Butein from *Rhus verniciflua* Protects Pancreatic β Cells against Cytokine-Induced Toxicity Mediated by Inhibition of Nitric Oxide Formation

Gil-Saeng Jeong, Dong-Sung Lee, Mi-Young Song, Byung-Hyun Park, Dae-Gill Kang, Ho-Sub Lee, Kang-Beom Kwon, and Youn-Chul Kim

Received June 21, 2010; accepted October 12, 2010

Butein (3,4,2'-4'-tetrahydroxychalcone), a plant polyphenol, is a major component in isolate of *Rhus verniciflua* Stokes (Anacardiaceae). It is shown to exert various potent effects such as antioxidant, anti-inflammatory induction of apoptosis among many properties. In this study, we investigated the effect of butein on cytokine-induced β-cell damage. Pre-treatment with butein is shown to increase the viability of cytokine-treated INS-1 cells at concentrations of 15—30 μM. Butein prevented cytokine-mediated cell death, as well as nitric oxide (NO) production, and these effects correlated well with reduced levels of protein expression of the inducible nitric oxide synthase (iNOS). Furthermore, the molecular mechanisms by which butein inhibits iNOS gene expression appeared to be through the inhibition of nuclear factor-κB (NF-κB) translocation. In a second set of experiments, rat islets were used to demonstrate the protective effects of butein and the results were essentially the same as those observed in Beutin pretreated INS-1 cells. Butein prevented cytokine-induced NO production, iNOS expression, and NF-κB translocation and inhibition of glucose-stimulated insulin secretion (GSIS). In conclusion, these results suggest that butein can be used for the prevention of functional β-cell damage and preventing the progression of Type 1 diabetes mellitus (T1DM).

Key words  butein; *Rhus verniciflua*; pancreatic β-cell; cytokine; nuclear factor-κB; glucose-stimulated insulin secretion

*Rhus verniciflua* Stokes (Anacardiaceae) has been widely used as a traditional herbal medicine in east asian countries such as Korea, China, and Japan, for its various pharmacological properties. *R. verniciflua* is known to possess diverse biological activities such as antioxidant, antiobesity,

anti-inflammatory, anti-apoptotic activity. Recent studies have suggested that an extract of *R. verniciflua* inhibits oxidative stress by scavenging the reactive oxygen species (ROS). It is a documented fact that an overproduction of ROS can have damaging effects on intracellular proteins. Antioxidant and anti-inflammatory activities of *R. verniciflua* is attributed to abundance of phenolics and flavonoids contents of *R. verniciflua* extract. Butein (3,4,2'-4'-tetrahydroxychalcone), a plant polyphenol, is one of the major active component of *R. verniciflua* traditionally been used in Korea, Japan, and China for the treatment of pain, parasitic infestations, and thrombotic diseases. Previous studies have shown that butein has various pharmacological effects, such as antioxidant and anti-inflammatory property, endothelium-dependent vasodilation, induction of apoptosis in B16 melanoma cells and human promyelocytic leukemia cells, inhibition of diabetes complications and inhibition of enzymes such as protein kinase and glutathione reductase. Although effects of butein in streptozotocin-induced diabetic rat tissues and its effect on the complications of diabetes have been previously studied but the effect and mechanism of butein action on pancreatic β-cells is yet to be determined at the molecular level.

Type 1 diabetes mellitus (T1DM), previously known as insulin-dependent diabetes mellitus (IDDM) is considered due to an autoimmune-mediated apoptosis or necrosis of insulin-producing pancreatic β-cells and destruction of Langerhans islets as evidenced by infiltration of pancreatic islets by mononuclear cells of the immune system, mainly T-lymphocytes and macrophages, leading to destruction of β-cells in type 1 diabetes. Previous studies have suggested that destruction of β-cells is as a result of islet-infiltrating cells and exposure to inflammatory cytokines, such as interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ). IL-1β is key in β-cell expression of inducible nitric oxide synthase (iNOS), production of nitric oxide (NO) and oxygen free radicals is the toxic component which in turn is potentiated by two other proinflammatory cytokines, TNF-α and IFN-γ. Thus, these cytokines are responsible for causing apoptosis of rat and human pancreatic β-cell. Cytokines exert their primary effects through the nuclear factor-κB (NF-κB) pathway. NF-κB is a key signaling mediator for cytokines such as IL-1β and IFN-γ, and it has been proposed that it also regulates transcription of the iNOS gene. NF-κB is initially located in the cytoplasm of unstimulated cells as inactive heterotrimeric complex composed of p50, p65 and inhibitory κB (IκB-α). A variety of inducers cause the dissociation of this heterotrimeric complex, presumably via the phosphorylation of IκB-α by IκB kinase, allowing NF-κB (p50·p65) dimers to be released from the heterotrimeric complex. Following which, the active NF-κB (p50·p65) dimers translocate into the nucleus and promotes the transcription of target genes resulting in gene expression.

