Mechanisms of Genistein Protection on Pancreas Cell Damage in High Glucose Condition

Wen-Wen Zhong¹, Yang Liu² and Chun-Lin Li¹

Abstract

Aim An adequate β cell number is important to prevent the onset and development of type 2 diabetes. The aim of this study was to determine if phytoestrogen genistein has protective effects against high glucose-induced cell apoptosis in human pancreas cells, and to try to determine the possible mechanism for this protection.

Methods Human pancreatic β cells were subjected to normal (5 mM) or high glucose (25 mM) with and without the presence of 100nM genistein, and also in the presence and absence of the pure anti-estrogen ICI-182780 (100 nM). Bcl-2 siRNA transfection was performed to investigate if the effect of genistein was also Bcl-2 dependent. Cell proliferation and apoptosis were determined by Tritiated Thymidine Incorporation Assay and Cell Apoptosis Detection ELISA. Estrogen receptor and Bcl-2 mRNA expression was measured by Real-time Quantitative PCR.

Results High glucose concentration caused cell proliferation inhibition and apoptosis in cultured human pancreatic β cells, and these effects were significantly reversed by genistein (P<0.01). Estrogen receptor beta was expressed in the cultured cells, and genistein protection was blocked by ICI-182780 administration as well as Bcl-2 siRNA transfection.

Conclusion Phytoestrogen gave protection against high glucose-induced pancreatic cell damage through estrogen receptor beta and Bcl-2 dependent pathways.

Key words: genistein, pancreas cell, high glucose, estrogen receptor, apoptosis, Bcl-2


Introduction

Diabetes mellitus has become a major threat to human health. The primary pathophysiological mechanisms of diabetes include insulin resistance and islet β cell dysfunction. Reduced β cell function is the key to diabetes onset, and the structure of β cell is the physical basis which determines the β cell function. Detailed investigation of β cell structure and function will lead to a further understanding of diabetes pathogenesis and treatments. The proliferation and apoptosis of β cells remain a dynamic balance at normal basis, and disturbance of this balance leads to diseases (diabetes or tumors). Under particular circumstances such as aging, obesity and insulin resistance, when the human body needs more insulin, β cells increase insulin synthesis and secretion by cell hyperplasia and hypertrophy. Reduced proliferation or increased apoptosis of β cells leads to diabetes. It has been demonstrated that the number of β cells is significantly reduced in type 2 diabetes. An adequate β cell number is very important to prevent the onset and development of type 2 diabetes. In vitro studies have also revealed that a high glucose concentration could inhibit cell growth and induce apoptosis in many cell types, such as human aortic and vein endothelial cells via direct cytotoxicity and oxidative stress.

Phytoestrogens are a group of biologically active plant substances with chemical structures similar to the endogenous estrogen, allowing them to bind to estrogen receptors (ER), though with lower affinity (1-4). Epidemiologically, there is a lower incidence of cardiovascular disease in Asian

¹Department of Geriatric Endocrinology, The Chinese PLA General Hospital, China and ²Department of Geriatric Hematology, The Chinese PLA General Hospital, China

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Correspondence to Dr. Chun-Lin Li, lcl301@yahoo.com.cn
countries that have a high soy phytoestrogen consumption in their diet (5). The anti-oxidative properties of phytoestrogens may be responsible in part for their potential protective effects. Genistein at physiological concentrations reduced free radical damage to MC3T3-E1 cells more effectively than either vitamin E or C (6). Glucose-triggered oxidation of LDL can be effectively prevented by genistein (7); genistein and equol were shown to protect against H2O2-induced DNA damage in human lymphocytes, more than anti-oxidant vitamins and estradiol (8). Genistein also plays a role in the scavenging of reactive oxygen species and lipid peroxidation (9). Daidzein has been shown to increase catalase mRNA expression and activate the catalase promoter region (10), and phytoestrogens can increase total glutathione levels, thus decreasing intracellular oxidant levels and preventing oxidative DNA damage (11).

This study was performed to determine if human pancreatic β cells (HPCs) were also susceptible to high glucose-induced cell damage, and if isoflavone genistein could provide cyto-protection in vitro. Blockage of estrogen receptor and Bcl-2 expression were performed to determine if these effects were through an estrogen receptor or via an alternative pathway.

