Prunetin Protects Against Dexamethasone-Induced Pancreatic B-Cell Apoptosis via Modulation of p53 Signaling Pathway

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Abstract

Long-term administration of dexamethasone results in insulin resistance and pancreatic β-cell apoptosis. Prunetin (an O-methylated isoflavone, a type of flavonoid) is demonstrated to protect diabetes, but the molecular mechanism of this protection is still unclear. This study thus aims to investigate how prunetin protects against dexamethasone-induced pancreatic β-cell apoptosis. Rat insulinoma (INS-1) cells were cultured in medium with or without dexamethasone in the presence or absence of prunetin or pifithrin-α, a p53 inhibitor. Cell apoptosis was measured by Annexin V/propidium iodide staining. Dexamethasone significantly induced INS-1 apoptosis but dexamethasone plus prunetin significantly reduced INS-1 apoptosis. Dexamethasone-treated INS-1 upregulated p53 protein expression; the induction of p53 was also reduced in the presence of RU486, a glucocorticoid receptor (GR) inhibitor. This suggested that dexamethasone induced P53 via GR. Dexamethasone-treated INS-1 significantly increased p53, Bax, and Rb protein expressions, whereas treatments of dexamethasone plus prunetin or pifithrin-α significantly decreased these protein expressions. In addition, dexamethasone significantly decreased B-cell lymphoma 2 (Bcl2), while dexamethasone plus prunetin or pifithrin-α significantly increased Bcl2. Dexamethasone significantly increased caspase-3 activity while co-treatment of dexamethasone plus prunetin or pifithrin-α significantly decreased caspase-3 activity to the control level. Taken together, our results revealed that prunetin protected against dexamethasone-induced pancreatic β-cells apoptosis via modulation of the p53 signaling pathway.

Keywords

flavonoids, steroid, glucocorticoid receptor, p53, apoptosis, pancreatic β-cell

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Dexamethasone is one of the synthetic steroid drugs which have potent glucocorticoid (GC) effects. GC has been used for treatment in several diseases including asthma and autoimmune diseases.¹ One well-known effect of GC is hyperglycemia in people with or without diabetes.² Long-term GC usage is associated with a high incidence of new onset steroid-induced diabetes (NOSID).³ However, the pathophysiology of NOSID is still unclear. It is well known that GC induces insulin resistance, increases hepatic glucose production, and inhibits glucose uptake into muscle. The major effect of GC is the induction of gluconeogenesis in the liver. There are several mechanisms that GC induces gluconeogenesis in the liver. First, GC enhances breakdown of peripheral fat and protein into free fatty acids and amino acids, which are delivered to the liver as substrates for gluconeogenesis.² Second, GC increases liver gluconeogenesis by inducing expression and actions of key enzymes including phosphoenolpyruvate carboxykinase and glucose-6-phosphatase.² Lastly, GC reduces the inhibitory effect of insulin on gluconeogenesis in the liver. GC-induced insulin resistance alone cannot cause diabetes since the impairment of pancreatic β-cell function plays a crucial role in the development of diabetes.² This notion suggested that GC might induce pancreatic β-cell dysfunction to develop NOSID.

The role of GC on pancreatic β-cells has been investigated. Acute effect of GC was shown to decrease insulin secretion in vitro in both pancreatic β-cell line and mouse pancreatic islet.¹⁰,¹¹ These studies demonstrated that GC reduced insulin secretion through several mechanisms, including decreased glucose uptake and oxidation, membrane depolarization, and
calcium-induced insulin exocytosis.\textsuperscript{10-12} Also, the patient who took GC drug had the reduction of insulin secretion.\textsuperscript{13} A defect of insulin biosynthesis and an induction of pancreatic β-cell apoptosis were found in chronic exposure of pancreatic β-cell to GC.\textsuperscript{14} GC induced pancreatic β-cell dysfunction through the glucocorticoid receptor (GR).\textsuperscript{15} This notion was supported by the evidence in mice that diabetes developed after specific overexpression of GR in pancreatic β-cells.\textsuperscript{15} Also, a similar finding was demonstrated in humans that increased GR sensitivity was related to metabolic syndrome.\textsuperscript{16} It is possible that dexamethasone might bind to GR and stimulate the p53 pathway. The induction of p53 could lead to pancreatic β-cell apoptosis.

