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The role of autophagy in advanced glycation end product-induced proliferation and migration in rat vascular smooth muscle cells

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ABSTRACT

Objective(s): To investigate the role of autophagy in advanced glycation end products (AGEs)-induced proliferation and migration in rat vascular smooth muscle cells (VSMCs).

Materials and Methods: After culture, VSMCs were treated with 0, 1, 10, and 100 μg/ml concentrations of AGEs. Autophagy specific protein light chain 3 (LC3)-I/II was determined by western blotting, autophagosomes were observed with electron microscopy, cell proliferation was quantified using the methyl thiazolyl tetrazolium (MTT) assay, and cell migration was evaluated using Transwell migration and scratch assays.

Results: Compared to the control group, the level of LC3- II/I in AGEs treatment group was up-regulated, and the number of autophagosomes was also increased. Furthermore, in concentration of 100 μg/ml AGEs, the extent of proliferation and migration was significantly increased compared to the control group. However, pretreating cells with autophagy inhibitor 3-MA could attenuate these effects.

Conclusion: Our study demonstrated that AGEs-induced autophagy accelerated AGEs-stimulated proliferation and migration in VSMCs.

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Introduction

Advanced glycation end products (AGEs) have been observed in a series of pathological conditions, particularly in type 2 diabetes (1). AGEs come through intracellular and extracellular spaces as a result of the maillard reaction (2). Following binding to the receptor for AGEs (RAGEs), a series of events occur leading to the proliferation and migration of vascular smooth muscle cells (VSMCs), including enhanced reactive oxygen species (ROS) production, up-regulated cytokines or growth factors, and decreased nitric oxide bioavailability, which accelerate atherosclerosis in a crowd of type 2 diabetic patients with or without coronary heart disease (3-5).

It is well known that autophagy is an evolutionarily driven process that includes degrading damaged cytoplasmic organelles and long lived proteins (6). The process is important for maintaining energy balance by recycling cellular products at critical times. When autophagy is induced, the expression of the autophagy marker light chain 3-II (LC3-II) and the ratio of LC3-II to LC3-I increases notably, whereas sequestosome 1 (SQSTM1/p62) decreases (7). Autophagy is known to be involved in the pathologic progression of several cardiovascular diseases, including atherosclerosis (8), cardiomyopathy (9), cardiac ischemia/reperfusion (10), and heart failure (11). Autophagic vacuolization and expression of the autophagy marker LC3-II have been discovered during the progression of atherosclerosis (12), which may be due to oxidative stress, endoplasmic reticulum (ER) stress, hypoxia, and inflammation. It has been shown that the inhibition of autophagy enhances plaque necrosis and NADPH oxidase-mediated oxidative stress, which indicates that autophagy plays a protective role in advanced atherosclerosis (9).

In accordance with previous findings, we demonstrated that AGEs could induce autophagy through the Akt and ERK signaling pathways in VSMCs (13). However, the role of autophagy in AGEs-induced proliferation and migration has not yet been fully elucidated. The objective of this study was to evaluate the influence of autophagy on the AGEs-induced migration and proliferation of VSMCs.

Materials and Methods

Materials

Monoclonal rabbit antibodies, including an-ti-SQSTM1/p62 and Beclin-1 were obtained from Cell Signaling Technology (MA, USA). The dilution ratio was 1:1000 (5 μl: 5 ml). A polyclonal rabbit anti-LC3 antibody was obtained from Sigma (St Louis, MO, USA). The dilution ratio was 1:3000 (1.7 μl: 5.1 ml). Horseradish peroxidase (HRP)-marked anti-GAPDH antibody was purchased from Kangchen (Shanghai, China). The dilution ratio was 1:1000 (5 μl: 5 ml).

Preparation of AGEs

AGEs were prepared according to the reported methods in previous literature (14). For this purpose, 50 mg/ml bovine serum albumin (BSA) was incubated with glucose generated in non-enzymtic glyction in the

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dark at 37 ˚C for 16 weeks. Under the same condition, glycoprival was incubated and acted as the control group. The unincorporated sugars were removed by dialyzing against PBS after incubation. Limulus amebocyte lysate assay (Sigma, St. Louis, MO, USA) was used to evaluate the level of endotoxin.