**Materials and Methods**

**Reagents**

All reagents and chemicals were obtained from Sigma, Poole, UK unless otherwise stated. All reagents for tissue culture were obtained from Invitrogen, Life Technologies, Paisley, Scotland, UK. The Cell Death ELISA was obtained from Roche Diagnostics, Ltd., Manheim, Germany.

**Cell culture**

Human pancreas was obtained from Department of Surgery, and digested by collagenase. Islets were purified by mesh sieve, cultured in RPMI Media 1640 for 24 hours, and further digested with trypsin and DNAase to obtain single cell suspension. β cells were separated and purified in a florescence-activated cell sorter. HPCs were cultured in RPMI Media 1640. The experiments were performed on seeds seeded into either 12-well plates or 96-well plates at a density of 2.5x10^4/mL. Cells were incubated at 37°C in an atmosphere of 5% CO2. Cells were divided into two major groups: with and without Bcl-2 siRNA transfection. Control cells received normal 5 mM glucose medium; high D-glucose-treated cells had this medium supplemented with D-glucose to achieve 25 mM glucose in total. Cells were treated with 100 nM genistein, with or without the pure anti-estrogen ICI182780 (100 nM). Cells were then incubated for 24 hours. For each experimental group, cells were seeded in five parallel wells for specific detection, and results were confirmed by two other repeated tests.

**Cell death detection ELISA**

To detect the DNA fragmentation of apoptotic cells, the Cell Death Detection ELISA assay was used according to the manufacturer’s protocol. Briefly, cells were lysed with 200 μL lysis buffer and centrifuged at 4,000 rpm for 10 min, and 20 μL supernatant of each sample was transferred into a streptavidin-coated microtiter plate (MTP). Immunoreagent mixture (80 μL) was added, washed and 100 μL ABTs substrate solution was added. A MTP microplate reader (Anthos 2010, Anthos Labtech Instruments, Salzburg, Austria) was used to measure the absorbance at the wavelength of 405 nm with 492 nm as a reference.

**Tritiated thymidine incorporation assay**

Each sample received 0.25 uCi of [H]-TdR (Amersham International, Buckinghamshire, UK) and was incubated for 3 hours at 37°C. After incubation the media was removed and cells washed with phosphate-buffered saline, 0.5 mL of 10% trichloroacetic acid was then added to each sample for 1 hour to precipitate the DNA. Unbound thymidine was removed by a gentle wash with 4°C phosphate-buffered saline. The DNA was then dissolved in 500 μL of 0.1% Triton X100 in 10% sodium hydroxide. The final lysates were transferred to a scintillation vial with 10 mL of Ecoscint H (National Diagnostics, Yorkshire, UK) for counting.

**Bcl-2 siRNA transfection**

The Bcl-2 siRNA was transfected to the cells using the lipofectamine method according to the manufacturer’s protocol (Invitrogen). Briefly, for each 10 cm² plate surface area, the mixture of 10 μL Bcl-2 siRNA and 10 μL lipofectamine was incubated for 20 minutes at room temperature to allow the siRNA/lipofectamine complexes to form before adding to the cells, which were given 800 μL of serum/antibiotics-free medium after washing with the same medium twice. The final concentration of Bcl-2 siRNA was 100 nM.

**Taqman quantitative PCR for Bcl-2 and estrogen receptor mRNA expression**

Cell total RNA was extracted using Trizol reagent and treated with DNase to remove genomic DNA. RNA was then reverse-transcribed to cDNA using M-MLV Reserve Transcriptase. The GeneAmp 5700 sequence detection system (Applied Biosystems Ltd., Warrington, Cheshire, UK) for PCR was used to measure the relative level of gene expression quantitatively. Each sample was amplified with primers and probes for the target gene as well as the housekeeping gene, human β-glucuronidase (hGUS). The primers and probes were designed using Primer Express software (Applied Biosystems) and synthesized by MWG-Biotech (Ebersberg, Germany). The sequences of primer set were submitted to a BLAST search at http://www.ncbi.nlm.nih.gov/BLAST/ to confirm their uniqueness. The internal probe was labeled at the 5’ ends with the reporter fluorochrome 6-carboxyfluorescein (FAM) and at the 3’ ends with the
Figure 1. HPCs incubated in 5 mM or 25 mM glucose-containing medium were exposed to 100 nM genistein for 24 hours. Cell proliferation was detected by tritiated thymidine incorporation assay (A) and cell apoptosis was measured using the cell death ELISA (B). *p<0.01 as compared with the counterpart of each group in 5 mM glucose; **p<0.01 compared with control cells in 25 mM glucose (mean±SD).

