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AGEs trigger autophagy in diabetic skin tissues and fibroblasts

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ABSTRACT

Objective: Accumulation of advanced glycation end products (AGEs) contributes to the development of diabetic ulcers. Recent evidence indicates that AGEs administration enhanced autophagy in many cell types. As a positive trigger of autophagy, the effect of AGEs on autophagy in skin tissues and fibroblasts remains unknown.

Methods: Skin tissues were isolated from Spreqne–Dawley rats and immunohistochemical staining was performed to analyze the location of LC3 and FOXO1 in skin tissues. Then primary cultured foreskin fibroblast cells with treated with AGEs and the effect of AGEs on autophagy was investigated. Protein level expressions of LC3, Beclin-1 and FOXO1 in fibroblasts were analyzed by Western blotting. Autophagic flux is detected with autophagy inhibitor chloroquine and mRFP-GFP-LC3 tandem construct.

Results: Compared with skin from normal rats, immunohistochemical staining shows a predominant LC3 localization in fibroblasts cytoplasm in diabetic rats. Elevated expression of FOXO1 also existed in diabetic rats dermis fibroblasts when compared with normal rats in immunohistochemical analysis. In human skin fibroblasts cells, AGEs administration stimulated the autophagy related LC3-II/LC3-I and Beclin-1 expressions and increased autophagy flux. In mRFP-GFP-LC3 puncta formation assays, both autolysosome and autophagosome were increased in human fibroblasts after treatment with AGEs. Fibroblasts exposed to AGEs also have increased FOXO1 expression compared with control group.

Conclusion: AGEs could induce autophagy at least in part via regulating the FOXO1 activity in diabetic skin tissues and fibroblasts.

1. Introduction

Diabetic ulcers represent a main public health, which is strongly associated with significant costs and increased risk of death [1]. Advanced glycation end products (AGEs) are derived from the nonenzymatic reaction of glucose and proteins. AGEs and its receptor RAGE plays a crucial role in diabetes [2,3]. Evidence suggests that excessive accumulation of AGEs contributes to the development of diabetic dermopathy and foot ulcers [4,5]. Our previous studies have also shown that AGEs play an important role in the pathogenesis of diabetic skin tissues and impairment of diabetic wound healing [6,7]. However, the exact molecular mechanism of AGEs on wound healing in diabetes remains unclear.

Recent evidence showed that AGEs is a positive trigger of autophagy. Except act as a pro-survival mechanism, autophagy is now considered to be a death execution mechanism and consequence of excessive autophagy would lead to cell function impairment and autophagic cell death (i.e. type II programmed cell death) [8–10]. Previous studies on the role of autophagy in the pathophysiologic of diabetes were emphasis on the autophagic process in β cells [11]. In addition to the data on the role of autophagy in islet β cells, there is growing evidence that autophagy can impact on the skeletal muscle and adipose tissue in diabetes.

The FOXO family is a subclass of Forkhead transcription factors characterized by a winged helix DNA-binding domain known as a Forkhead box [12]. FOXOs plays a pivotal role in triggering autophagy in a variety of cell types [13]. A current study shows that FOXO1-mediated autophagy is achieved via its nuclear accumulation and transcriptional activity activation, which leading to a dual effect on autophagy induction, one by promoting the expression of autophagy-related genes and the other by suppressing of

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mechanistic target of rapamycin (MTOR) [14]. More recently, AGEs was found to regulate FOXO1 and involved in the regulation of diabetic wound healing. In study by Zhang et al. [15], they found that FOXO1 has different role in normal and diabetic status, which could promote healing of wound in normal conditions but impede reepithelialization in diabetic mice. The results suggest that FOXO1 may be a potentially therapeutic target to treat diabetic wounds.

In total, AGEs administration enhanced autophagy in many cell types while suppression of its receptor RAGE markedly reduced the formation of autolysosome and improved the cell viability to AGEs exposure [16–18]. On these bases, we assumed that there were AGEs-mediated cell function impairment through activation of autophagy during conditions of diabetic wound healing, and FOXO1 may involve in this process. The present study was to explore the role of AGEs in autophagy in diabetic skin tissues and skin fibroblasts, and to investigate the preliminary molecular mechanisms responsible for the interplay between AGEs and autophagy.

2. Methods and materials

2.1. Cell culture and treatment

Human primary foreskin fibroblast cells, kindly provided by Dr. Cheng (Sun Yat-sen University, China), were cultured in DMEM (Gibco, USA) containing 10% fetal bovine serum (FBS, Hyclone, USA) in a CO_2 incubator (37 °C, 5% CO_2). After reaching 80% confluence, cells were incubated overnight in serum-free DMEM containing 0.5 mg/ml BSA (Sigma,USA) or AGEs-bovine serum albumin (AGEs-BSA, Merk Millipore) [19]. Fibroblasts were treated by 25, 50, 100 mg/L AGEs (AGEs group) or BAS only (control group) for 48 h.

