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Xiaokeping-induced autophagy protects pancreatic β-cells against apoptosis under high glucose stress

Yanyang Wu[a,b,c,d,1], Yongquan Hu[a,1], Haiyan Zhou[a], YunLin Wei[e], Xincong Kang[a], Dongbo Liu[a,b,c,d,*]

[a] Hunan Agricultural University, Changsha, 410128, Hunan, PR China
[b] Hunan Provincial Key Laboratory of Crop Germplasm Innovation and Utilization, Hunan Agricultural University, Changsha, 410128, Hunan, PR China
[c] Hunan Co-Innovation Center for Utilization of Botanical Functional Ingredients, Changsha, 410128, Hunan, PR China
[d] State Key Laboratory of Subhealth Intervention Technology, Changsha, 410128, Hunan, PR China
[e] Kunming University of Science and Technology, Yunnan, PR China

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ABSTRACT

Xiaokeping (XKP), a prescribed Traditional Chinese Medicine (TCM), has been used to treat patients with type II diabetes mellitus for many years; however, the molecular mechanism of its effects is unknown. As the only insulin producer, the pancreatic β cell plays an important role in diabetes. Whether XKP influences the viability of pancreatic β cells remains to be substantiated. In the present study, autophagy/apoptosis analyses were used to evaluate the therapeutic effect of XKP on pancreatic β-cells induced by high glucose levels and to investigate a potential causal molecular mechanism of XKP effect on the cells. The pancreatic β-cell lines MIN-6 were divided into four groups: control, high glucose (33.3 mmol/L), high glucose with XKP, high glucose with XKP and 3-Methyladenine (3-MA). Immunofluorescence assay was employed to determine autophagosome formation and flow cytometry was used to determine apoptotic rates of the β cells by the detecting expression of autophagy- and apoptosis-related proteins. High glucose increased the apoptotic rate of β-cells from 5.37% to 23.24%; however addition of XKP mitigated the rate at 10.92%. Data indicate that autophagy of β-cells was induced by XKP via the mammalian target of rapamycin (mTOR) pathway. Where the autophagy inhibitor 3-MA was added, the apoptotic rate was 23.94%, similar to the high glucose group rate. The results suggest a potential cyto-protective effect of XKP from high glucose toxicity by its induction of autophagy which may be linked to mTOR-mediated autophagy.

1. Introduction

Diabetes mellitus (DM) is a global chronic metabolic disease characterized by high blood glucose concentration. Diabetic patients exhibit either insulin deficiency due to β-cell failure or insulin resistance where the body lacks a normal response to insulin [1]. The pancreatic β-cell plays an important role in diabetes pathology because it produces insulin. Both type I diabetes mellitus (T1DM) and type II diabetes mellitus (T2DM) are characterized by progressive β-cell failure [2]. In T1DM, β-cell failure is typically caused by viral infections or cytokines, such as interleukin-1β (IL-1β) and interferon-γ (IFN-γ), that induce a progressive autoimmune assault against β-cells [3]. The pathogenesis of T2DM is more variable because of the different degrees of β-cell failure and insulin resistance [4]. Apoptosis is the primary form of β-cell death in both types of DM [2]. That high blood glucose concentration is the most significant characteristic of diabetic patients [5] is supported by diabetic models establishing that glucose toxicity as a contributor to the development of insulin resistance, impaired insulin secretion and pancreatic β-cell failure [6].

Autophagy, a conserved intracellular, self-protective mechanism under stressful conditions, is necessary to maintain the architecture and function of mammalian cells [7]. Through a lysosomal degradation pathway, autophagy helps host cells degrade longevity proteins, damaged mitochondria and other aging orto produce small molecules of organic matter (e.g. amino acids) to ensure the survival of cells and maintain cell homeostasis [8]. Autophagy is either positively mediated by the adenosine 5'-monophosphate (AMP)-activated protein kinase (AMPK) pathway [9], or negatively mediated by the mammalian target of rapamycin (mTOR) pathway [10].

The cross-talk between autophagy and apoptosis is complex [11].