quencher fluorochrome 6-carboxytetramethylrhodamine (TAMRA). For Bcl-2, forward primer: 5’- TTG GCC CCC GTT GCT T -3’; reverse primer: 5’- CGG TTA TCG TAC CCC GTT CTC -3’; probe: 5’-AGC GTG CGC CAT CTT TCC CAG-3’. For ERα, forward primer: 5’-CAA GGG AAG TAT GGC TAT GGA ATC-3’; reverse primer: 5’-CTC ACA GGA CCA GAC TCC ATA ATG -3’; probe: 5’-CCT GAA GCA TAG TCA TTC CAC ACT GCA GAC-3’. For ERα46, forward primer: 5’- AAG AGG GTG CCA GGC TTT G-3’; reverse primer: 5’-GTC CAA GAG CAA GTT AGG AGC AA-3’; probe: 5’-CTA GAG ATC CTG ATG ATT GGT CTC GTG TTCG3-3’. For ERβ, forward primer: 5’-CGA CAA GGA GTT GGT ACA CAT GA-3’; reverse primer: 5’-AAC AGG CTG AGC TCC ACA AAG-3’; probe: 5’-CAG CTG GGC CAA GAA GAT TCC CG-3’. DEPC-treated water was used as a non-template control and non-reverse transcribed samples were used to confirm that positive results were not due to amplification of genomic DNA. The results were analyzed using GeneAmp 5700 software, and the baseline and threshold were set manually. The PCR cycle consisted of an initial cycle of 50°C for 2 minutes followed by 95°C for 10 minutes, then 50 repeated cycles of 95°C for 15 seconds (denaturation) and 60°C for 1 minute (primer annealing and extension).

Statistical analysis

Statistical analysis was performed on at least three replicates using ANOVA with Tukeys post hoc analysis (SPSS version 11, SPSS UK Ltd, Surrey, UK). Data are expressed as mean ± standard deviation (SD). Values of P<0.05 were considered statistically significant.

Results

High glucose concentration significantly reduced cell growth and increased endothelial cell DNA fragmentation compared to control (P<0.01); 24-hour incubation of genistein significantly reduced cell apoptotic DNA fragmentation by 47.6% and restored cell proliferation by 62.2%, respectively (P<0.01). Detailed data are shown in Fig. 1.

The pure anti-estrogen ICI182780 significantly reversed high glucose-induced cell DNA synthesis inhibition and apoptosis by 78.9% (Fig. 2A) and 72.2% (Fig. 2B), respectively. Quantitative PCR showed that only ERβ and not ERα or the soluble type of ERα, ERα46, was expressed in this system (Fig. 2C). The time course of 5 mM and 25 mM glucose showed no effect on the expression of the ERβ mRNA over 30 min to 72 hours, and anti-estrogen ICI 182780 per se did not cause any significant changes in cell growth and viability (data not shown).

Bcl-2 mRNA was persistently expressed in HPCs. Bcl-2 siRNA transfection inhibited Bcl-2 expression, but did not induce more apoptosis in high glucose condition statistically. In the cells without Bcl-2 mRNA expression, the protective
Figure 2. HPCs incubated in 25 mM glucose were exposed to 100 nM genistein and/or anti-estrogen ICI182780 for 24 hours. Cell proliferation and cell apoptosis were detected by tritiated thymidine incorporation assay (A) and cell death ELISA (B). Estrogen receptor mRNA expression was detected by Quantitative PCR (C). *p<0.01 compared with high glucose (HG)-treated group; **p<0.01 compared with high glucose + genistein (HG+G) treated group; AE: anti-estrogen ICI182780 (mean±SD).

