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Original article

7, 8-Dihydroxycoumarin (daphnetin) protects INS-1 pancreatic β-cells against streptozotocin-induced apoptosis

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ABSTRACT

Background and objective: Daphnetin (7, 8-dihydroxycoumarin), a natural coumarin compound, is known to exhibit antioxidant and anti-inflammatrory effects. However, the underlying mechanisms of its anti-apoptotic and antidiabetic effects yet not been examined. Therefore, the present work studied the anti-apoptotic and anti-diabetic effects of daphnetin by *in vitro* experiments.

Methods: The rat insulinoma (INS-1) cells were pre-treated with daphnetin at different concentrations (1, 10, 20 and 40 µM) for 24 h followed by exposition to streptozotocin (STZ) (3 mM) for 12 h. Effects of daphnetin and STZ on INS-1 cells were determined by MTT assay, glucose stimulated insulin secretion (GSIS) assay, lipid peroxidation, antioxidant status (SOD, CAT, GPx, and GST) Apoptosis staining (DAPI, Hoechst 33342, AO/EB and ROS) was performed by fluorescence microscopy, and Bcl-2, Bax and NF-κB protein expression was detected by Western blotting.

Results: MTT assay indicated that the viability of INS-1 cells was significantly reduced with exposure to STZ for 12 h as compared to control cells, while pre-treated with daphnetin for 24 h resulted in a significant improvement of cell viability. The effects daphnetin treatment in INS-1 cells on insulin secretion was tested and results showed that the pre-treatment of daphnetin could improve GSIS. Further, daphnetin pre-treatment significantly reduced the levels of lipid peroxidation markers and also improved antioxidant enzymes' activities in STZ-induced INS-1 cells. Western blotting assay revealed that daphnetin could suppress apoptosis through up-regulation of anti-apoptotic Bcl-2 protein expression and the down-regulation of pro-apoptotic Bax and nuclear factor NF-κB protein levels.

Conclusion: The results showed that daphnetin might be used in treating diabetes due to its insulin stimulating property and subsequent regulation of apoptotic pathway.

Introduction

Diabetes mellitus (DM) is a metabolic disorder that constitutes a huge global health burden due to its co-morbidities including obesity and its complications such as diabetic nephropathy, neuropathy, and retinopathy. According to the estimation of World Health Organization (WHO), more than 415 million people have been diagnosed as diabetic patients in 2015, which is projected to rise to 642 million people in 2040 (Cavan et al., 2015). The excessive loss of pancreatic β-cell mass mainly due to apoptosis is a major cause for the development of diabetic hyperglycemia in both type 1 and type 2 DM. In recent years, the apoptosis of β-cells has become an active area of research. Several lines of evidence from autopsy suggested that β-cell mass in diabetic patients is significantly reduced and its reduction is associated with increased apoptosis (Jurgens et al., 2011). Some studies on animal and human beings have showed that the increase of β-cell apoptosis is initiated by a variety of stimuli such as inflammatory cytokines and chronic hyperglycemia (Thomas et al., 2009).

In diabetes, oxidative stress generates oxygen free radicals, inhibits the antioxidant enzymes activity levels and increased the formation of lipid peroxides. Tangvarasittichai (2015) reported that the level of malondialdehyde, a by-product of lipid peroxidation and reactive oxygen species (ROS) increased in diabetes. STZ can induce apoptosis in pancreatic β-cells, which leads to increased

Abbreviations: ANOVA, analysis of variance; AO, acridine orange; CAT, catalase; DCFH-DA, 2′,7′-dichlorodihydrofluorescein diacetate; DMSO, dimethyl sulfoxide; EB, ethidium bromide; ECL, enhanced chemiluminescence; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; GPx, glutathione peroxidase; GSH, reduced glutathione; GSIS, glucose-stimulated insulin secretion; LHP, lipid hydroperoxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide; NF-κB, nuclearfactor-κB; PBS, phosphate-buffered saline; ROS, reactive oxygen species; RPMI, Roswell Park Memorial Institute medium; SEM, standard error of mean; SOD, superoxide dismutase; STZ, streptozotocin; TBARS, thiobarbituric acid reactive substances; WHO, World Health Organization.

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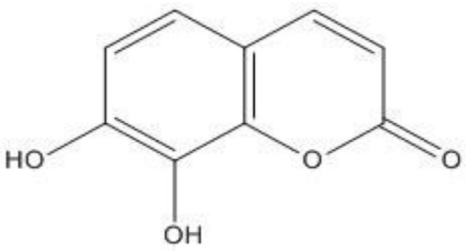

Fig. 1. Structure of daphnetin.

production of ROS and enhanced oxidative stress in β-cells. Under normal physiological conditions, there is a balance between ROS production and scavenging by endogenous antioxidants. Persistent and elevated levels of ROS can directly and indirectly disrupt this balance by altering the important biological molecules such as proteins, lipids and DNA (Birben et al., 2012). Particularly the β-cells are more susceptible to oxidative stress due to the fact that they express major antioxidants such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) at low levels.

