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Effects of phycocyanin on INS-1 pancreatic β-cell mediated by PI3K/Akt/FoxO1 signaling pathway

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ABSTRACT

The level of methylglyoxal (MG), which is a side-product of metabolic pathways, particularly in glycolysis, is elevated in diabetes. Notably, the accumulation of MG causes a series of pathological changes. Phycocyanin (PC) has been demonstrated to show insulin-sensitizing effect, however, the underlying molecular mechanism remains elusive. The aim of this study was to investigate the protective effects of PC on INS-1 rat insulinoma β-cell against MG-induced cell dysfunction, as well as the underlying mechanisms. PC was preliminarily verified to time-dependently activate PI3-kinase (PI3K) pathway, but the PI3K-specific inhibitor Wortmannin blocked the effect of PC. Glucose-stimulated insulin secretion (GSIS) was impaired in MG-treated INS-1 cells. Furthermore, MG induced dephosphorylation of Akt and FoxO1, resulting in nuclear localization and transactivation of FoxO1. Nevertheless, these effects were all effectively attenuated by PC. The ameliorated insulin secretion was related to the changes of FoxO1 mediated by PC, which demonstrated by RNA interference. And, the dosage used in the above experiments did not affect β-cell viability and apoptosis, although long-term MG induced cell apoptosis and mitochondrial dysfunction. In conclusion, PC was capable to protect INS-1 pancreatic β-cell against MG-induced cell dysfunction through modulating PI3K/Akt pathway and the downstream FoxO1.

1. Introduction

Type 2 diabetes (T2D) is characterized by hyperglycemia, insulin resistance (IR), and impaired β-cell function [1,2]. Numerous studies have shown that, advanced glycation end-products (AGEs) contribute to the development of IR, inflammation, and all types of diabetes mellitus [3]. Methylglyoxal (MG), known to be the precursor substances of AGEs, accumulates in the plasma of diabetes patients [4]. Indeed, MG cannot only be involved in the pathogenesis of many complications of type 2 diabetes, but also promotes the obstacle of β-cell characterized by the impairment of insulin function [5–9].

β-Cell dysfunction is thought to have a primary role in the pathogenesis of T2D [10]. Insulin receptor and its downstream signal proteins constitute a complex signal transduction pathway in islet β-cell, regulating insulin secretion, maintaining β-cell growth, proliferation and survival [11,12]. Several reports have highlighted the importance of PI3K signaling in cellular physiology [13,14]. Moreover, the role of PI3K has been examined in the context of islet biology: a critical node between the insulin receptor substrates and Akt [15].

FoxO factors expressed in β cells are transcriptional effectors of insulin signaling, and the PI3K/Akt signaling can inhibit their activity through phosphorylation-dependent nuclear exclusion [16,17]. The activity of FoxO1 is regulated by various external stimuli (including insulin, growth factors, and oxidative stress) through modifying its subcellular localization and posttranslational modifications [18–20]. In response to insulin signaling, Akt phosphorylates FoxO1, resulting in the nuclear reduction and inactivation of FoxO1. Furthermore, FoxO1 nuclear reduction has been thought to be the possible mechanism for the inhibition of FoxO1-mediated transcription [18,21].

Phycocyanin (PC), a kind of dark blue protein isolated from *Spirulina platensis*, has a wide range of clinical applications such as free radical scavenging, anti-inflammatory and protecting DNA from oxidative damage. PC has been previously verified to effectively enhance insulin sensitivity, ameliorate insulin resistance of peripheral target tissues and regulate glycolipids metabolism in KKAy mice [22]. In addition, PC can counteract alloxan's negative effects in alloxan-injured mice [23]. Until now, it is not clear how does PC ameliorate insulin resistance and improves insulin

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sensitivity and whether its work is mediated by insulin transduction pathway. On the other hand, little is available about the effects of PC on MG-induced cell dysfunction.

In this study, after exposed to PC in normal/MG condition, rat insulinoma INS-1 cells were utilized as cell models to investigate the protective actions of PC and the potential molecular mechanisms. We demonstrated that PC protected INS-1 cells against MG-induced dysfunction through modulating the PI3K/Akt/FoxO1 signaling pathway. Furthermore, we showed that the effects of PC might be FoxO1-dependent. On the other hand, we investigated the effect of PC on the long-term MG-treated INS-1 cells. The results gain new insight into the mechanism of anti-diabetic effect of PC.

2. Materials and methods

2.1. Reagents

Phycocyanin was extracted from the *S. platensis*. The processes of extraction and purification included homogenization, centrifugation, precipitation with ammonium sulphate, DEAE-Sepharose Fast Flow chromatography, Capto Q chromatography, SOURCE 30 Q chromatography and high performance liquid chromatography (HPLC) [24]. Methylglyoxal was obtained from sigma (St. Louis, MO, USA). Antibodies against FoxO1, phosphorylated (p)-FoxO1 (Ser256), Akt, p-Akt (Thr308), tubulin and PCNA were from Cell Signaling Technology (Beverly, MA, USA). RPMI 1640 medium were obtained from Gibco (Grand Island, NY, USA) and FBS from Hyclone (Logan, UT, USA). The BCA protein assay kit and Total RNA Extractor (Trizol) kit were obtained from Generay (Shanghai, China). PI3K inhibitor Wortmannin, MAPKK inhibitor PD98059, Hochest 33342 stain kit and JC-1 assay kit were obtained from Beyotime (Shanghai, China). Apoptosis detection kit was from BD (Franklin Lake, New Jersey, USA). The Luciferase Assay System was purchased from Promega (Madison, WI, USA). Insulin ELISA kit was from R&D (Germany), siRNA of FoxO1 and scrambled siRNA were purchased from Biomics (Nangtong, China). The TaqMan One-step PCR Master Mix Reagents kit was purchased from ABI (Foster City, CA, USA).

