Anti-apoptotic Function and Mechanism of Ginseng Saponins in Rattus Pancreatic β-Cells

Fang Chen,†,‡ Yuewen Chen,†,‡ Xincong Kang,‡ Zhiguang Zhou,‡ Zhixu Zhang,‡,* and Dongbo Liu†,‡,*

†Horticulture and Landscape College, Hunan Agricultural University; Changsha 410128, China; ‡State Key Laboratory of Sub-health Intervention Technology, State Administration of Traditional Chinese Medicine; Changsha 410128, China; *Key Laboratory for Crop Germplasm Innovation and Utilization of Hunan Province; Changsha 410128, China.

Received May 20, 2012; accepted June 18, 2012

Apoptosis is the main form of β-cell death in diabetes. Ginseng has been used as an anti-diabetic herb for several thousand years in Asia with ginsenoside Rg1 and ginsenoside Rb1 as important active ingredients. In this study, we demonstrated ginsenoside Rg1 and Rb1 protect β-cells from high glucose/cytokine-induced pancreatic β-cell apoptosis via inhibiting nitric oxide (NO) production and regulating apoptosis-related genes. Among these genes, Bax, Fas and Caspase-3 gene expressions were up-regulated by high glucose, whereas only Bax and Caspase-3 gene expression were elevated by cytokines. In these two stimuli-induced apoptotic cells, Rg1 down-regulated Fas gene expression, while Rb1 decreased Caspase-3 gene expression. As a conclusion, Fas signal pathway and mitochondria stress is mostly related to anti-diabetes function of ginsenoside Rg1, while Caspase-3 pathway is essential when Rb1 is present.

Key words apoptosis; ginsenoside; high glucose; cytokine; diabetes

Type 1 diabetes mellitus (T1DM) is an autoimmune disease caused by absolute insulin deficiency due to destruction of the pancreatic β-cells. Type 2 diabetes mellitus (T2DM) is a metabolic disorder of non-insulin-dependent and slow in onset characterized by a progressive decline in β-cell function and chronic insulin resistance.1,2) Both types are characterized by progressive β-cell failure and recent treatments are focusing on enhancing endogenous β-cell function and/or regeneration.3) Because apoptosis is probably the main form of β-cell death,4) apoptosis-caused β-cell mass decreasing and function impairing have provided a hopeful target in diabetes treatment.5) It is suggested that cytokines, lipotoxicity and glucotoxicity are three main stimuli for β-cell apoptosis.4,6,7) High glucose and cytokine have been shown to induce β-cell death via apoptosis and/or necrosis.8–10) Chronic exposure to elevated levels of glucose causes β-cell dysfunction and may induce β-cell apoptosis in T2DM. Exposure to high glucose has dual effects, triggering initially "glucose hypersensitization" and later apoptosis, via different mechanisms.11) After prolonged exposure to cytokines, functional impairment results in T1DM.12) Cytokines may induce direct deleterious effects on β-cells via generation of toxic oxygen free radicals and nitric oxide (NO), as well as sensitizing β-cells to T-cell-mediated cytotoxicity in vivo by up- and down-regulating multiple genes.12,13)

There are three key apoptosis signaling pathways in β-cells, namely mitochondria pathway, death signal pathway and endoplasmic reticulum (ER) stress pathway. In the process of mitochondria pathway, free radicals, Bcl-2 family chronic lymphocytic leukemia (CLL)/lymphoma-2 (Bcl-2) family-regulated, apoptotic proteases-activating factor 1 (ApaF-1) and cysteine proteases cysteine aspartase-9 (Caspase-9) are required14,15), death signal pathway involves Fas or tumor necrosis factor (TNF)R1-activated and demanded Fas-associated protein with death domain (FADD) and Caspase-8.16,17) The ER stress pathway is mediated by transcription factor C/EBP homologous protein (CHOP)/growth arrest and DNA damage induced gene (GADD)153 and Caspase-12.18–20) The major functional players in apoptosis are caspase, adaptor proteins, and proteins from the TNF receptor (TNF-R) family and the Bcl-2 family.14 Each of the three pathways converges to a common execution phase of apoptosis which requires proteolytic activation of Caspase-3 through both extrinsic and intrinsic signaling pathway.21–23) Members of Bcl-2 family are pro-apoptotic or anti-apoptotic,14,24) The pro-apoptotic/anti-apoptotic balance of Bcl-2 family members, such as Bcl-2/Bcl-2-associated X protein (Bax),25) will effect apoptosis by cytochrome c release and Caspase activation.24) Fas is a cell-surface protein mediating apoptosis. Cytokines can induce up-regulation of Fas expression in β-cells, making them susceptible to apoptosis in the presence of agonistic anti-Fas antibodies, or interaction with Fas ligand (Fasl)-expressing T-cells.26–28) It is well known that cytokine such as interleukin (IL)-1β, TNF-α and interferon (IFN)-γ, could induce expression of inducible nitric oxide synthase (iNOS) and then lead to NO formation within pancreatic β cells, which contributes to a major extent to β-cell necrosis and apoptosis.12,29–31)