Western blot analysis

Cells were obtained from every group of VSMCs after different treatments were administered and then solubilized on ice in an ice-cold radio immunoprecipitation assay (RIPA) buffer for 30 min. Total protein concentrations were assessed with a BCA Protein Assay kit. (Thermo Scientific, Rockford, IL, USA). Then, 30–50 μg of denatured total protein was separated by 15% and 10% SDS-PAGE and transferred to PVDF membranes. Non-specific binding sites were blocked with 5% non-fat milk in a Tris-buffered solution (10 mM Tris-HCl [pH 7.6], 100 mM NaCl and 0.1% Tween 20) for 1 hr at room temperature. The membranes were incubated with the primary antibody at 4 ˚C overnight. The membranes were washed for three times, and then incubated with HRP -conjugated secondary antibodies for 2 hr at 37 ˚C. Finally, the membranes were detected by enhanced chemiluminescence (ECL) reagents (Amersham, Haemek, Israel) and scanned by Image Quant LAS (Bioscience, Buchinghamshire, UK). The grayscale value was determined using imaging software J2x (Rawak Software, Germany).

Cell viability assay

Rat A7R5 VSMCs were briefly incubated at 4,000 cells in triplicate in 96-well plates and left to adhere overnight. After incubation with fresh medium containing 3-Methyladenine (3-MA; 2 mM) or AGEs (100 μg/ml) for 48 hr, 10 μl of MTT solution (5 mg/ml) was added, and the cells were further cultured for 4 hr at 37 ˚C overnight. The medium was then discarded, and 150 μl of DMSO was added to each well. The absorbance at 450 nm was measured with a microplate reader.

Electron microscopy

Rat A7R5 VSMCs were treated with 100 μg/ml AGEs for 6 hr, and a 1 mm^3 tissue block was taken. After being fixed in 2.5% glutaraldehyde and 1% osmium acid, in sequence, the tissue blocks were processed with a graded series of ethyl alcohol and embedded in EPON618. After that, 70 nm thin slices were prepared, laid on copper mesh, and then stained with heavy metals, uranyl acetate, and lead citrate. The ultrastructures of the rat hippocampus, especially synaptic structure and autophagic vacuoles, were observed and photographed using transmission electron microscopy (TEM; H7650, Hitachi, Japan).

In vitro scratch wound assay

Rat A7R5 VSMCs were grown to 90% confluence on 6-well plates. The confluent monolayer was scratched with a 200 μl sterile pipette tip. The scratch wound was incubated with serum-free DMEM containing the indicated chemical(s) and AGEs for 24 hr. After incubation, the area of the cells that had migrated across the denuded area was evaluated by measuring the area of the scratch wound at 0 and 24 hr. The result was expressed as the percentage of recolonization in the denuded zone.

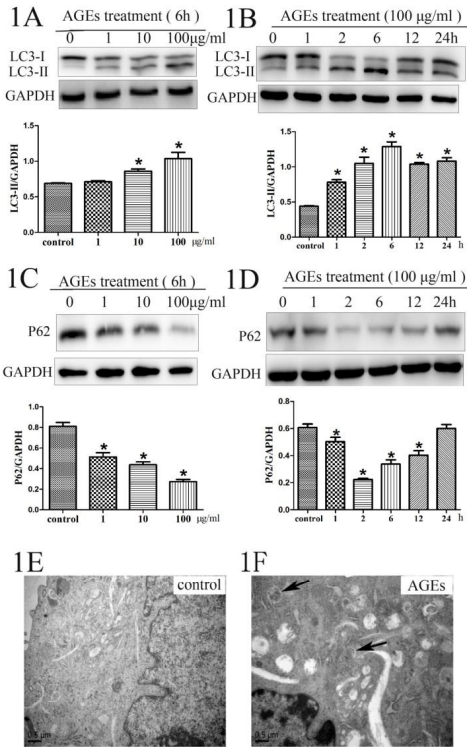

Figure 1. (1B&1D) After treatment with advanced glycation end products (AGEs), western blot analysis of sequestosome 1 (SQSTM1/P62) and light chain 3-II (LC3-II) protein levels. Treating with 100 μg/ml AGEs on vascular smooth muscle cells (VSMCs) for 0, 1, 2, 6, 12, and 24 hr. (1A & 1C) Cells were treated for 6 hr with 0, 1, 10, and 100 μg/ml AGEs. (1E&1F) Representative electron micrographs of the VSMCs treated with 100 μg/ml AGEs for 6 hr. Typical autophagosomes were found easily in the cells treated with AGEs, rather than in the control group. **P*<0.05 vs. control