* Corresponding author at: Hunan Agricultural University, Changsha, 410128, Hunan, PR China.
E-mail address: chinasaga@163.com (L. Dongbo).
[1] These authors contributed equally to this work.

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Notably, numerous death stimuli are capable of activating either the autophagy or apoptosis and several genes that are critical for execution of their respective pathway can function in the other pathway. In addition, the autophagy and apoptosis pathways share common regulator and components, and can positively or negatively regulate and modify each other [12]. Thus, the exact relationship between autophagy and apoptosis depends on specific conditions.

Xiaokeping (National Medicine Approved NO. Z44020069), a commercially avaliable TCM that is licenced by China Food and Drug Administration (CFDA), has been used in the clinical treatment of T2DM in China for many years [13]. Xiaokeping consists of 12 Chinese medicinal herbs: *Ginseng* radix (1.08%), *Coptidis* rhizoma (1.08%), *Trichosanthis* radix (26.88%), *Asparagi* radix (2.72%), *Astragali* radix (26.88%), *Salviae miltiorrhizae* radix et rhizoma (8.03%), *Lycii* fructus (6.45%), *Astragali complanati pollen* (8.03%), *Puerariae lobatae* radix (8.03%), *Anemarrhenae* rhizoma (5.38%), *Galla chinensis* (2.72%), and *Schisandrae chinensis* fructus (2.72%). It contains numerous chemicals such as alkaloids, flavonoids, quinones, triterpene glycosides, and organic acids. Studies have reported that XKP can inhibit diabetic nephropathy in streptozotocin-induced rats by blocking signaling of transforming growth factor-β1 (TGF-β1) / similar mothers against decapentaplegic homolog 7 (Smad7) [14]. However, the molecular mechanism of XKP underlying its effects in clinical treatment of T2DM is not clear and the interaction between autophagy and apoptosis in relation to pancreatic β cells under high glucose stress is unknown. The objective of the present study was to investigate potential mechanisms of XKP in diabetes therapy for secondary development of XKP.

2. Material and methods

2.1. Cell culture

A mouse pancreatic beta cell line, MIN-6 (a gift from Dr. Huang Gan), was cultured in 25 mmol/L glucose and Dulbecco's modified Eagle's medium (DMEM, Gibco, 8116008) supplemented with 10% fetal bovine serum (FBS, BI, 1507772) and 1% 100× L-Glutamine (TransGen, FG201-01). Cultures were grown in a high humidity environment with 5% carbon dioxide (CO_2) and at a temperature of 37 ℃.

2.2. Preparation of XKP

We obtained XKP from Guangzhou Zhongyi Pharmaceutical Limited Company (National Medicine Approved Number: Z44020069, standard code: 86900428000227). The assortment of herbs in XKP was ground into powder and 1 g of powder was then soaked in 50 ml of ultrapure water with ultrasonic treatment for 1 hand subsequently heated to 80 ℃ for 8 h. The mixture was condensed to 10 ml and filtrated through a 0.22 μm sterilizing filter (Millex, SLGP033RB). The filtrate was stored in a microorganism-free tube at 4 ℃ within three days.

2.3. Flow cytometry

Apoptotic cells were quantified by flow cytometry [15] using the Propidium Iodide (PI)-Annexin V/fluorescein isothiocyanate (FITC) Apoptosis Detection Kit (BD Pharmingen, 5043815). A total volume of 2 ml of MIN-6 suspension cells at 2×10^5 cells/ml were seeded in each well of a 6-well microtitre plate and incubated at 37 ℃ for 24 h. The cells were grown in different media for each treatment group: basic medium basic medium with 33.3 mmol/L glucose; basic medium with 1 μl/ml XKP basic medium with 33.3 mmol/L glucose and 1 μl/ml XKP mixture; basic medium with 33.3 mmol/L glucose, 1 μl/ml XKP and 2 mmol/L 3-MA. After 36 h at 37 ℃, the cells were washed with 1 ml of phosphate buffered saline (PBS) solution and trypsinized with 350 μl of trypsin-EDTA to be collected into the same centrifuge tube for each well and was then centrifuged at 1000 rpm for 10 min. The supernatant was discarded; the precipitant was washed with 1 ml of PBS solution and centrifuged at 1000 rpm for 10 min. We repeated the wash process twice. The precipitant was resuspended in 100 μl annexin binding buffer (1×) filtered by 70 μm Cell Strainers (Biologix, 15-1070), and then transferred to a new tube. In each tube, 5 μl of Annexin V-FITC was added and the tube was placed in ice for 10 min. Then 5 μl of PI was added and tubes were incubated for five more minutes in ice. Another 400 μl annexin binding buffer (1×) was added to each tube and the solution was analysed by flow cytometry (BD FASCCanto II) with Cell Quest software (BD Sciences, America).