Ginseng, often described as the “king herb,” has been used as an anti-diabetic herb for several thousand years in Asia. The anti-diabetic actions of ginseng might mediate through a variety of mechanisms including actions on improving systemic insulin sensitivity,32) increasing the activity of a glucose transporter protein,33) and reducing glycolgenolysis, thus restraining hyperglycemia.34) Moreover, ginseng treatment increased insulin release from pancreatic β-cells, which is probably caused by increasing β-cell stimulation and insulin synthesis.35–37) Ginseng consists of a number of active constituents, such as polysaccharides, peptides, polyacetylenic alcohols and fatty acids,38) of these, and most pharmacological properties are mainly attributed to ginsenosides.39) Ginsenosides Rb1 and Rg1 hold important positions in stimulatory and inhibitory effects on central nervous system.40,41) Rg1 also

The authors declare no conflict of interest.

*To whom correspondence should be addressed. e-mail: chinasaga@163.com

© 2012 The Pharmaceutical Society of Japan
plays a role of anti-apoptosis in neonatal rats with hypoxia ischemia brain damage, and could inhibit Caspase-3 activation by strengthening and stabilizing hypoxia inducible factor-1α signal pathway.\textsuperscript{43} Rb1 was shown to protect cells from apoptosis induced by UVB radiation through the induction of DNA repair.\textsuperscript{43} Rb1 also could repress cell death induced by 6-hydroxydopamine in SH-SY5Y neuroblastoma cells,\textsuperscript{44} and protect dopaminergic cells from oxidative stress.\textsuperscript{45, 46} Within pancreatic β-cells, there is few reported result concerning the anti-apoptotic function of Rb1 and Rg1 in the treatment of diabetes.

The aim of the study was to investigate the effects of ginsenosides Rb1 and Rg1 on stimuli-induced pancreatic β-cell apoptosis and explore its mechanisms regarding signal transduction pathway.

MATERIALS AND METHODS

Cells Culture and Drug Treatment Rattus pancreatic β-cell line, Rin-m5F, was obtained from tumor cells bank of Chinese Academy of Medical Sciences (CAMS). Rin-m5F cells were cultured in RPMI1640 medium with 12% fetal calf serum, which adjusted to contain 2 mM l-glutamine, 1.5 g/L sodium bicarbonate, 10 mM glucose, 10 mM HEPES-N-2-hydroxyethyl) piperazine-N'-2-ethanesulfonic acid (HEPES), and 1.0 mM sodium pyruvate. Cells were subcultured every 3–4 d, two to three times weekly. Rin-m5F cells (after passages 4) were seeded into 6-well plate at a density of 1 \times 10^5 per well and grown for 48 h, then divided into 9 groups: control group (contained 10 mM glucose), high glucose group (contained 25 mM glucose), cytokines group (contained 10 mM glucose, 50 U/mL IL-1β, 1000 U/mL TNF-α, 1000 U/mL IFN-γ), high glucose + ginsenoside Rg1 group (contained 25 mM glucose and 20 mM Rg1), high glucose + ginsenoside Rb1 group (contained 25 mM glucose and 20 mM Rb1), high glucose + Rg1 + Rb1 group (contained 25 mM glucose, 20 mM Rg1 and 20 mM Rb1), cytokines + Rg1 group (contained cytokines motioned before and 20 mM Rg1), cytokines + Rb1 group (contained cytokines and 20 mM Rb1), cytokines + Rg1 + Rb1 group (contained cytokines, 20 mM Rb1 and 20 mM Rb1), and cultured for 48 h.

