Crude extract of *Ceriporia lacerata* has a protective effect on dexamethasone-induced cytotoxicity in INS-1 cells via the modulation of PI3K/PKB activity

JI-HYE KIM[1], YU-KYOUNG PARK[1], JI-EUN KIM[2], SAM-PIN LEE[3], BYOUNG-CHEON KIM[2] and BYEONG-CHURL JANG[1]

[1]Department of Medical Genetic Engineering, Keimyung University School of Medicine, Dalseo-gu, Daegu 704-701; [2]Worldbiotech Co, Ltd., Sangju, Gyeongbuk 742-912; [3]Department of Food Science and Technology, Keimyung University, Dalseo-gu, Daegu 704-701, Republic of Korea

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Abstract. Excessive and/or long-term glucocorticoid therapy reduces β-cell mass and induces hyperglycemia, which contribute to the development of steroid-induced diabetes. *Ceriporia (C.) lacerata* is one of the white-rot fungi and has been used in bioremediations, such as lignocellulose degradation, in nature. The pharmacologic effect of *C. lacerata* on steroid-induced β-cell toxicity is not known. In this study, we evaluated the effect of a crude extract from a submerged cultivation of *C. lacerata* on the survival and apoptosis of INS-1 rat insulin-secreting cells exposed to dexamethasone (Dex), a synthetic diabetogenic glucocorticoid. Treatment with the *C. lacerata* crude extract (CLCE) largely blocked the Dex-induced reduction in survival and apoptosis of INS-1 cells. Moreover, CLCE treatment inhibited Dex-induced protein kinase B (PKB) dephosphorylation without affecting Dex-induced extracellular signal-regulated protein kinase-1/2 dephosphorylation and MKP-1 upregulation. Importantly, the protective effect of CLCE on Dex-induced cytotoxicity in INS-1 cells was attenuated by LY294002, an inhibitor of PI3K/PKB. CLCE treatment, however, did not protect the INS-1 cells from the cytotoxic effects triggered by other insults, such as interleukin-1β (an inflammatory cytokine), streptozotocin (a diabetogenic drug), thapsigargin (a calcium mobilizing agent), and tunicamycin (an ER stress inducer). Collectively, these findings demonstrate for the first time the ability of CLCE to specifically protect INS-1 cells from Dex-induced cytotoxicity through the modulation of the PI3K/PKB pathway. It is suggested that CLCE may be applied for the prevention and/or treatment of steroid diabetes in which reduction of β-cell survival and induction of β-cell apoptosis play pathogenic roles.

Correspondence to: Dr Byeong-Churl Jang, Department of Medical Genetic Engineering, Keimyung University School of Medicine, 1095 Dalgubeol-daero, Dalseo-gu, Daegu 704-701, Republic of Korea
E-mail: jangbc12@kmu.ac.kr

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Introduction

Glucocorticoids, due to their anti-inflammatory and immunosuppressive activity, are widely used as therapeutic tools for the treatment of many inflammatory and/or immune diseases, including rheumatoid arthritis, organ transplantation, leukemia, multiple sclerosis or neurological disorders. However, there is increasing evidence that glucocorticoid therapy is associated with numerous toxic and/or side effects, including steroid-induced diabetes (1,2). It has been demonstrated that long-term and/or high-dose glucocorticoid therapy induces hyperglycemia, in part, through the inhibition of insulin synthesis and secretion, insulin resistance of peripheral tissues, and increased hepatic glucose production, which may contribute to the incidence and/or development of steroid diabetes (3,4). Considering that steroid diabetes is also linked to steroid-induced β-cell toxicity, research towards the better understanding of the mechanisms underlying steroid-induced β-cell toxicity and identification of new drugs or substances that prevent and/or protect against steroid-induced β-cell toxicity is critical.

Previous investigations have shown that dexamethasone (Dex), a synthetic glucocorticoid, reduces the survival and induces the apoptosis of thymocytes, leukemia and insulin-secreting cells (5-9), and the effect is mediated through the release of mitochondrial cytochrome *c* (5,6), the activation of caspases and generation of reactive oxygen species (8), the inhibition of nuclear factor-κB (9,10), and/or the inhibition of phosphorylation of insulin receptor substrate-2 and protein kinase B (PKB) (11). A large body of evidence also demonstrates that Dex treatment leads to alteration of the expression and/or activities of many intracellular signaling proteins (12-14), including mitogen-activated protein kinase (MAPK) phosphatase-1 (MKP-1) and the family of MAPKs, which are involved in gene expression, cell survival and/or apoptosis. Notably, a recent study revealed that Dex treatment inhibited the phosphorylation of extracellular signal-regulated protein

kinase-1/2 (ERK-1/2), one member of the MAPK family, and stimulated expression of MKP-1, contributing to a reduction in pancreatic β-cell proliferation in islets from early lactating mothers (15). These results suggest that Dex-induced β-cell toxicity may be attributed, to a small extent, to the ability of Dex to modulate the expression and/or activities of these signaling proteins.

