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Inhibition of islet amyloid polypeptide fibril formation by selenium-containing phycocyanin and prevention of beta cell apoptosis

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ABSTRACT

Human islet amyloid polypeptide (hIAPP) fibril is the major constituent of amyloid deposits in pancreatic islets of type 2 diabetes. Misfolding and hIAPP fibril formation are thought to be important in the pathogenesis of diabetes. Studies have showed that selenium-containing phycocyanin (Se-PC) inhibited the fibrillation of hIAPP to form nanoscale particles, which is mainly by interfering with the combination between hIAPP. Small nanoscale oligomers tended to grow into larger nanoparticles and the size of nanoparticles increased with the incubation time. By interfering with the fibrillation of hIAPP and altering the structure, Se-PC alleviated hIAPP-induced cell apoptosis. Meantime, generation of ROS produced during the fibrillation process was inhibited, which was proposed to be the main factor for the hIAPP-cytotoxicity in beta cells. Taken together, Se-PC inhibited hIAPP fibrillation, thus suppressed the formation of ROS to show protective effect on hIAPP mediated cell apoptosis. Our studies provide useful information for our understanding of the interaction mechanisms of Se-PC on hIAPP structure and protective mechanisms on hIAPP cytotoxicity, presenting useful candidate for anti-diabetes drug development.

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1. Introduction

Amyloidosis characterize by misfolding of normal proteins and deposition of amyloid fibrils is associated with serious diseases, including amyloid-β peptide (Aβ) with Alzheimer's disease (AD), islet amyloid polypeptide (IAPP) with type 2 diabetes mellitus (T2DM), and prion protein (PrP) with the spongiform encephalopathies [1]. Therein, human islet amyloid polypeptide (hIAPP) is a 37-residue peptide synthesized and co-secreted along with insulin in pancreatic beta cells [2–5]. hIAPP, normally soluble and natively unfolded in its monomeric state, shows the propensity to aggregate into β-sheet-rich fibrils with the existence of partially folded intermediate that tends to form discrete-structured oligomers followed by pre-formed oligomers assembling to amyloid fibrils [6,7]. Amyloid fibril is the major constituent of amyloid deposits in pancreatic islets of T2DM and the deposition of amyloid in the pancreatic islets is a characteristic pathological feature of T2DM, leading to β-cell dysfunction and loss of islet β-cell mass [8–11]. The identity of the toxic species associated with amyloid diseases is widely debated and the controversial mechanisms underlying hIAPP cytotoxicity involve with oxidative stress, membrane destabilization and soluble oligomer formation [12–14]. Although the molecular mechanisms by which different proteins assemble into highly ordered fibrillar deposits and cause disease remain topics of debate, small molecules can bind to IAPP extended structures, resulting in alteration of the distribution of conformers and inhibition of oligomerization and fibrillation of hIAPP [15]. It is postulated that inhibition of amyloid fibril formation is a potentially key therapeutic approach towards diabetes. Thus, to antagonize hIAPP-induced cytotoxicity, attempts have been made by the exploration of series of fibrillation inhibitors such as peptide-based inhibitors [16], coordination compounds [17,18], natural products [19,20], small molecule inhibitors [21], macromolecular crowding [22] and nanoparticles [23].

The trace element selenium (Se) is an essential nutrient element for human health and selenium-containing compounds and particles exert extensive biological functions [24–27]. Early studies indicated that inorganic Se acted as an insulin mimic and sodium selenate improved glucose homeostasis in type 1 and type 2 diabetic animals [28–30]. Meanwhile, Se inhibited amyloid fibril formation from hIAPP [31]. Supplementing Se was perceived as an effective strategy to prevent and treat diabetes, whereas high Se intake may also lead to adverse effects [32]. Phycocyanin (PC), a

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blue photosynthetic pigment purified from Spirulina, is a popular dietary nutritional supplement demonstrating strong anti-oxidative, anti-inflammatory, hepatoprotective and neuro-protective activities in both *in vitro* and *in vivo* studies [33–35]. Our previous studies have demonstrated that PC protected INS-1E pancreatic beta cells against hIAPP-induced apoptosis through attenuating oxidative stress and mitochondrial dysfunction [36,37]. Considering the favorable protective effects of PC and the anti-diabetic effect of Se, selenium-containing phycocyanin (Se-PC) with good biological activity and low toxicity is used for the exploration. It is of our interest in the present study to deliberate the effect of Se-PC on hIAPP morphology and cytotoxicity to further demonstrate the mechanism of Se-PC protection function.