Discussion

Toxicity of hyperglycemia contributed to reduced β cell number and dysfunction of islet β cells, leading to a worsened blood glucose control and additional requirement of insulin in maintaining blood glucose within normal range clinically (12). High glucose-induced cell damage via different pathways, including an increase in the production of free radicals leading to oxidative stress (13), and inhibition of synthesis or depletion of endogenous cellular antioxidant defenses (14, 15), and was shown to affect a number of cell types such as endothelial cells (13, 16, 17). Our data supported other research that high blood glucose contributes to increased pancreas cell apoptosis and decreased cell growth in vitro, and this also provides an experimental proof to the decreased number and dysfunction of pancreas islet β cells.

Genistein significantly inhibited high glucose-induced cell apoptosis by 47.6%, indicating that genistein could provide protective effects against toxicity of hyperglycemia in cultured human pancreatic cells. This is in accord with reports in the literature showing the protective effect of genistein on free radical-induced oxidative damage in MC3T3-E1 osteoblast-like cells and the protection of H2O2-induced human lymphocytes DNA damage (18-20). The results from proliferation assays mirrored the effects on apoptosis, with genistein protecting against the reduction in proliferation from HG-induced oxidative stress. Restored pancreas cell growth and liability might contribute to restored islet β cell count and function, therefore, it was suggested that administration of phytoestrogen genistein might play a positive role in hyperglycemia toxicity alleviation and blood glucose management in diabetes.

The protective effects of genistein were reversed by the pure antiestrogen ICI182780 indicating that the protective effects of these phytoestrogens were being mediated through the estrogen receptor alone, and that no additional mechanism of action through an alternative pathway was occurring. The anti-estrogen ICI182780 is commonly used to block the activation of ER (21, 22). Our findings are consistent with the literature, and the protective effect of genistein on pancreas beta cell cytotoxicity has been reported (23). The mRNA of ERβ but not α was found to be expressed in HPCs in our system. Whether this is due to the techniques
employed or the culture conditions used, it is unclear why ERs have been found by some but not by others.

Blockage of Bcl-2 expression by specific siRNA transfection reduced the protected effect of genistein, and the transfection did not induce more cell damage per se. This indicated that the protection by genistein was highly associated with its modulatory effect on the expression of the pro-survival protein Bcl-2. Exposure of pancreas cells to genistein resulted in an upregulation of Bcl-2 expression, which in turn would have helped in the maintenance of the mitochondrial membrane potential as well as the control of cytochrome c release and caspase cascade activation, which finally result in an increased cell metabolic activity and viability and a reduced apoptotic level in oxidative stress damaged HPCs.

Blockage of Bcl-2 expression and ERβ function both could reduce the genistein protection suggesting a relation between estrogen receptor and Bcl-2. Genistein modulated Bcl-2 expression probably via activating and regulating the transcriptional effect of ERβ, and the modulation of ERβ expression by genistein seemed to be regulated via the receptor itself. The bcl-2 promtoter was demonstrated to contain two estrogen response elements, and 17β-oestradiol induced bcl-2 transcription in human breast cancer MCF-7 cells (24), therefore, the modulation of Bcl-2 expression by genistein was very likely to be attributed to the direct transcriptional effect of ERβ which is activated simultaneously by genistein treatment.

In conclusion, genistein protected against high glucose-induced cell apoptosis and inhibition of proliferation in human pancreas cells that expressed ERβ, by an estrogen-dependent mechanism, and these effects were reversed by the antiestrogen ICI182780. Hypothetically, the protective properties of phytoestrogen may give clinical pancreatic β cell protection through the activation of estrogen receptor, by which the proliferative activity, viability, pro-survival protein Bcl-2 and even insulin-secreting property of pancreatic β cells may be restored in the condition of diabetes or hyperglycemia.

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The authors state that they have no Conflict of Interest (COI).

References
2. Miksicek RJ. Interaction of naturally occurring non-steroidal estrogens with expressed with recombinant human estrogen receptor. J...