Prunetin, an O-methylated isoflavone, is a phytoestrogen, which is found in licorice,\textsuperscript{17} red cherry,\textsuperscript{18} legumes, and soy bean.\textsuperscript{19} Prunetin has anti-inflammatory effects both in vitro and in vivo.\textsuperscript{20,21} It inhibited the expression of inflammatory cytokines, such as interleukin-6.\textsuperscript{22} Furthermore, prunetin exerted the anti-adipogenic effects in obese animals.\textsuperscript{23} Administration of prunetin significantly reduced body weight gain, visceral fat pad weights, and plasma glucose level.\textsuperscript{23} It decreased glucose levels while increased AMP-activated protein kinase (AMPK) activation and triglyceride levels in the study of a model organism.\textsuperscript{24} Also, it stimulated glucose uptake in muscle cells via increased AMPK-Akt pathways.\textsuperscript{25} Prunetin is well known as a potent tyrosine kinase inhibitor in vitro,\textsuperscript{26} and is capable of blocking growth factor action.\textsuperscript{27} The protective effect of prunetin against dexamethasone action on pancreatic β-cells is still unknown. We hypothesized that prunetin might prevent pancreatic β-cell apoptosis from dexamethasone treatment via inhibition of the p53 signaling pathway. The aim of this study was thus to verify the protective effect of prunetin against dexamethasone-induced pancreatic β-cells apoptosis by inhibition of the p53 signaling pathway.

Materials and Methods

**INS-1 Cell Culture**

Rat insulinoma (INS-1) cells (received as a gift from Professor C. Newgard) were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C in humidified air containing 5% carbon dioxide and the culture medium was changed every 2 days.

**Analysis of Cell Apoptosis by Using Annexin V-Fluorescein Isothiocyanate and PI Staining**

INS-1 (6.5 × 10^5 cells per plate) cells were cultured in the presence or absence of 0.1 µM dexamethasone, with or without 10 µM prunetin in the presence and absence of 10 µM pifithrin—p53 inhibitor. After incubation for 72 hours, the cells were collected and stained with annexin V-fluorescein isothiocyanate and propidium iodide (PI) (BD Biosciences, USA). Apoptotic cells were then analyzed by a FACSort flow cytometer (Becton Dickinson, USA).

**Nuclear and Cytoplasmic Protein Extraction**

Nuclear and cytoplasmic proteins were extracted using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit (Pierce, USA). Briefly, INS-1 cells were added with 10 µM of prunetin, 10 µM of imatinib, and 10 µM of pifithrin-α for 48 hours. Then, INS-1 cells were washed and trypsinized with 0.05% trypsin edetated ethylenediaminetetraacetic acid. After incubation, the cell pellet was collected by centrifugation at 4°C and was added with ice-cold Cytoplasmic Extraction Reagent I. Then, the cell pellet was incubated on ice for 10 minutes. After incubation, ice-cold Cytoplasmic Extraction Reagent II was added. The cell pellets were collected after centrifugation at 4°C. The supernatant which is cytoplasmic protein was transferred and stored at −70°C.

**Caspase-3 Activity Assay**

INS-1 cells were cultured in the presence or absence of 0.1 µM dexamethasone, with or without 10 µM prunetin in the presence and absence of 10 µM pifithrin for 72 hours. A caspase-3 assay was performed in accordance with the manufacturer’s protocol (Promega, Madison, WI, USA). Briefly, INS-1 were seeded into a 96-well plate at 1 × 10^4 cells per well and allowed to attach overnight. At 72 hours of treatment, 100 µL of caspase-Glo reagent was added to each well and gently mixed on a plate shaker for 30 seconds. The plates were then incubated at 37°C for 30 minutes in the dark. A substrate for luciferase (aminoluciferin) was released after caspase-3 enzyme cleavage. The luminescence was measured by a plate-reading luminometer (Synergy H1 Hybrid Multi-Mode Microplate Reader, Bio-Tek, Winooski, VT, USA). The change in the luminescence signal is directly proportional to caspase-3 activity.

**Western Blot Analysis**

The cells were lysed in a radioimmunoprecipitation assay buffer (Pierce, USA) and were quantified for total protein by a micro bicinchoninic acid protein assay kit. Total proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membrane (Bio-Rad, USA). After that, the membrane was blocked with 5% skimmed milk. The membrane was incubated with one of the following primary antibodies: anti-p53 (Santa Cruz Biotechnology, USA), anti-Bax (Santa Cruz Biotechnology, USA), anti-Rb (Santa Cruz Biotechnology, USA), and anti-Bcl2.
Dexamethasone with or without 10 µM of RU486 (a GR protein expression via GR, INS-1 cells were cultured with 0.1 µM early apoptosis of pancreatic β-cells (Figure 1(c & d)).