2. Materials and methods

2.1. Materials

Human islet amyloid polypeptide was synthesized using t-boc chemistry and purified by reverse phase high performance liquid chromatography in the Yale University. Phycocyanin was purified from Spirulina by fast protein liquid chromatography methods as described previously. Thioflavin T was purchased from Shanghai Godo Industrial Co., Ltd. 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), thiazolyl blue tetrazolium bromide (MTT), 4′,6-diamidino-2-phenyindole (DAPI), phalloidin, 2′,7′-dichlorofluorescein diacetate (DCFH-DA), propidium iodide (PI), and 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylimidacarbocyanine iodide (JC-1) were purchased from Sigma. Terminal transferase dUTP nick end labeling (TUNEL) assay kit was obtained from Roche Applied Science (Basel, Switzerland). PathScan® Cleaved poly (ADP-ribose) polymerase (PARP) (Asp214) Sandwich ELISA Kit and cleaved caspase-3 (c-caspase-3) antibody were obtained from Cell Signaling Technology (Beverly, MA). Caspase-3 substrate (Ac-DEVD-AMC) was acquired from Biomol (Germany). RPMI-1640 medium and fetal bovine serum (FBS) were purchased from Gibco BRL (Gaithersburg, MD). The water used in all experiments was ultrapure, supplied by a Milli-Q water purification system from Millipore.

2.2. Thioflavin T (ThT) fluorescence assay

20 μM hIAPP was respectively incubated with 2.5 μM and 5 μM Se-PC solution at 37 °C for 48 h. Thioflavin T fluorescence assay was performed by combining 40 μl of incubated solution with 160 μl of 50 μM ThT in PBS (phosphate buffered saline, containing NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, KH_2PO_4 2 mM). Fluorescence measurements were recorded in 96-well black microplate (Corning Costar Corporation, USA) by fluorescence microplate reader (Spectra Max M5, Bio-Tek) at room temperature. The excitation wavelength was set to 440 nm, and emission was monitored at 482 nm.

2.3. Particle size analysis

The experiments were performed using the Zetasizer Nano ZS particle analyzer (Malvern Instruments Limited). 1 ml of 10 μM hIAPP solution with 5 μM or 10 μM Se-PC was incubated in PBS for 48 h at 37°C. Samples were placed in the disposable granularity cuvette for the measurements with a fixed detector angle of 173°.

2.4. Circular dichroism (CD) assay

30 μM hIAPPand10 μM Se-PC was incubated in PBS at 37 °C for 48 h, followed by centrifuging at 10,000 rpm for 15 min to remove large precipitates. Supernatants were transferred for testing. CD spectra were recorded on a Chirascan circular dichroism spectrum analyzer (Applied Photophysics Limited, UK) over the wavelength range of 190–260 nm under a constant flow of nitrogen gas. Measurements were carried out in cells of 0.1 cm path at room temperature. The spectra represent the average of 3 scans. 200 μl samples were added each time.

2.5. Atomic force microscopy (AFM)

20 μM hIAPP was incubated with or without 5 μM Se-PC at 37 °C for 48 h. 10 μl incubated solution was dripped on mica plate for the observation after air dried. hIAPP samples and paraformaldehyde fixed cell samples were characterized by AFM. Experiments were performed by Bioscope Catylyst Nanoscope-V (Veeco instruments, USA) with nominal resonance frequency of 150 kHz and nominal spring constant of 5 N/m.

2.6. Transmission electron microscopy (TEM)

20 μM hIAPP was respectively incubated with or without 5 μM Se-PC at 37 °C for 48 h. 10 μl incubated solution was dripped on polyporous carbon film-coated copper grids for the observation after air dried. The images were visualized at an accelerating voltage of 80 kV under TEM microscope (Hitachi H-7650).

2.7. Preparation of calcein-containing lipidosome

The calcein-containing lipidosome was prepared in reference to an existing method [38]. 100 mg DPPC and 10 mg calcein were dissolved in 5 ml chloroform/methanol completely followed by drying to lipid membrane with rotary evaporator.10 ml PBS was added to suspend mixed micelles under 50 °C, then dialyzed in dialysis tube with 8000 molecular weight cut-off for 24 h.