2.4. Immunofluorescence staining

Immunofluorescence staining was used to assess autophagosome formation. MIN-6 cells were cultured in 12-well plates which placed round glass coverslips in advance, treated with different conditions of the basic medium, basic medium with 33.3 mmol/L glucose, basic medium with 1 μl/ml XKP mixture; basic medium with 33.3 mmol/L glucose and 1 μl/ml XKP mixture; or basic medium with 33.3 mmol/L glucose, 1 μl/ml XKP mixture and 2 mmol/L 3-MA. After 36 h of incubation at 37 ℃, the medium was discarded. The cells were washed with PBS three times, fixed with 4% paraformaldehyde for 10 min, and then washed with PBS three times. After blocking with blocking liquid (PBS plus 10% FBS) for 30 min, cells were incubated at 37 ℃ for 1 h with a primary antibody, anti-LC3 (microtubule-associated protein 1 light chain 3) polyclonal antibody (MBL, PM036), and then washed three times with blocking liquid. Cells were subsequently incubated 37 ℃ for 1 h with a secondary antibody, anti-rabbit IgG Alexa Fluor 488 conjugate (CST, 4412), and then washed three times with PBS. Imaging was performed with a confocal laser scanning microscopy (Zeiss LSM 710).

2.5. Western blotting

Western blotting was used to assess expression of different proteins. MIN-6 cells were cultured in 6-well plates in one of the same five treatments as described in the previous section. After discarding the medium, cells were washed with PBS three times, then lysed in 200 μl 2% Sodium dodecyl sulfate (SDS) per well. The extracts were warmed to 100 ℃ for 10 min and then mixed with 6× protein loading buffer (TransGen, J21020) and warmed to 100 ℃ for 10 min again. The extracts were separated by SDS-PAGE (5% Stacking Gel, 12～15% Running Gel, 70 V 30 min, 110 V 40 min), and then transferred to a polyvinylidene difluoride membrane (70 V 30～60 min). After blocking with 5% nonfat milk in PBST (PBS plus 0.2% Tween-20) for 1 h, the membrane was incubated with multiple primary antibodies: anti-LC3 (MBL, PM036), Anti-SQSTM1/p62 (sequestosome 1) antibody (MBL, PM045), Anti-S6K (ribosomal protein S6 kinase) antibody (CST, 2708), Anti-Phospho-S6K antibody (CST, 9206), Anti-AMPK antibody (CST, 4811), Anti-Phospho-AMPK (Thr 172) antibody (CST, 4188), Anti-procaspase-3 antibody (CST, 9662S), Anti-cleaved-caspase-3 antibody (CST, 9661 T), and Anti-GAPDH (glyceraldehyde-3 -phosphate dehydrogenase) antibody (Yataihengxin, ZB002) at 4 ℃ overnight. Recycling primary antibodies, washing with PBST three times, the membrane was incubated for 1 h at room temperature with a secondary antibody of goat anti-mouse IgG1 (Southern biotech, 1070-05) or goat anti-rabbit IgG (Southern biotech, 4050-05), and then washed with PBST three times. ECL western blotting detection reagents (Pierce, 32106) were used for the detection step.

2.6. Statistical analysis

All data were analysed by SPSS Software. All samples were carried out in triplicate and triplicate data were expressed as means ± S.D. for the three different experiments. One-way ANOVA was used to measure statistical differences between the means within each experiment. The significant statistical difference of p-value < 0.01 was used.