White-rot fungi, including *Ganoderma*, have been used for medicinal purposes for centuries in China, Japan and Korea (16). *Ceriporia (C.) lacerata* is one of the white-rot fungi and has been used in bioremediations in nature, such as lignocellulose degradation (17). The pharmacological effect of *C. lacerata* on steroid-induced β-cell toxicity is not known. In this study, we evaluated the effect of a crude extract from a submerged cultivation of *C. lacerata* on the survival and apoptosis of INS-1 rat insulin-secreting cells treated with Dex.

Materials and methods

Materials. RPMI-1640 medium, heat-inactivated fetal bovine serum (FBS) and penicillin-streptomycin were purchased from WelGENE, Inc. (Daegu, Korea). Antibody against procaspase-9 was purchased from Enzo Life Science, Inc. (Farmingdale, NY, USA). Antibody against phospho-eukaryotic initiation factor-2α (p-eIF-2α) was purchased from Epitomics (Burlingame, CA, USA). Antibodies against X-linked apoptosis of protein (XIAP) and interleukin-1β (IL-1β) were purchased from R&D Systems (Minneapolis, MN, USA). Antibodies against p-extracellular signal-regulated protein kinase-1/2 (p-ERK-1/2), p-PKB, T-ERK-1/2 (total ERK-1/2), T-PKB (total PKB) and T-eIF-2α (total eIF-2α) were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibody against poly(ADP-ribose) polymerase (PARP) was purchased from Roche Diagnostics (Mannheim, Germany). LY294002 and streptozotocin (STZ) were purchased from Biomol (Plymouth Meeting, PA, USA). Antibodies against p-pancreatic endoplasmic reticulum kinase (p-PERK), T-PERK (total PERK), activation of transcription factor-6 (ATF-6), MKP-1, glucose-regulated protein 78 (GRP78), goat anti-rabbit immunoglobulin G-horseradish peroxidase (IgG-HRP), and goat anti-mouse IgG-HRP were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Enzyme-linked chemiluminescence (ECL) Western detection reagents were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Proteinase inhibitor cocktail (100X) was purchased from Calbiochem (Madison, WI, USA). Bradford reagent was purchased from Bio-Rad (Hercules, CA, USA). Other reagents, including Dex, and the actin antibody were purchased from Sigma (St. Louis, MO, USA).

Preparation of a crude extract of C. lacerata (CLCE). *C. lacerata* was initially grown on a potato dextrose agar medium and was transferred to 50 ml of a seed culture (potato/dextrose broth) medium (both were from Difco Laboratories, Inc., Detroit, MI, USA) by punching out a portion (20 mm diameter). Culture broth (~50 ml) was aseptically homogenized at 10,000 x g for 3 min and inoculated 2% (v/v) into a culture medium with the following composition (g/l): glucose, 20; peptone, 2; yeast extract, 2; K_2HPO_4, 1; KH_2PO_4, 0.5; $MgSO_4$, 0.5. The pH value was adjusted to 5.0 before sterilization. The submerged culture was then carried out in a 5-liter jar fermentor (Fermentec Co., Ltd., Cheongwon-gun, Korea) containing 4 liters of the medium (at 300 x g, 25˚C) for 10 days. The total culture broth cultivated for 10 days was lyophilized and ground into powder. The powder (5 g), including mycelium, was added to distilled water (100 ml) and then shaken at 150 x g at 25˚C for 6 h. Samples were centrifuged at 10,000 x g for 20 min, and the resulting supernatant was filtered through a Whatman filter paper no. 2 (Whatman International Ltd., Maidstone, UK). The culture filtrate was mixed with 4 volumes of isopropyl alcohol, strirred vigorously and left overnight at 4˚C, following centrifugation at 10,000 x g for 20 min. The precipitate of the crude extract was lyophilized and the weight was estimated.

Cell culture. INS-1 rat insulin-secreting cells were grown in RPMI-1640 supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C under a humidified condition in 95% air and 5% CO_2.

Cell count analysis. INS-1 cells were seeded in 24-well plates ($1x10^5$/500 μl/well) overnight. INS-1 cells were then treated without or with Dex in the presence or absence of CLCE plus LY294002 for 24 h. Alternatively, INS-1 cells seeded in 24-well plates ($1x10^5$/500 μl/well) were treated for 24 h without or with Dex in the absence or presence of CLCE (with no heating) or heated CLCE (95˚C, 1 h). The number of surviving cells, which were not stained by trypan blue dye, was counted under a microscope. Approximately, <100 cells were counted for the analysis.