In the present study, we identified butein to possess cytoprotective effects on cytokine-induced pancreatic β-cell and rat islets destruction. Our data showed that butein inhibited cytokine-induced NO production, iNOS expression, NF-κB signaling and damage to glucose-stimulated insulin secre-
tion. Collectively, these all results indicate that butein from *R. verniciflua* may be used to prevent functional β-cell death and thus may be useful as an antidiabetic agent.

**MATERIALS AND METHODS**

**Chemicals and Reagents** Butein (>95% pure) was isolated from the bark of *R. verniciflua* as described by Kang *et al.*37 Dried the bark of *R. verniciflua* were purchased from the University Oriental herbal drugstore, Iksan, Korea, in March 2004, and a voucher specimen (No. WP 04-12) was deposited at the Herbarium of the College of Pharmacy, Wonkwang University (Korea). Butein (NNMBP1) was deposited at the New Natural Material Bank of the College of Pharmacy, Wonkwang University (Korea). Quercetin (>98% pure) (Sigma-Aldrich, St. Louis, MO, U.S.A.) was used as a positive control. Roswell Park Memorial Institute medium-1640 (RPMI-1640), fetal bovine serum (FBS), and antibiotics were obtained from Gibco BRL (Gaithersburg, MD, U.S.A.). Tissue culture plates (96-well) and other tissue culture dishes were obtained from Falcon (Biosciences, Oxford, U.K.). All other chemicals, including 3′-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO), were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Butein was dissolved in DMSO to make a 30 mM solution (stock solution) and then diluted with the medium solution. The final DMSO concentration in each experimental and control well was kept constant at 0.1%. This final DMSO concentration showed no relevant effects on cellular growth and survival in our assay. IL-1β and IFN-γ were obtained from R&D (Minneapolis, MN, U.S.A.). Primary antibodies, including iNOS, IκB-α, p-IκB-α, p65 and responsible secondary antibodies used for Western blot analysis were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). All reagents were purchased from Sigma unless otherwise noted.

**Cell Culture** INS-1 cells, a rat pancreatic β-cell line, were obtained from Prof. Kang-Beom, Kwon (WonKwang University, Iksan, Korea) and maintained at 5×10^5 cells/ml culture in RPMI-1640 supplemented with containing 5.5 or 11 mM glucose, 10% heat inactivated FBS, 10 mM N-(2-hydroxyethyl)piperazine-N′-2-ethanesulfonic acid (Hepes), pH 7.4, 10.2 mM L-glutamine, 50 mM sodium pyruvate, 2.5 mM β-mercaptoethanol, streptomyacin (100 μg/ml), and penicillin G (100 IU/ml) and incubated at 37°C in a humidified atmosphere containing 5% CO₂ and 95% air.

**MTT Assay for Cell Viability** Effects of various experimental modulations on cell viability were evaluated by determining the mitochondrial reductase function on the basis of their ability to reduce tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), into formazan crystals.38 The formation of formazan is proportional to the number of functional mitochondria in living cells. After treatment with cytokines for 48 h, cells were washed twice with phosphate buffered saline (PBS), and then MTT solution was added to a concentration of 50 μg/ml in each well. After 4 h incubation at 37°C, the medium was discarded and the formazan blue that formed in the cells was dissolved in 150 μl of DMSO. Optical density was determined with a microplate reader at 570 nm. The optical density of formazan formed in control (untreated) cells was taken as 100% viability.