Transwell migration assay

Cell migration was evaluated using a 24-well Transwell plate that contained polycarbonate 8.0-μm pore membrane filter inserts. The cultured VSMCs were trypsinized and suspended in the upper wells (1×10^5 cells in 200 μl of serum-free DMEM containing 1% FBS), whereas the lower wells were filled with DMEM containing 10% FBS. The AGEs and experimental drugs were added to the upper wells. The cells were allowed to migrate across the porous filters at 37 ˚C in 5% CO_2 for 24 hr. After being fixed with 4% formaldehyde (w/v) for 20 min and immersed into a crystal violet staining solution for 10 min, the cells that had migrated to the lower side of the filter were examined with microscopy. The number of migrated cells was then quantified using OD 560 nm measurement.

Statistical analysis

Data from at least three independent experiments were expressed as the mean ± SEM. A t-test was used to analyze the variance. The level of statistical significance was set as $P<0.05$.

Results

AGEs-induced autophagy was enhanced in rat A7R5 VSMCs

After treatment with 100 μg/ml AGEs for various time periods (0, 1, 2, 6, 12, and 24 hr), Western blotting was performed and the results showed that the expression of

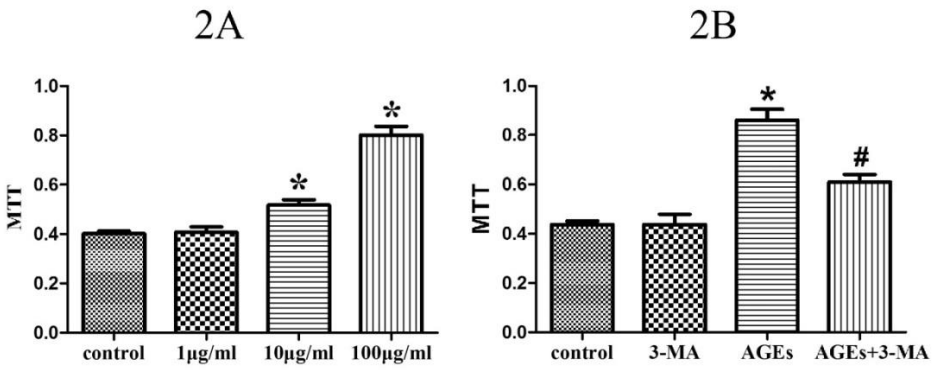

Figure 2. Comparing proliferation of the vascular smooth muscle cells (VSMCs) treated with advanced glycation end products (AGEs) (1, 10, and 100 μg/ml) (2A) and AGEs + 3-Methyladenine (3-MA) (2B) for 48 hr. Increased proliferation by AGEs was inhibited using a pretreatment with 3-MA. These results indicate that autophagy accelerated the AGE-induced proliferation of VSMCs. **P*<0.05 vs control; #*P*<0.05 vs. AGEs

autophagy-related proteins, LC3-II to LC3-I, was notably increased, peaking at 6 hr, whereas the expression of SQSTM1/p62 was decreased relatively, with a low peak at 6 hr (Figure 1B & 1D).Cells were also treated with AGEs at various concentrations (0, 1, 10 and 100 μg/ml) for 6 hr. The expression of LC3-II increased in a dose-dependent manner in the AGE-treated cells and the level of SQSTM1/P62 decreased in the same way (Figure 1A & 1C).

To visualize autophagy inducted in AGE-treated VSMCs directly, we used TEM to examine autophagosomes. We treated VSMCs cells with 100 μg/ml AGEs for 6 hr. Apparently, we found that autophagosome contained cellular material or membranous structures in the AGEs treatment group (Figure 1F). While, in the control group, autophagosomes were rarely detected (Figure 1E). The results demonstrated a dose-and time-dependent effect of treatment with AGEs on the expression levels of SQSTM1/P62 and LC3-II.