Apoptosis Assay by Fluorescence Activated Cell Sorting (FACS) Apoptosis was determined quantitatively by flow cytometry using the Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) Kit (KeyGEN Biotechnology, Nanjing, China) according to the manufacturer’s instruction. After 48 h treatment, culture supernatants were collected for floating dying and apoptosis cells. Adherent cells were rinsed with phosphate buffered saline (PBS) and harvested by brief trypsinization. Cell fractions were combined, washed with PBS, and then double-stained with FITC-conjugated Annexin V and PI for cellular staining for 15 min at room temperature. Flow cytometric analyses were performed on a FACS Calibur™ (Becton Dickinson, U.S.A.) within 1 h. The apoptotic cells were measured respectively in the upper right or lower right quadrant of the FACS histogram. In the early stages of apoptosis, the cells were stained by Annexin V-FITC, but excluded by PI (FITC+/PI-), because the cell membrane remained intact. While in the late stages, plasma membrane integrity is broken, and it enables PI to exist and bind to DNA and makes cells positive for both Annexin V-FITC and PI (FITC+/PI-).

Insulin Secretion Assay Supernatants were collected after 48 h exposure, insulin concentration was determined by direct chemiluminescent immunoassay using ADVIA® Centaur XP System (Siemens, Germany). The steps of measurement were conducted according to the instruction of insulin detection kit (Siemens, Germany).

Nitric Oxide (NO) Measurement Biologically produced NO is rapidly oxidized to nitrite and nitrate in aqueous solutions.\textsuperscript{47} In this study, NO production was measured as nitrite concentration in cell-free culture supernatants using the NO detection kit (Jiancheng Bioengineering Institute, Nanjing, China) by colorimetric assay. After 48 h treatment, 100 µL of culture supernatant was collected for examining the NO concentration. The absorbance was measured at 540 nm by using a spectrophotometer (UV-1800, Shimadzu, Japan). NO concentration was calculated using the absorbance by the formula described in the instruction.

Isolation of RNA, cDNA Synthesis and Real-Time Reverse Transcription Polymerase Chain Reaction (RT-PCR) Total RNA was isolated using TRIzol reagent (CoWin Biosciences, Beijing, China). The quality of the isolated RNA was assessed by electrophoresis on 1% agarose gels based on the integrity of 28S, 18S and 5S bands after ethidium bromide staining. The total RNA concentration was quantified by measuring absorbance at 260 nm. cDNA was synthesized from RNA by the reverse transcription of 0.5 µg of total RNA using the ReverAid™ First Strand cDNA Synthesis Kit (Fermentas, Canada) in a total volume of 20 µL. The reaction mixture

<table>
<thead>
<tr>
<th>Target genes</th>
<th>Primer</th>
<th>Amino acid sequences (5'–3’)</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcl-2</td>
<td>Forward primer</td>
<td>TGGGATGCCTTTGTGGGAACCT</td>
<td>125 bp</td>
</tr>
<tr>
<td>Bax</td>
<td>Forward primer</td>
<td>CAGGTATGACCACTGATGATG</td>
<td>128 bp</td>
</tr>
<tr>
<td>Fas</td>
<td>Forward primer</td>
<td>GACGCTGAGAGGATGATTGCT</td>
<td>125 bp</td>
</tr>
<tr>
<td>iNOS</td>
<td>Forward primer</td>
<td>GTTGGCAAGGCTAAGGATG</td>
<td>120 bp</td>
</tr>
<tr>
<td>Caspase-3</td>
<td>Forward primer</td>
<td>TGGGATTCTTTGTGGGAACCT</td>
<td>112 bp</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward primer</td>
<td>GGAGCAAAAAAGGGCAACCA</td>
<td>111 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
<td>TTCTCTTGGAGGACCATT</td>
<td>111 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
<td>CCTACCCACCTCCAGTATTG</td>
<td>111 bp</td>
</tr>
</tbody>
</table>
was then incubated at 42°C for 1 h, 70°C for 5 min. Real-time PCR was performed on the StepOnePlus™ Real-Time PCR System of Applied Biosystems. The ratus’ cDNA sequences of Bcl-2, Bax, Fas, Caspase-3, iNOS and GAPDH were obtained from GenBank, and then designed primers by Primer Express 3.0 software (ABI). The primer sequences were showed in Table 1. For quantitative real-time PCR, each reaction was run in triplicate and contained the following: 3.2 µL Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen, U.S.A.), 0.6 µL Forward primer and Reverse primer (10 µM), 0.2 µL ROX Reference Dye (Invitrogen, U.S.A.) and 0.4 µL cDNA template in a final reaction volume of 10 µL. Cycling parameters were 50°C for 2 min and 95°C for 2 min to activate DNA polymerase, and then followed by 40 cycles of 95°C for 15 s and annealing at 60°C for 30 s. After amplification, the melting curve analysis was performed as 95°C for 15 s, 60°C for 30 s and 95°C for 15 s to confirm formation of the expected PCR products. Products from all assays were subjected to 2% agarose gels electrophoresis to confirm specificity. The emission data were quantified using the cycle threshold (Ct) value. Data were normalized to GAPDH and presented as the mean fold change as compared to controls.