**Nitrite Measurement** Biologically produced NO is rapidly oxidized to nitrite and nitrate in aqueous solutions.39 Therefore, NO production was evaluated by measuring nitrite concentrations in the cell-free culture supernatant using a colorimetric assay. After treatment with cytokines for 24 h, 100 μl aliquots of the culture supernatants were incubated with 100 μl of a modified Griess reagent (1 : 1 mixture of 1% sulfanilamide in 30% acetic acid and 0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride in 60% acetic acid) at room temperature for 5 min, at this time, the absorbance at 540 nm was measured using a spectrophotometer. The concentration of NO was determined using a linear standard curve generated from serial dilutions of sodium nitrite in working medium.

**Isolation of Islets and Glucose-Stimulated Insulin Secretion (GSIS) Assay** Pancreatic islets were isolated from male Sprague-Dawley rats using the collagenase digestion method, as described previously.40 After treatment with cytokines for 24 h, islets were then washed three times in Krebs-Ringer bicarbonate buffer (25 mM Hepes, 115 mM NaCl, 24 mM NaHCO₃, 5 mM KCl, 1 mM MgCl₂, 2.5 mM CaCl₂, and 0.1% bovine serum albumin, pH 7.4) containing 3 mM D-glucose, and insulin secretion was then measured by static incubation of the islets for 30 min in the presence of either 5.5 or 20 mM D-glucose. The insulin content of the medium was then determined by ELISA (Linco Research, St. Charles, MO, U.S.A.).

**Western Blot Analysis** Western blot analysis was performed by lysing cells in 20 mM Tris–HCl buffer (pH 7.4) containing a protease inhibitor mixture (0.1 mM phenylmethanesulfonyl fluoride, 5 mg/ml aprotinin, 5 mg/ml peptatin A, and 1 mg/ml chymostatin). Protein concentration was determined with the Lowry protein assay kit (P5626; Sigma). The homogenates, which each contained 20 μg of protein, were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 7.5% (for iNOS, p65, and Lamin B) or 12% resolving (for IκBα and p-IκBα) and 3% acrylamide stacking gels, and then transferred to a Hybond enhanced chemiluminescence (ECL) nitrocellulose membrane (Biorad). Next, the membrane was blocked with 5% skimmed milk and then incubated for 4 h with 1 μg/ml of primary antibody for iNOS, IκBα, p-IκBα, p65, β-actin, Lamin B (Santa Cruz Biochemicals, Santa Cruz, CA, U.S.A.). Horseradish peroxidase-conjugated immunoglobulin G (IgG) was used as a secondary antibody followed by ECL detection (Amersham).

**Preparation of Nuclear Extracts** After treatment with cytokines for 1 h, cells were harvested, washed with ice-cold PBS buffer, and kept on ice for 1 min. The suspension was mixed with buffer A (10 mM HEPES, pH 7.5, 10 mM KCl, 0.1 mM ethylene glycol bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA), 0.1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride, 5 mg/ml aprotinin, 5 mg/ml pepstatin, and 10 mg/ml leupeptin) and lysed by three freeze–thaw cycles. Cytosolic fractions were obtained by centrifugation at 12000×*g* for 20 min at 4°C. The pellets were resuspended in buffer C (20 mM HEPES, pH 7.5, 0.4 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 5 mg/ml aprotinin, 5 mg/ml pepstatin, and...
10 mg/ml leupeptin), kept on ice for 40 min, and centrifuged at 14000×g for 20 min at 4°C. The resulting supernatant was used as the soluble nuclear fraction. The sample was then centrifuged for 20 min at 12000×g. Aliquots of the nuclear extracts were stored at −80°C, and the protein concentration was determined using the Bradford method.41)

**Statistical Analysis** Data are expressed as mean±S.D. of at least three independent experiments. To compare three or more groups, one-way analysis of variance (ANOVA) followed by Newman–Keuls post hoc test was used. Statistical analysis was performed with Prism software, version 3.03 (GraphPad Software Inc.). Differences associated with a p<0.05 were considered statistically significant.