Autophagy accelerated AGE-induced proliferation of VSMCs

To examine the relation between autophagy and AGEs-induced proliferation of VSMCs, we investigated the effect of AGEs on VSMC proliferation. The results showed that cells cultured with AGEs (100 μg/ml) showed increased proliferation over a 48 hr period (Figure 2A). However, pretreating cells with 3-MA (2 mmol/l), an autophagy inhibitor, for 30 min could inhibit the AGE-enhanced proliferation of VSMCs (Figure 2B). These results indicate that autophagy accelerated the AGE-induced proliferation of VSMCs.

AGEs accelerated the migration of VSMCs

The migration capacity of VSMCs was evaluated using Transwell migration and an *in vitro* scratch assay. As illustrated in Figure 3A, VSMCs were treated with AGEs at various concentrations (0, 1, 10, and 100 μg/ml) for 12 hr. A significantly larger number of cells migrated to the lower side of the porous membrane in the Transwell chambers in a dose-dependent manner under treatment with AGEs compared with the control group. This effect was also observed in the *in vitro* scratch assay (Figure. 3B). Incubation with AGEs at various concentrations (0, 1, 10, and 100 μg/ml) for 24 hr promoted the closure of a linear scratch compared with the control group, in a dose-dependent manner. The results showed that AGEs

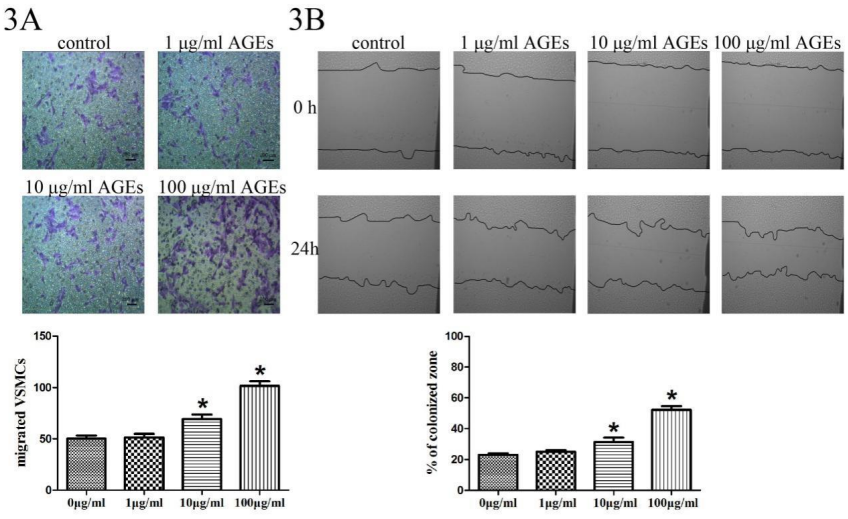

Figure 3. (3A) Cells were treated with 0, 1, 10, and 100 μg/ml advanced glycation end products (AGEs) for 12 hr. Transwell filters were stained with a crystal violet solution to visualize the migrated cells (×40). (3B) Cells were treated with 0, 1, 10, and 100 μg/ml AGEs for 24 hr. To quantify the migrated vascular smooth muscle cells (VSMCs) serving as benchmarkers, black spots were drawn to ensure the capture of the same zone throughout the experiments. The percentages of colonized zones were evaluated and used to compare the differences among the groups (×40).**P*<0.05 vs. control