2.2. Skin experiment and hematoxylin–eosin (HE) staining

Skin tissues were isolated from Spreqne–Dawley rats as our previously publication and introduced briefly as follows [20]. Spreqne–Dawley rats each were randomized into control and streptozotocin (STZ, Sigma, USA) injection group. STZ-treated rats were considered to be diabetic when the concentration of blood glucose is equal to or higher than 16.7 mmol/L. Skin tissues from the skull were obtained and fixed in 4% formaldehyde and then stained with hematoxylin and eosin to examine their morphology.

2.3. Immunohistochemistry

Immunohistochemical staining was performed to analyze the location of LC3, Beclin-1 and FOXO1 in skin tissues. Paraffin sections of skin tissues were detected using primary antibodies from Abcam LC3 antibody (1:1000); Beclin-1 antibody (1:200), and FOXO1 antibody (1: 500). The binding of the primary antibodies was revealed by biotin-labeled second antibody after washed away with PBS, and then detected with diaminobenzidine tetrahydrochloride (DAB) visualization. Positive staining appeared as brown. Controls for immune-specificity were included in all experiments and the primary antibody was replaced with PBS.

2.4. Western blotting

After washed twice with PBS, whole cell extracts were prepared by lysis buffer with PMSF to yield whole cell extracts. For western blot analysis, cell lysates were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. Antibodies to LC3, Beclin-1 and FOXO1 were from Abcam (LC3, 1:1000; Beclin-1, 1:200; FOXO1, 1:500). Primary antibodies were detected with goat anti-rabbit secondary antibodies (1:5000; Abgent). As an inhibitor of the lysosomal pH gradient, chloroquine diphosphate (Abcam, ab142116) was choice as a pharmacological inhibitor of autophagy. Immunoreactive bands were detected by enhanced chemoluminescence (ECL) technique using the Immobilon Western Chemiluminescent HRP Substrate (Merk Millipore) while values were corrected with the absorbency of the internal control (β-actin).

2.5. Tandem mRFP-GFP-LC3 fluorescence microscopy

Fibroblast cells were co-transfected with adenovirus expressing mRFP-GFP-LC3 (Hanbio, China) for 48 h, and then treated with different concentration of AGEs or only BSA, with or without chloroquine to inhibit autophagosome-lysosome fusion. The cells examined and analyzed by using a confocal microscope (Zeiss LSM510 Meta, Germany) and representative cells were photographed.

3. Results

3.1. AGEs induce autophagy in diabetic skin tissues

The results in human skin fibroblasts are consistent in vivo experiment. As shown in Fig. 1A, hematoxylin–eosin (HE) staining showed histological appearance of rat skin tissue. Fibroblasts staining with HE are sparse, separated by collagen bundles, and recognized by their oval nucleus (Black arrows). Compared with skin from normal rats, immunohistochemical staining shows a predominant fibroblasts cytoplasmic localization of LC3 in full-thickness dermis of diabetic rats with excessive AGEs accumulation (Fig. 1B & C).

3.2. AGEs induce autophagy in human skin fibroblast cells

3.2.1. AGEs increase LC3 and Beclin-1 expressions and autophagy flux level

Human skin fibroblasts were treated with AGEs and autophagy was investigated. LC3 shows two bands as LC3-II and LC3-I, respectively. The relative expression of LC3-II to LC3- I ratio (LC3-II/LC3-I) and expression of Beclin-1 on western blot assay is used to evaluate the degree of autophagy. Our results showed that the AGEs administration stimulated the expressions of LC3-II/LC3-I and Beclin-1 in dose-dependent manners in human skin fibroblasts cells (Fig. 2A.). Autophagy flux was further determined by chloroquine, which is an inhibitor that blocks autophagosome and lysosome fusion. As shown in Fig. 2B., AGEs administration led to further increase of LC3-II/LC3-I in the presence of chloroquine, suggesting that AGEs increases autophagy flux level.

3.2.2. Visualized mRFP-GFP-LC3 puncta formation assays

Autophagic flux is also traced with an mRFP-GFP-LC3 tandem construct. The mRFP (red) and GFP (green) signals of tandem fluorescent LC3 (mRFP-GFP-LC3) show different localization patterns. Autophagosomes and autolysosomes are labeled with yellow (mRFP and GFP) and red (only mRFP) signals, respectively. When autophagic flux is increased, both autophagosomes (LC3-labeled yellow puncta) and autolysosomes (LC3-labeled red puncta) are increased. When autophagosome-lysosome fusion into autolysosomes is blocked, only autophagosomes (LC3-labeled yellow puncta) are increased. At present, compared with BSA administration group, both autolysosome (LC3-labeled red puncta) and autophagosome (LC3-labeled yellow puncta) were increased in fibroblasts after treatment with AGEs. However, compared with BSA administration group, only increases of autophagosome (LC3-labeled yellow puncta) in fibroblasts were detected after treatment with chloroquine, no matter with or without AGEs