**RESULTS**

The cytotoxicity of butein (Fig. 1) to INS-1 cells was measured by MTT assay. As shown in Fig. 2A, cell viability was not significantly reduced by butein up to the concentration of 30 μM. Thus, INS-1 cells were treated with butein in the concentration range of 3.75—30 μM during follow-up experiments. The apoptotic or necrotic destruction of pancreatic β-cells is thought to be related at least in part by cytokines such as IL-1β, TNF and IFN-γ.32,33) INS-1 cells (5×10⁴ cell/ml) were pretreated with the indicated concentrations of butein (Fig. 2B) for 3 h, and then treated with IL-1β (2 ng/ml) for 48 h. Cell viability was also determined using MTT assay. As shown in Fig. 2A, cell viability was not significantly reduced by butein up to the concentration of 30 μM butein for 3 h, and then treated with IL-1β alone, whereas cells when pretreated with butein (3.75—30 μM) showed significantly reduced overexpression of protein levels of iNOS (Fig. 3A). Butein significantly inhibited cytokine-mediated NO production in a dose-dependent manner, with maximum inhibition occurring at 30 μM (Fig. 3B). Quercetin, as the positive control, significantly reduced cytokine-mediated overexpression of iNOS protein and iNOS-derived NO production (Figs. 3A, B). These results suggest that the cytoprotective effect of butein against the effects of IL-1β is due to the inhibition of NO production via inhibition of iNOS expression.

NF-κB has been implicated in the transcriptional regulation of cytokine-induced NO production and iNOS expression.42—44) In the present study, we estimated the effect of butein on IL-1β-stimulated translocation of NF-κB from the cytosolic compartment to the nucleus in INS-1 cells. IL-1β (2 ng/ml)-treated INS-1 cells showed increased IκBα degradation and phosphorylation in the cytosol, and p65 subunit levels in the nucleus (Fig. 4) when compared to unstimulated control cells. However, IL-1β (2 ng/ml)-induced NF-κB activation was markedly inhibited by pretreatment with butein (Fig. 4), which suggests that butein inhibited iNOS expression through reduction of NF-κB activation. In another study it was observed that IκBα is a major participant in IL-1β and IFN-γ-induced NF-κB activation in rat pancreatic β-cells.45) Therefore, in this study we investigated alterations of IκBα levels in the cytosol fraction after cytokine treatment. IL-1β-treated INS-1 cells showed increased IκBα degradation and phosphorylation of protein levels (Fig. 4). The increased IκBα degradation and phosphorylation in the cytosol, and p65 subunit levels in the nucleus due to cytokine treatment was markedly suppressed by treatment with butein.

To support the physiological importance of the results obtained from the cell line studies, we demonstrated the protective effects of butein using pancreatic islets isolated from male Sprague-Dawley rats. Rat islets (30 islets total) were pretreated with the indicated concentrations of butein for 3 h,
and then treated with IL-1β (2 ng/ml) and IFN-γ (100 U/ml) for 24 h. At a non-cytotoxic concentration (3.75—30 μM), rat islets were challenged with IL-1β and IFN-γ in the presence or absence of the butein and the levels of iNOS (Fig. 5A) expression and iNOS-derived NO (Fig. 5B) production were measured. Pretreatment of the rat islets with butein for 3 h resulted in decreased iNOS expression (Fig. 5A) and reduced iNOS-derived NO (Fig. 5B) production. Butein significantly prevented combination cytokines-mediated iNOS expression and NO production with maximum inhibition occurring at 30 μM (Figs. 5A, B). To examine whether butein inhibited NO production via inhibition of iNOS protein expression, we used Western blot analysis. And also, the positive control, quercetin at 50 μM, significantly reduced cytokine-mediated overexpression of iNOS protein and iNOS-derived NO production in pancreatic islets (Figs. 5A, B).

In the present study, results consistently showed the protective effects of butein on cytokines-induced destruction of pancreatic β-cells. To provide functional data, we tested whether butein is protective against IL-1β and IFN-γ-induced impairment of glucose-stimulated insulin secretion in rat islets. After 24 h of exposure to the IL-1β and IFN-γ, insulin secretion was observed in response to 20 mM glucose (Fig. 6). Control islets secreted insulin at a concentration of 25.03 ± 2.3 ng/10 islets, whereas insulin secretion from combination cytokines-treated islets decreased significantly to 10.3 ± 3.5 ng/10 islets (Fig. 6). However, pretreatment with the indicated concentrations of butein for 3 h blocked the effects of the combination cytokines, and restored islets insulin secretion to levels similar to that of the control in concentration of 30 μM (Fig. 6).