Prunetin or pifithrin-α significantly decreased the percentage out dexamethasone whereas co-cultured dexamethasone with pancreatic β-cells when compared with the control condition with- Dexamethasone significantly increased early apoptosis of pancreatic β-cells (Figure 1(a)). Cell morphology corresponded to cell death significant when compared with that of the control condition (Figure 1(b)). P53 protein expression of INS-1 cells treated with prunetin or pifithrin-α alone was not significantly different from that of INS-1 cells cultured in the control condition. This result suggests that the protective effect of prunetin against dexamethasone-induced pancreatic β-cell apoptosis may be through the inhibition of p53 signaling pathway.

Results

The Effective Dose of Dexamethasone That Induced Cell Death in INS-1 Cells

To identify the dose of dexamethasone induced pancreatic β-cell death, INS-1 cells were incubated with or without dexamethasone at 0.01, 0.1, 1, and 10 µM for 72 hours. Then, the numbers of cell death were measured by PI staining. The percentage of cell death was increased in correspondence with the dose of dexamethasone when compared with the control condition. Dexamethasone at dose 0.1, 1, and 10 µM significantly increased the percentages of cell death when compared with those cultured without dexamethasone. Only dexamethasone at 0.01 µM, the number of cell death was increased but not significant when compared with that of the control condition (Figure 1(a)). Cell morphology corresponded to cell death results (Figure 1(b)).

Prunetin and Pifithrin-α Decreased Cell Apoptosis in INS-1 Treated with Dexamethasone

Our preliminary data from RT² polymerase chain reaction (PCR) array suggested that prunetin reduced pancreatic β-cell apoptosis via reduction of p53 (data not shown). To verify whether prunetin exerted its effect through p53, 10 µM of prunetin or 10 µM of pifithrin-α-p53 inhibitor was added into INS-1 cells with 0.1 µM dexamethasone. After 72 hours, cell apoptosis was examined by annexin V/PI staining. Dexamethasone significantly increased early apoptosis of pancreatic β-cells when compared with the control condition without dexamethasone whereas co-cultured dexamethasone with prunetin or pifithrin-α significantly decreased the percentage early apoptosis of pancreatic β-cells (Figure 1(c & d)).

To examine whether dexamethasone upregulated p53 protein expression via GR, INS-1 cells were cultured with 0.1 µM dexamethasone with or without 10 µM of RU486 (a GR inhibitor), and nuclear p53 protein was measured by Western blot analysis. Dexamethasone significantly induced nuclear p53 whereas RU486 reduced nuclear p53 protein (Figure 2(a)).

Effects of Prunetin on P53 Protein Expression in Pancreatic β-Cells Treated with Dexamethasone

To confirm that prunetin protected against pancreatic β-cell apoptosis from dexamethasone via reduction p53, p53 protein expression was assessed by Western blot analysis. INS-1 cells were treated with prunetin or pifithrin-α in the presence or absence of 0.1 µM dexamethasone. P53 protein expression was significantly increased in INS-1 cells treated with dexamethasone when compared with those cultured without dexamethasone. On the contrary, prunetin or pifithrin-α incubated with dexamethasone significantly brought the level of p53 protein expression back to the level of the control condition (Figure 2(b)). P53 protein expression of INS-1 cells treated with prunetin or pifithrin-α alone was not significantly different from that of INS-1 cells cultured in the control condition. This result suggests that the protective effect of prunetin against dexamethasone-induced pancreatic β-cell apoptosis may be through the inhibition of p53 signaling pathway.

Effect of Prunetin and Pifithrin-α on Apoptotic Downstream Proteins of P53 Pathway in Pancreatic β-Cells Treated with Dexamethasone

To examine whether prunetin protected against dexamethasone-induced pancreatic β-cell apoptosis via decreased apoptotic downstream proteins of p53 pathway, Bax and Rb protein expressions were assessed by Western blotting analysis. INS-1 cells were pretreated with 10 µM of prunetin or 10 µM of pifithrin-α. Then, the cells were treated with or without 0.1 µM dexamethasone. As shown in Figure 3(a and b), INS-1 cells treated with dexamethasone significantly increased both Bax and Rb protein expression when compared with that of the control condition while co-treatment significantly decreased both Bax and Rb protein expression when compared with dexamethasone alone. There was no significant change in Bax and Rb protein expression in INS-1 cells treated with prunetin or pifithrin-α when compared with control. This result suggested that prunetin protected against dexamethasone-induced pancreatic β-cell apoptosis by decreased Bax and Rb protein expression.