2.8. Cell culture and treatments

INS-1 rat insulinoma cell line was a gift from Dr. P. Maechler (University of Geneva, Switzerland). Briefly, cells were maintained in RPMI-1640 medium supplemented with 10% FBS, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 10 mmol/L HEPES, 50 μmol/L mercaptoethanol, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humid atmosphere (95% relative humidity, 5% CO_2). The passage number of the INS-1 cells used in this study was 65–74. For peptide treatment, lyophilized hIAPP was dissolved in HFIP, which was removed by evaporation under N_2. The hIAPP solution used to cultured cells was freshly prepared. The cells were seeded in 96-well microplate at a density of 5×10^4 cells per well for MTT and LDH assays. Cells were seeded in 6-well plates at a density of 9×10^5 cells per well for other assays. Cells were incubated with 20 μM hIAPP for 24 h in the absence or presence of 2.5 μM or 5 μM Se-PC to examine the effects of hIAPP on INS-1 cells.

2.9. MTT Assay

Cell viability was determined by measuring the ability of cells to transform MTT to a purple formazan dye [39]. INS-1 cells seeded in 96-well microplate were cultured in a humidified atmosphere for 24 h at 37 °C, and then the cells were incubated with 20 μM hIAPP alone or indicated concentration of Se-PC for 24 h. Morphological changes in INS-1 cells observed by phase-contrast microscopy (magnification, 100 ×). After incubation, 20 μl/well of MTT solution (5 mg/ml in PBS buffer) was added, followed by 5-h incubation. The medium was aspirated and replaced with 150 μl/well of DMSO to dissolve the formazan salt. The color intensity of the formazan solution was measured at 570 nm using a microplate spectrophotometer (Spectro Amax TM 250) to reflect the cell growth condition.

2.10. LDH Assay

Release of LDH indicates the change of membrane permeability, which can reflect the damage extent of cell membrane. Cells were precipitated by centrifugation (1500× g) for 10 min at room temperature at the end of the treatments with samples mentioned above. Supernatants were transferred to a 96-well plate, and LDH activity was assayed using the cytotoxicity detection kit according to the manufacturer's instructions. Cells treated with 1% Triton X-100 were used as high control and media without cells served as a low control. Results were expressed as % cytotoxicity [(experimental value − low control)/(high control − low control) × 100].

2.11. Flow cytometric analysis

The cell cycle distribution was analyzed by flow cytometry [40]. Briefly, the treated cells were harvested and fixed with 70% ethanol overnight at −20°C, followed by incubated with propidium iodide (PI) for 4 h in darkness. The stained cells were analyzed by Epics XL-MCL flow cytometer (Beckman Coulter, Miami, FL). Apoptotic cells with hypodiploid DNA content were measured by quantifying the sub-G1 peak in cell cycle pattern.

2.12. TUNEL, DAPI and phalloidin staining assay

DNA fragmentation, as one of the marks during cell apoptosis, can be detected by TUNEL assay. This assay was performed as previously described [41]. Briefly, treated INS-1 cells cultured on cover glass in 6-well plates were fixed with 4% paraformaldehyde for 1 h at room temperature. After washed in PBS, cells were permeabilized with 0.1% Triton X-100 for 2 min, and then incubated with 50 μl TUNEL reaction mixture for 60 min at 37 °C. The nuclei of the cells were double stained with 1 μg/ml of DAPI for 15 min and actin filaments were labeled with 5 μg/ml phalloidin for 30 min. Stained cells were washed with PBS and observed on a fluorescence microscopy (Nikon Eclipse 80i).

2.13. Immunofluorescence

Cleavage of caspase-3 was analyzed by immunofluorescence [42]. Briefly, cells were incubated with hIAPP and Se-PC for 24 h and fixed with 3.7% formaldehyde in PBS for 15 min. After rinse several times with PBS, the cells were permeabilized with 0.2% Triton X-100 in PBS at room temperature for 5 min. Permeabilized cells were then blocked with 0.1% BSA and subsequently incubated with primary antibody of cleaved caspase-3 (1:200) overnight at4 °C and Alexa-488 labeled anti rabbit IgG antibody (1:250) for 1 hat room temperature. Nuclei and actin filament were stained with DAPI and phalloidin respectively for 15 min after secondary antibody incubation. After washing with PBS, cover slips were mounted onto microscope slides using ProLongantifade mounting reagent (Molecular Probes). The slides were analyzed using a fluorescence microscopy (Nikon Eclipse 80i).

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