2.2. Purity determination of PC

To determine the purity of PC, high performance liquid chromatography analysis was performed. Besides, SDS polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed on 15% polyacrylamide gel. Moreover, UV Spectrophotometry analysis was taken at wavelengths of 280, 620 and 652 nm and the A620/A280 ratio was calculated.

2.3. Cell culture

The insulin-secreting INS-1 cells were maintained with RPMI 1640 containing 11 mM glucose supplemented with 10%(v/v) fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, 0.11 g/L L-glutamine, 0.11 g/L sodium pyruvate, and 50 μM β-mercaptoethanol in humidified 5% (v/v) CO_2, 95% (v/v) air at 37 °C. Cells were washed with PBS and starved for 8 h in serum-free medium containing 0.2% (w/v) BSA before treatment. Wortmannin or PD98059 were added in certain experiments before the addition of 5 μM Phycocyanin (PC).

2.4. MTT assay

A modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay was used in determination of cell viability. Briefly, INS-1 cells (5×10^4 per well) were seeded in flat-bottomed 96-well culture dishes for 24 h, starved for 8 h, and then treated or not with MG for 30 min or PC for 24 h at the indicated different concentrations. The MTT assay was performed as described previously [25]. The absorbance of the samples was measured at 570 nm (630 nm as a reference).

2.5. Western blot assay

INS-1 cells were seeded and treated with indicated substances in each experiment. Whole cell extracts were prepared by RIPA lysate (Beyotime, Shanghai, China). Nuclear and cytoplasmic fractions were extracted with the nuclear and cytoplasmic protein extraction kit (Sangon, Shanghai, China). The Western blot assay was performed as described previously [26]. Quantification of western blot was performed by measuring the optical densities of the respective bands using Image J (NIH, Bethesda, MD, USA).

2.6. Flow cytometric analysis of apoptosis

INS-1 cells were seeded into six-well culture dishes, treated with indicated substances, and then digested with Trypsin (no EDTA). The apoptosis analysis was performed as described previously using apoptosis detection kit (BBI) [27].

2.7. Transient transfection and luciferase reporter assay

The forkhead responsive element (FHRE)-luciferase reporter plasmid (Addgene plasmid 1789) was used for transcriptional activity of FoxO1. A plasmid expressing the gene encoding β-galactosidase driven by the cytomegalovirus (CMV) promoter (Clontech Laboratories, Palo Alto, CA, USA) was transiently transfected as internal control. After seeded in glass bottom dishes and incubated for 24 h, INS-1 cells were transfected with 2.5 μg FHRE-Luc diluted in 250 μL Opti-MEM® medium per dish by Lipofectamine®3000 Reagent (Invitrogen) according to the manufacturer's protocol, and after incubated for 24 h, the medium was replaced with fresh medium. Then cells were treated as described in the figure legends. Finally, cells were harvested for luciferase reporter assays as described according to the manufacturer's instructions.

As for FoxO1 subcellular distribution, GFP-FoxO1 (Addgene plasmid 17551) was transiently transfected as above. Following treatments, cells were fixed gently to preserve GFP fluorescence using 4% paraformaldehyde for 15 min at room temperature followed by several washes with PBS. Cells were then permeabilized with 0.2% (vol/vol) Triton X-100 for 20 min at 4 °C and washed with PBS. Nuclei were stained with DAPI (50 ng/mL in PBS) for 10 min at room temperature. Confocal microscope was used for imaging cells.

2.8. siRNA transfection

siRNA transfection was performed using SuperFectin™ II siRNA transfection reagent (Pufei) according to the manufacturer's instructions. SuperFectin™ II siRNA reagent (2.5 μL/well) and FoxO1 siRNA or scrambled siRNA were diluted in transfection stock buffer provided with the transfection kit to give a final concentration of 50 nM siRNA. Experiments were started 24 h following transfection. The following Silencer Select Pre-designed siRNA for FoxO1, 5′-GGAGAUACCUUGGAUUUUAU-U-3′ and 5′-AAUAAAAUCCAAGGUAUCUCC-3′ were obtained from Biomics. The knockdown efficiency was assessed by Western blot analyses.

2.9. Quantitative real-time RT-PCR analysis

INS-1 cells were seeded into six-well culture dishes and treated with substances as indicated. Thereafter, cells were lysed and total RNA was isolated using a commercial kit, and quantitative real-time PCR was performed as described previously [28]. The