Effect of Prunetin and Pifithrin-α on Antiapoptotic Protein–Bel2 in Pancreatic β-Cells Treated with Dexamethasone

To examine the effect of prunetin on the increment of antiapoptotic protein–Bel2 protein expression, INS-1 cells were pretreated with 10 µM of prunetin or 10 µM of pifithrin-α. Then, the cells were treated with or without 0.1 µM
Dexamethasone, and Bcl2 protein expression was determined by Western blotting. Dexamethasone significantly decreased Bcl2 protein expression when compared with that of the control condition. Co-treatment significantly increased Bcl2 expression when compared with dexamethasone alone. There was no difference in Bcl2 expression in INS-1 cells treated with prunetin or pifithrin-α alone when compared with those of the control condition (Figure 4(a)). This result suggested that prunetin protected against dexamethasone-induced pancreatic β-cell apoptosis by increased Bcl2 protein expression.

Effect of Prunetin and Pifithrin-α on Caspase-3 Activity in Pancreatic β-Cells Treated with Dexamethasone

To examine the effect of prunetin on the downstream protein of mitochondria-induced pancreatic β-cell apoptosis, caspase-3 activity was measured by luminescent assay. Dexamethasone significantly increased caspase-3 activity compared with the control condition. Co-treatment significantly decreased caspase-3 activity when compared with dexamethasone alone. Prunetin or pifithrin-α alone had no...
effect on caspase-3 activity when compared with the control condition (Figure 4(b)). This result suggested that prunetin and pifithrin-α protected against mitochondria induced pancreatic β-cell apoptosis.

Discussion
Several synthetic GCs have been clinically used for the treatment of inflammatory and autoimmune diseases. One
serious adverse effect in the excessive use of GCs is “steroid-induced diabetes”, which is related to systemic insulin resistance and decreased pancreatic β-cell mass. The condition of insulin resistance results in decreased glucose consumption and high blood glucose level, which in turn induces insulin secretion and increases insulin synthesis. The long-term increase in insulin synthesis can cause endoplasmic reticulum stress and pancreatic β-cell apoptosis. Similarly, GC treatment can also cause pancreatic β-cell death.

This study thus aimed to examine the effect of dexamethasone-induced pancreatic β-cell apoptosis and investigated the antagonistic effect of prunetin—an isoflavone phytoestrogen that protected against dexamethasone-induced pancreatic β-cell apoptosis.

The results of the present study demonstrated that dexamethasone markedly increased pancreatic β-cell death in a dose-dependent manner at the concentration ranging from 0.1 to 10 µM (Figure 1). These are comparable to those of other studies, which showed that the concentrations at 0.1 and 1 µM of dexamethasone induced mitogen-inducible gene 6 (Mig6), leading to the pancreatic β-cell death in a dose-dependent manner. In addition, the concentration of dexamethasone at 1 µM significantly increased the death of pancreatic β-cell line from hamster. The concentrations of dexamethasone at 1 and 10 µM also inhibited MC3T3-E1 proliferation and induced cell death in a dose-dependent manner. These findings were agreeable with that of the present study that dexamethasone 0.1 µM induced apoptosis of INS-1 cells. Consistent with the present study, it was reported that half-maximal inhibitory concentration value of dexamethasone was 0.092 µM in INS-1 cells. Moreover, the patients’ plasma levels of dexamethasone for mean diurnal levels ranged from 100 to 510 nmol/L.

The result of our preliminary study showed that p53 was upregulated in INS-1 cells cultured with dexamethasone by apoptotic real-time PCR array (data not shown). Thus, this study aimed to examine the role of p53 in dexamethasone-induced pancreatic β-cell apoptosis and to investigate the role of prunetin in the prevention of dexamethasone-induced pancreatic β-cell apoptosis. Dexamethasone markedly increased INS-1 cell apoptosis and nuclear p53 protein expression while prunetin and pifithrin-α (a specific p53 inhibitor) co-incubated with dexamethasone reduced both apoptosis and nuclear p53 expression. Prunetin and pifithrin-α could rescue dexamethasone-induced pancreatic β-cell apoptosis and decreased nuclear p53 expression in a similar manner. Thus, these results suggested that dexamethasone might induce pancreatic β-cell apoptosis via p53 signaling pathway, and prunetin could protect against dexamethasone induced-pancreatic β-cell apoptosis via suppressed nuclear p53. Our results were similar to those of the previous studies that dexamethasone inhibited cell modulation by p53 activation in neural cell lines, rat glioma cells, and human lung carcinoma cells. Moreover, knockdown of p53 prevented dexamethasone-induced senescence in tenocytes.