**Statistical Analysis**  All the experiments were repeated 3 times and the data were presented as mean±S.E.M. Statistical differences between the groups were analyzed by using the one-way analysis of variance (ANOVA) followed by Tukey’s test of SPSS19.0 statistical software. A value of p<0.05 was considered significant and p<0.01 highly significant.

**RESULTS**

**Ginsenosides Reduced the Percentages of Apoptotic Cells Induced by High Glucose/Cytokines** After 48h stimulation, high glucose or cytokines increased β-cells apoptosis by 2.50 and 4.74 fold, respectively. However, after coculturing with Rg1 or Rb1, the percentages of apoptotic cells were largely reduced, and Rb1 is more effective than Rg1. When the two ginsenosides were put together, anti-apoptotic effect was more evident (Fig. 1).

**Ginsenosides Protected High Glucose/Cytokines-Impaired Insulin Secretion** After exposure to high glucose or cytokines, the insulin concentrations of supernatant significantly decreased to 60.48% or 63.33% compared to control group. Ginsenoside Rg1 or Rb1 protected high glucose/cytokines-impaired insulin secretion. However, two ginsenosides together did not show additional effect on this protective function (Fig. 2).

**Ginsenosides Inhibited High Glucose/Cytokines-Induced iNOS Expression and Consequent NO Production** As shown in Fig. 3, the relative expression levels of iNOS and NO production induced by high glucose were increased to 2.14 and 2.05 fold respectively, compared to control group. The two values were greatly reduced under treatment of ginsenosides. Moreover, similar result was observed in the cytokines-induced apoptotic cells.

**Ginsenosides Affected Bcl-2, Bax, Fas and Caspase-3 Genes Expression Levels of β-Cells Induced by High Glucose** After exposure to high glucose, relative expression levels of Bax, Fas and Caspase-3 genes increased by 1.46, 1.51 and 2.32 fold, however, ginsenoside Rg1 reduced 30.14% and 66.89% of high glucose-induced Bax and Fas gene expression, respectively, no negative regulation of Caspase-3 gene expression was observed. On the other hand, Rb1 performed significant reductions in Bax, Fas and Caspase-3 gene expression induced by high glucose stimulation (Fig. 4).

**Ginsenosides Affected Bcl-2, Bax, Fas and Caspase-3 Genes Expression Levels in Cytokine-Induced Cells** After exposed to cytokines for 48h, β-cell showed increase of Bax, Fas and Caspase-3 gene expression up to 1.39, 1.20 and 2.18 fold respectively compared to control group. Meanwhile, Bcl-2 gene relative expression level decreased to 0.64. Rg1 down-regulated Bax and Fas gene expression, nevertheless, Rb1 reduced Bax and Caspase-3 gene expression but not Fas gene expression (Fig. 5).
Fig. 2. Effects of Ginsenosides on Insulin Secretion in Rin-m5F Cells Induced by High Glucose/Cytokine

A, cells were induced by high glucose and treated with or without ginsenosides; B, cells were induced by cytokines and treated with or without ginsenosides. Data are shown as mean±S.E.M. of 3 independent experiments. **p<0.01 vs. control group; #p<0.01 vs. high glucose/cytokine group.

Fig. 3. Effects of Ginsenosides on NO Production and iNOS Gene Expression in Rin-m5F Cells Induced by High Glucose/Cytokines

A and C, Rin-m5F cells were induced by high glucose and treated with or without ginsenosides; B and D, cells were induced by cytokines and treated with or without ginsenosides. A and B showed concentration of NO, and C and D showed iNOS mRNA expressions. Data are shown as mean±S.E.M. of 3 independent experiments. **p<0.01 vs. control group; #p<0.05, ##p<0.01 vs. high glucose/cytokine group.

Fig. 4. Effects of Ginsenosides on Expression of Apoptosis-Related Genes in High Glucose-Induced Rin-m5F Cells

The results are reported as mean±S.E.M. of 3 independent experiments. *p<0.05, **p<0.01 vs. control group; #p<0.05, ##p<0.01 vs. high glucose group.