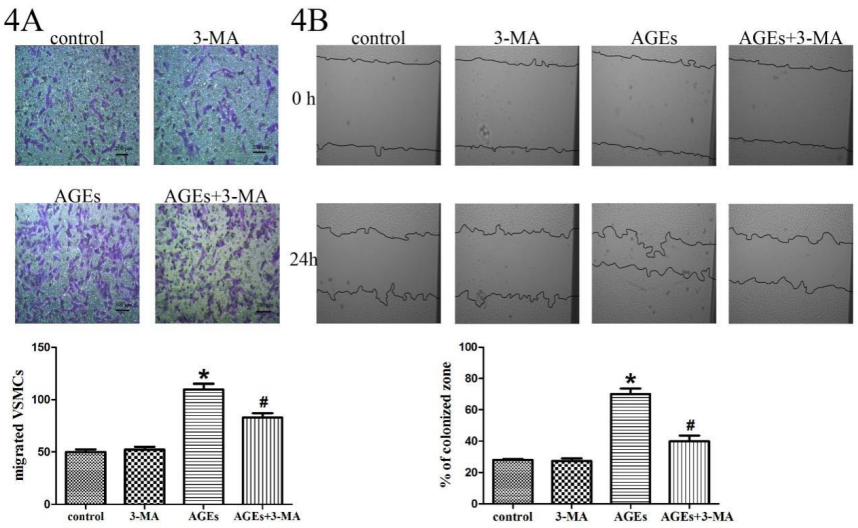

Figure 4. A comparison of the migration of the vascular smooth muscle cells (VSMCs) treated with 100 μg/ml advanced glycation end products (AGEs) for 12 hr (4A) and 100 μg/ml AGEs + 2 mmol/l 3-Methyladenine (3-MA) (4B) for 24 hr. Increased migration by AGEs was inhibited by a pretreatment with 3-MA. These results indicate that autophagy accelerated AGE-induced migration of VSMCs. **P*<0.05 vs control; #*P*<0.05 vs. AGEs

accelerated the migration of VSMCs.

Autophagy promoted AGEs-induced migration of VSMCs

To make sure whether autophagy is participated in AGEs-induced migration of VSMCs, we detected the effect of the 3-MA autophagy inhibitor on the migration of VSMCs cultured in AGEs. The VSMCs were pretreated with 3-MA (2 mmol/l) at the indicated concentrations for 30 min before treatment with AGEs (100 μg/ml). The autophagy inhibitor 3-MA abrogated the AGEs-enhanced migration of VSMCs (Figure 4A and 4B), indicating that autophagy promoted AGE-induced migration of VSMCs.

Discussion

Lots of studies have shown that the interactions between AGEs and RAGE induce the activation of the nuclear factor kappa B (NF-kB) signaling pathway and the production of ROS, leading to VSMC proliferation and migration, which is an important mechanism involved in the development of atherosclerotic lesions in diabetes mellitus (15, 16). Increasing evidences indicate that VSMCs are the major producers of extracellular matrix and they relax or contract to change the local blood pressure and the volume of the vessel (17). AGEs-induced proliferation and migration of VSMCs are important in the pathogenesis of atherosclerosis (18). Nevertheless, the relevant underlying mechanism has not yet been fully elucidated.

Autophagy is either a cellular defense mechanism or it is potentially deleterious. In different cells and under different conditions, autophagy may play contrasting roles in the course of apoptosis or pathophysiology of cardiovascular diseases. Autophagy could also be induced by ischemia or enhanced by reperfusion. Autophagy plays a protective role during the progress of ischemia, whereas it may have damaging effect during the reperfusion process (19). A study conducted by Zhang *et al*. showed that using lentivirus-mediated RNA interference knockdown of mTOR could delay the process of atherosclerosis and stabilize plaques by inhibition of macrophages number through autophagy in apoE deficient mice (20). Other previous studies have demonstrated that the activation of autophagy is involved in the process of AGE-related diabetic cardiovascular complications. AGEs can trigger autophagy in cardiomyocytes through the RAGE/PI3K/AKT/mTOR signaling pathway (21). Zhang and colleagues reported that apelin could inhibit migration and proliferation of rat pulmonary arterial smooth muscle cells (PASMCs) through activation of Akt/mTOR signaling and inhibition of autophagy under hypoxia (22). In addition, our previous study demonstrated that AGEs could induce autophagy in VSMCs (13). However, it is not clear whether autophagy could promote VSMCs migration and proliferation under AGEs pretreatment. Our present study showed that AGEs enhanced the migration and proliferation of VSMCs in a dose-dependent manner. Furthermore, we used 3-MA, an autophagy inhibitor, which could moderate AGEs-induced migration and proliferation of VSMCs. Hence, we conclude that AGEs induced autophagy and accelerated proliferation and migration of VSMCs stimulated by AGEs.

Conclusion

It now appears that the regulation of autophagy contributed to the improvement of the proliferation and migration of VSMCs resulting from damage caused by AGEs. Inhibition of autophagy may exert a protective effect on vasculopathy in patients with diabetes mellitus. The underlying molecular mechanisms through which autophagy regulates migration and proliferation of VSMCs are intricate, and further studies are needed to clarify underlying mechanisms.

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