Nuclear factor kappa B (NF-κB) is actually a family of structurally-related proteins that are involved in the control of a large number of normal cellular and body functions, such as immune and inflammatory responses, developmental processes, cellular growth and apoptosis. Inhibition of NF-κB signaling in mouse β-cells *in vivo* protects against multiple low-dose STZ-induced diabetes, while mice with constitutive NF-κB activation in β-cells spontaneously develop insulitis and immune mediated diabetes (Salem et al., 2014). In addition, NF-κB activation is also pro apoptotic to β-cells. Conversely, the NF-κB activation in β-cells from non-obese diabetic (NOD) mice accelerates the disease (Kim et al., 2007). Currently available therapies for diabetes include insulin and various oral antidiabetic agents such as sulfonylureas, biguanides and glinides. Many of them have a number of serious adverse effects; therefore, the search for more effective and safer hypoglycemic agents is one of the important areas of investigation (Vinayagam et al., 2016). Current studies involved in developing effective, economic ways to manage the side-effects of hyperglycemia can be to reduce the excess ROS generation by using bioactive natural antioxidants which are safe and easily accepted by the cells (Bahadoran et al., 2013).

7, 8-Dihydroxycoumarin (common name: daphnetin; molecular formula: $C_9H_6O_4$; Fig. 1) is a bioactive compound isolated from *Daphne koreana* Nakai and *Daphne odora* Thunb. (Thymelaeaceae) and further identified by various analytical approaches such as UV-Vis spectrophotometer, ^{1}H NMR, ^{13}C NMR, mass spectral data and CD spectroscopic methods (Huang et al., 1990; Peng et al., 2011). Chen et al. (2004) quantified daphnetin in *Daphne tangutica* Maxim. extracts by high performance liquid chromatography (HPLC), and studied its medicinal applications. Daphnetin showed to inhibit kinase activity *in vitro* and to display a significant free radical scavenging activity and inhibitory effects on lipid peroxidation. It also shows anti-cancer, antioxidant, anti-inflammatory, anti-hypoxic, neuroprotective, anti-proliferative, anti-diarrheal and antiparasitic activities (Thuong et al., 2010; Witaicenis et al., 2014). Wang et al. (2013) reported that daphnetin inhibited the proliferation of A549 human lung adenocarcinoma cells, induced their apoptosis via Akt/NF-κB signaling suppression, had significant antitumor effects and promote tumor apoptosis via multiple signaling pathways. In the light of the diverse pharmacological activities of daphnetin, it has a great potential to have other effects. Therefore, in this work, we investigated the protective effects of daphnetin against STZ-induced damage in rat insulinoma (INS-1) pancreatic β-cells since this activity has not been investigated yet.

Material and methods

Materials

Roswell Park Memorial Institute (RPMI)-1640 medium, fetal bovine serum (FBS), and phosphate buffered saline (PBS) were purchased from Thermal Fisher Scientific (Waltham, MA, USA), streptomycin and penicillin were purchased from Hyclone (Logan, UT, USA). STZ 4′, 6-diamidino-2-phenylindole dihydrochloride (DAPI), and propidium iodide were purchased from Sigma-Aldrich (St Louis, MO, USA). Natural daphnetin (Batch number: R-007-14073; CAS No.: 486-35-1; botanical source: root of *D. koreana*; purity $\geq$ 98.5% (RP-HPLC); MW: 178.14) was purchased from Chengdu Herb Purify Co., LTD (Chengdu, China). Glucose, dimethyl sulfoxide (DMSO), 3-(4, 5-dimethyl-2-thiaozolyl)-2, 5-diphenyl- tetrazolium bromide (MTT), 2-7-diacetyl dichlorofluorescein (DCFH-DH), and ethidium bromide (EB) were purchased from Aladdin (Shanghai, China). H33342 was purchased from Yuanye (Shanghai, China). Ethyl bromide and acridine orange were purchased from Malone Pharmaceutical (Dalian, China). Insulin by enzymes-linked immunosorbent assay (ELISA) kit was purchased from Millipore (Billerica, MA, USA). Monoclonal antibodies and secondary antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA).

Cell culture and experimental groups

INS-1 was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). INS-1 cells were cultured in RPMI-1640 with 10% FBS, penicillin (100 u/ml) and streptomycin (100 µg/ml) (Life Technologies, Carlsbad. CA, USA) in a humidified incubator containing 5% CO_2 at 37 °C. Cells were grown in 75 cm^2 tissue culture flasks and used for experiments when in exponential growth phase. Daphnetin was diluted in RPMI-1640 with 0.1% dimethylsulfoxide.

INS-1 cells were treated with 3 mM STZ (dissolved in citrate buffer, pH 4.4 and diluted in RPMI-1640) and then sterile-filtered before used. The INS-1 cells were divided into six experimental groups. Group 1: Untreated control cells, group 2: Cells were treated by STZ (3 mM) for 12 h, groups 3, 4, 5, and 6 cells were pre-treated with daphnetin at doses of 1 µM, 10 µM, 20 µM, and 40 µM for 24 h, and then treated with STZ (3 mM) for 12 h, respectively.

Cell viability assay

Cell viability was measured by MTT assay. INS-1 cells were seeded onto 96-well plates at a density of 5×10^3 cells /well and treated with daphnetin (1, 10, 20, 40 µM) for 24 h, and then cells were exposed to STZ (3 mM) for 12 h. After the incubation, MTT (0.5 mg/ml) was added to each well for 4 h. The purple formazan precipitate was dissolved with DMSO, and color intensity was measured at 570 nm with a microplate reader (Fluostar Omega, BMG Labtech, Offenburg, Germany).

Glucose stimulated insulin secretion assay

INS-1 cells were seeded into 24-well plates at a density of 1×10^5 cells/wells and pre-treated with daphnetin for 24 h and exposed to STZ for 12 h; then cells were rinsed and incubated with Hanks balanced Salt solution containing 2.8 mM and 25 mM glucose for 30 min at 37 °C, respectively. After incubation, the supernatant from each well was collected, the concentration of insulin was measured by rat insulin ELISA kit according to the manufacturer's instructions (Millipore, Billerica, MA, USA).