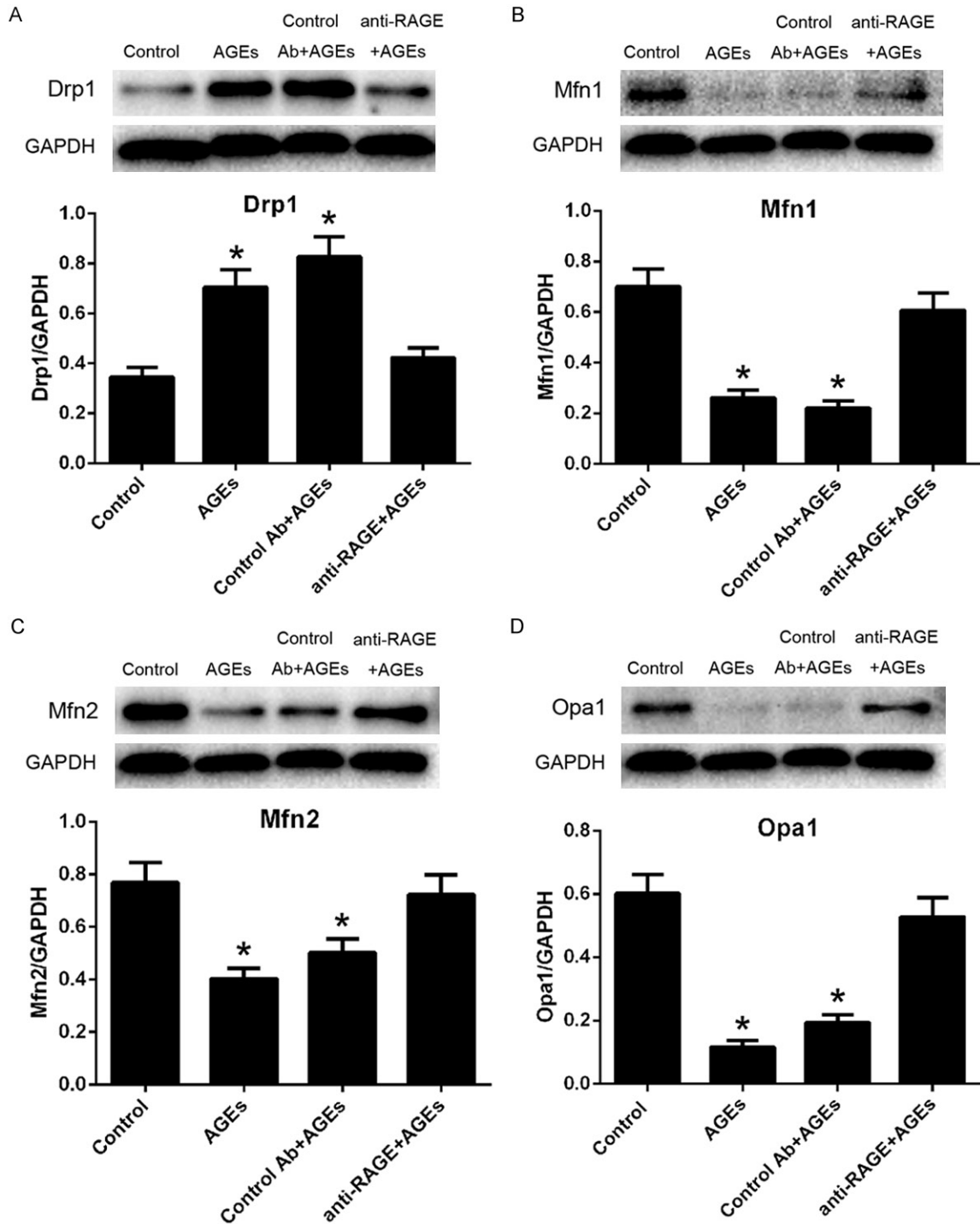
It is generally accepted that mitochondrial fusion is beneficial to maintain normal mitochondrial and cell function, whereas mitochondrial fission is detrimental. For example, overexpression of Mitofusin 2 promoting mitochondrial fusion inhibit oxidized low-density lipoprotein induced vascular smooth muscle cell proliferation and reduced atherosclerotic lesion formation in rabbit [27], inhibiting mitochondrial fission protects the heart against ischemia/reperfusion injury [28], and mitochondrial fission

inhibitor Mdivi can ameliorate pressure overload induced heart failure [29], while loss of mitofusin 2 promotes endoplasmic reticulum stress [30]. Our study found that AGEs can promote mitochondrial fission of HAECs by down-regulating the expression of Mfn1, Mfn2, Opa1 and up-regulating Drp1, indicating AGEs may induce endothelial cell dysfunction through disturbing balance of mitochondrial dynamics.

It is reported that increased production of reactive oxygen species in hyperglycemic conditions requires dynamic change of mitochondrial morphology [31], indicating mitochondrial dynamics plays an indispensable role in maintaining intracellular homeostasis and normal cellular function. It is reasonable that our study provided a new mechanism other than eliciting oxidative stress generation for AGEs induced endothelial cell dysfunction.

In conclusion, our study indicates that AGEs can influence mitochondrial dynamics in cultured human aortic endothelial cells through its receptor RAGE, promoting this dynamic balance of fusion and fission tilting to fission, with a significant increment of mitochondrial fission. This effect of AGEs may be attributable to its

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**Figure 8.** Pre-incubation with anti-RAGE antibody abolished AGEs-induced up-regulation of Drp1 and down-regulation of Mfn1, Mfn2, and Opa1. Pre-incubation with anti-RAGE antibody prevented AGEs induced up-regulation of protein expression of Drp1 (A) and down-regulation of protein expression of Mfn1, Mfn2, and Opa1 (B-D). GAPDH: glyceraldehyde-3-phosphate dehydrogenase. Data are expressed as Mean ± SD, N=3; \*P<0.01 versus control group.

role of up-regulating the expression of pro-fusional Drp1, down-regulating the expression of pro-fusional Mfn1, Mfn2, and Opa1. Considering

the high prevalence of diabetes and its cardiovascular complications, and the pivotal role of AGEs in the pathogenesis of these complica-

tions, there is no doubt that our study provides some valuable insights in coping with cardiovascular complications of diabetes.

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### Disclosure of conflict of interest

None.

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