**DISCUSSION**

*Rhus verniciflua* Stokes (Anacardiaceae) has been a traditional herbal medicine in East Asia and it is well known that *R. verniciflua* has been found to have various biological activities. 3—10) Previous studies have shown that butein isolated from *R. verniciflua* has various pharmacological effects, especially antioxidant and antiinflammatory activities. 12—14) In
this study, we present a possible mechanism by which butein exerts its cytoprotective action against cytokine-induced pancreatic β-cell destruction as pretreatment with butein from R. verniciflua resulted in protection against the development of Type 1 diabetes (T1DM).

T1DM is caused by pancreatic β-cells destruction as a result of immune cell infiltration and production of inflammatory cytokines such as IL-1β, TNF-α and IFN-γ. These cytokines have a direct role in inducing β-cells destruction or death. It is well known that IL-1β and/or IFN-γ stimulate iNOS expression and NO production in pancreatic β-cells, leading to an increase in β-cell destruction, possibly due to apoptosis, necrosis, and other processes. In other previous reports, inhibitors of iNOS-derived NO production is observed to protect insulin-secreting pancreatic β-cells against cytokines-mediated destruction, but the efficacy is dependent on the species and the cytokines used. There is a broad agreement that IL-1β and/or IFN-γ-induced pancreatic β-cells destruction is widely used as a model for T1DM. Therefore, we first examined that INS-1 cells, pretreated with the indicated concentrations of butein for 3 h, and then treated with IL-1β for 48 h. Treatment with IL-1β significantly reduced the INS-1 cell viability. However, pretreatment with 15 and 30 μM butein for 3 h increased the viability of IL-1β treated INS-1 cells. Thus, we also observed that treatment with cytokines, IL-1β and/or IFN-γ resulted in an increase in iNOS expression and NO production in INS-1 cells and rat islets, and butein effectively inhibited the destructive effects of these cytokines. These results indicate that iNOS expression and iNOS-derived NO production is a crucial factor in cytokine-induced toxicity of INS-1 cells and rat islets. Moreover, β-cells protective effect of butein against the effects of IL-1β is primarily due to the inhibition of NO production via reduction of iNOS expression. And also, quercetin was used as the positive control in this study. It is well known that quercetin is emerging as potent anti-inflammatory agents. In addition, the protective effects of quercetin against cytokine-induced pancreatic β-cells damage have previously been reported. Therefore, the process leading to the production of iNOS expression and NO production in response to treatment with cytokines and suppression of this process by butein was investigated in this study.

In pancreatic β-cells, inhibition of iNOS expression has been shown to prevent β-cells destruction, which suggests that increased NO production due to NF-κB activation is an important cellular signal in cytokine-induced β-cells destruction. NF-κB has been shown to control anti-apoptotic responses according to the modes of insults in β-cells and also it has been shown to regulate the expressions of pro-inflammatory genes that contribute to β-cells destruction especially genes such as iNOS. In addition, the promoters of other pro-inflammatory genes induced in β-cells also possess binding elements for NF-κB. The important role of NF-κB in β-cell destruction is underscored by the fact that suppression of NF-κB activation or translocation prevents IL-1β and/or IFN-γ-induced β-cell death and dysfunction both in vitro and in vivo experimental systems. Thus, to investigate whether NF-κB activation, a key signaling pathway leading to iNOS gene expression, could be an upstream target for the inhibitory effects of butein, we examined the effects of butein on the phosphorylation of 1xB-α and the nuclear translocation of NF-κB subunit, p65, in INS-1 cells. Our results clearly showed that phosphorylation and degradation of 1xB-α and the nuclear translocation of p65 induced by IL-1β were significantly attenuated after pre-treatment of INS-1 cells with butein.

In summary, we demonstrated that butein possesses a potent inhibitory effect on cytokine induced toxicity using both an insulinoma cell line and isolated rat pancreatic islets. We propose that the mechanism by which butein protects β-cells may be mediated by suppression of signaling in NF-κB pathways. The results of our study thus provide meaningful information not only on the mechanisms underlying β-cell death and dysfunction, but also on preventing the progression of T1DM. Thus, butein could be considered as an evolving novel therapeutic approach to salvage the pancreatic islet β-cell in T1DM.

Acknowledgements This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (MEST) (No. 2010-0029467).
REFERENCES

34) Ghosh S., Kariin M., Cell, 109, 81—96 (2002).