Fig. 5. Effects of Ginsenosides on Expression of Apoptosis-Related Genes in Cytokines-Induced Rin-m5F Cells

The results are reported as mean±S.E.M. of 3 independent experiments. *p<0.05, **p<0.01 vs. control group; #p<0.05, ##p<0.01 vs. cytokine group.
DISCUSSION

Apoptosis is probably the main form of β-cell death in T1DM and T2DM. In this study, we selected two of the main stimuli for β-cell apoptosis—high glucose and cytokines to construct apoptotic cell models. It has been reported that high glucose concentration impairs islet function by disturbing glucose metabolism in the mitochondria of β-cell and could induce apoptosis. In addition, high glucose could increase β-cell vulnerability to toxic damage by increasing the expression of potential autoantigens on the cell membrane surface. Cytokines (IL-1β, TNF-α and IFN-γ) may be directly cytotoxic to β-cells by inducing NO and oxygen free radicals, inhibiting insulin synthesis and secretion, destroying β-cells. Meanwhile, cytokines may sensitize β-cells to T-cell-mediated cytoxicity in vivo by up-regulating MHC class I expression on the β-cells (an action of IFN-γ), and inducing Fas expression of β-cells (actions of IL-1β, and possibly TNF-α and IFN-γ). Cytokines cause additional production of chemokines, further increasing insulitis and the consequent dysfunction and damage of β-cells.

In this study, we observed the loss of β-cells and reduction of cell function, which indicated the success of apoptotic β-cell model construction. Mechanisms underlying high glucose/cytokine-induced β-cell apoptosis include increasing NO production together with up- and down-regulation of apoptosis-related gene expressions. Among these genes, Bax, Fas and Caspase-3 gene expression were up-regulated by high glucose, whereas only Bax and Caspase-3 gene expression were elevated by cytokines. In addition, cytokines instead of high glucose down-regulated Bcl-2 gene expression. As a conclusion, Fas signal pathway is important in high glucose-induced apoptosis, while mitochondria pathway plays a key role in cytokine-induce apoptosis.

Ginsenoside Rg1/Rb1, separately or in combination, protected pancreatic β-cell from high glucose/cytokine-induced cell loss and function reduction. Both ginsenosides reduced iNOS gene expression and consequent NO production. However, the apoptosis-related genes involved in the protective function of the two agents are different. In high glucose-induced apoptotic cells, Rg1 down-regulated Fas gene expression, while Rb1 decreased Caspase-3 gene expression. Similar result was observed in the cytokines-induced apoptotic cells. Of note, in these cells, the impaired Bcl-2/Bax ratio (0.53) was protected by Rg1 with the value 1.04, which is comparable to control condition.

In conclusion, high glucose and cytokine induce pancreatic β-cells apoptosis via promoting NO production and regulating apoptosis-related genes. Both ginsenoside Rg1 and Rb1 protect β-cells from these two stimuli-induced apoptosis. The mechanism of anti-apoptotic effects of Rg1 is mostly related with Fas signal pathway and mitochondria stress, whereas Caspase-3 pathway is essential when Rb1 is present.

Acknowledgements This work was supported by a Grant of the New Century Excellent Research Award Program from Ministry of Education of the People’s Republic China (NECT-10-0141).

REFERENCES

33) Ohnishi Y, Takagi S, Miura T, Usami M, Kako M, Ishihara E, Yano
30) Liu DB, Darv
38) Lee FC. Facts about ginseng: The elixir of life
36) Waki I, Kyo H, Yasuda M, Kimura M. Effects of a hypoglycemic
28) Loweth AC, Williams GT, James RFL, Scarpel
34) Chung SH, Choi CG, Park SH. Comparisons between white ginseng
24) Friedlander RM. Apoptosis and caspases in neurodegenerative dis-
25) Hengartner MO, Horvitz HR. H. C. elegans cell survival gene ced-9
39) Attele AS, Wu JA, Yuan CS. Ginseng pharmacology: multiple con-
35) Eizirik DL, Pavlovic D. Is there a role for nitric oxide in
41) Benishin CG. Actions of ginsenoside Rbl on choline uptake in central
34) Attele AS, Zhou YP, Xie JT, Wu JA, Zhang L, Dey L, Pugh W, Rue
42) Wang D, Huang Y, Li Q, Xu S, Liu X. Anti-apoptotic effect of gins-
31) Eizirik DL, Pavlovic D. Is there a role for nitric oxide in β-cell dys-
40) Tsang D, Yeung HW, Tso WW, Peck H. Ginseng saponins: influence on neurotransmitter uptake in rat brain synaptosomes. Planta Met-
44) Sharma A, Bonner-Weir S, Weir G. Beta-cell adaptation to hyper-
37) Parkinso
20) Regulatory Corp.
47) Mo
20) Regulatory Corp.