The results of the present study suggested that dexamethasone activated p53-induced apoptosis in the pancreatic β-cell line.

It is known that p53 induced cell apoptosis by increasing proapoptotic protein, Bax, and decreasing antiapoptotic protein, Bcl-2.

**Figure 4.** Effect of prunetin and pifithrin-α treated with or without dexamethasone on Bcl-2 protein expression. (a) Above picture is a representative Western blot of β-actin proteins. The bar graph below demonstrates fold change in Bcl-2 protein normalized to β-actin protein. (b) Effect of prunetin and pifithrin-α treated with or without dexamethasone on caspase-3 activity. The bar graph below demonstrates fold change in caspase-3 activity. Data were presented as mean ± standard error of mean (N = 3). *P < 0.05, **P < 0.01, ***P < 0.001 significantly different from control. ##P < 0.01, ###P < 0.001 significantly different from dexamethasone treatment.
protein, Bcl2. In the present study, we showed that dexamethasone indeed increased Bax and decreased Bcl2 protein expression, while prunetin and pifithrin-α co-treatment with dexamethasone reduced Bax and increased Bcl2 protein expression. Activated Bax forms a pore at the outer mitochondrial membrane that leads to cytochrome c release. Then, cytochrome c activates its cascade and ensues cell apoptosis. The upregulation of Bax was found to be mediated by p53 that triggers the intrinsic mitochondria-mediated apoptotic pathway. Dexamethasone upregulated p53 and Bax were reported in transgenic mice. Also, dexamethasone was reported to induce neural cell apoptosis by promoting transcription of proapoptotic gene—Bax. Bcl-2 overexpression has been shown to inhibit β-cell apoptosis. Increased Bax and decreased Bcl2 produced cytochrome c release from mitochondria. Released cytochrome c induced caspase-3 activity, which leads to apoptosis. Similar results were shown in this study that dexamethasone increased caspase-3 activity while co-treatment decreased caspase-3 activity. Thus, our results suggested that prunetin and pifithrin-α protected against dexamethasone induced pancreatic β-cell via decreased Bax, increased Bcl2, and decreased caspase-3 activity.

The p53 also promotes cell apoptosis via induction of cell cycle arrest. P53 mediated growth arrest by dephosphorylation of Rb. Then, dephosphorylated Rb bound to transcriptional factor E2F as a complex, which inhibited deoxyribonucleic acid (DNA) synthesis and cell proliferation. While, in cell proliferation, Rb was phosphorylated and dissociated from transcriptional factor E2F; then, E2F moved to transactivate gene involved in DNA synthesis. Mice lacking both Rb and p53 frequently developed insulinomas and other islet tumors in several tissues. In addition, p53-activated Rb was an essential suppressor of mouse β-cell proliferation in vivo. Our study demonstrated that dexamethasone increased Rb expression while co-treatment had opposite effects. In agreement with our results, dexamethasone was shown to increase Rb protein expression in INS-1 cells. Therefore, our results suggested that dexamethasone induced pancreatic β-cell apoptosis through p53 signaling pathway via increased Rb protein expression, and prunetin and pifithrin-α prevent this effect. It is also possible that prunetin might promote cell proliferation. Prunetin promoted bone regeneration via activation of mitogen-activated protein kinase (MAPK) signaling in osteoblasts. Prunetin might act similarly to other phytoestrogens that stimulated the MAPK-extracellular signal regulated kinase pathway to promote cell differentiation. Upregulate antioxidant gene expression, and activation of non-genomic estrogen receptor-α signaling. Another possibility is that p53 might inhibit NF-E2-related factor 2 (NRF2). NRF2 induces the antioxidant response. This can promote oxidative stress and cell death. However, further study is required to clarify these issues.

In conclusion, this study demonstrated for the first time that prunetin protected against dexamethasone-induced pancreatic β-cell apoptosis via the reduction of p53 signaling pathway. A better understanding of the protective mechanism of prunetin will facilitate the development of novel drugs or supplements that prevent steroid-induced diabetes.

Declaration of Conflicting Interests
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