Measurement of DNA fragmentation. INS-1 cells were seeded in 6-well plates at a density of $0.5x10^6$ cells/well in a 2-ml volume the day before drug treatment. INS-1 cells were treated without or with Dex in the presence or absence of CLCE for 24 h. The conditioned cells were then harvested, washed, and lysed in buffer [50 mM Tris (pH 8.0), 0.5% sarkosyl, 0.5 mg/ml proteinase K and 1 mM EDTA] at 55˚C for 3 h, followed by addition of RNase A (0.5 μg/ml) and further incubation at 55˚C for 18 h. The lysates were centrifuged at 10,000 x g for 20 min. The genomic DNA in the supernatant was extracted with an equal volume of neutral phenol-chloroform-isoamyl alcohol mixture (25:24:1), and analyzed by electrophoresis on 1.7% agarose gel. The DNA was visualized and photographed under UV illumination after staining with ethidium bromide (0.1 μg/ml).

Preparation of whole cell lysates. To measure the effects of Dex and/or CLCE on the expression and/or activity of cell growth-, stress- or apoptosis-related proteins, including procaspase-9, PARP, MKP-1, PKB, ERK-1/2, PERK, eIF-2α, GRP78 and ATF-6, INS-1 cells were seeded in 6-well plates ($0.5x10^6$/2 ml/well) the day before drug treatment. INS-1 cells were then treated without or with Dex in the presence or absence of CLCE for 2, 4 or 8 h. Each time, the conditioned cells were washed twice with PBS and exposed to cell lysis buffer [50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 0.1% sodium dodecyl sulfate, 0.25% sodium deoxycholate, 1% Triton X-100, 1% Nonidet P-40, 1 mM EDTA, 1 mM EGTA and proteinase

inhibitor cocktail (1X)]. The cell lysates were collected in a 1.5-ml tube and centrifuged for 20 min at 4˚C at 12,000 x g. The supernatant was saved and protein concentrations were determined using Bradford reagent.

Western blot analysis. Proteins (50 μg) were separated by SDS-PAGE (10%) and transferred onto nitrocellulose membranes (Millipore). The membranes were washed with TBS (10 mM Tris, 150 mM NaCl) supplemented with 0.05% (vol/vol) Tween-20 (TBST) followed by blocking with TBST containing 5% (wt/vol) non-fat dried milk. The membranes were incubated overnight with antibodies specific for MKP-1 (1:2,000), p-PKB (1:2,000), T-PKB (1:2,000), p-ERK-1/2 (1:2,000), T-ERK-1/2 (1:2,000), p-PERK (1:2,000), T-PERK (1:2,000), p-eIF-2α (1:2,000), T-eIF-2α (1:2,000), GRP78 (1:2,000), procaspase-9 (1:2,000), PARP (1:2,000) or actin (1:5,000) at 4˚C. The membranes were then exposed to secondary antibodies coupled to horseradish peroxidase for 2 h at room temperature. The membranes were washed three times with TBST at room temperature. Immunoreactivities were detected by ECL reagents. Equal protein loading was assessed by the expression level of the actin protein.

Statistical analysis. Cell count analysis was carried out in triplicates and repeated three times. Data are expressed as the means ± standard error (SE). The significance of difference was determined by one-way ANOVA. All significance testing was based on a P-value of <0.05.

Results

Treatment with C. lacerata crude extract suppresses the Dex-induced reduction in the survival and apoptosis of INS-1 cells. Initially, the effect of CLCE on the survival of Dex-treated INS-1 cells was determined by cell count analysis. Compared with the control (column 1), treatment with Dex (100 nM, 24 h) largely reduced the survival of INS-1 cells (column 3) (Fig. 1A). However, treatment with CLCE at 5 or 10 μg/ml substantially blocked the reduction in INS-1 cell survival by Dex. Using a 10 μg/ml concentration, we next investigated whether CLCE modulates the Dex-induced apoptosis of INS-1 cells by measuring nuclear DNA fragmentation, an apoptotic marker. Compared with the control (lane 1), Dex treatment induced nuclear DNA fragmentation in INS-1 cells (lane 3) (Fig. 1B). However, CLCE treatment largely suppressed the Dex-induced nuclear DNA fragmentation in INS-1 cells.

Dex treatment leads to MKP-1 upregulation and PKB and ERK-1/2 dephosphorylation, but does not alter the expression and/or activity of GRP78, ATF-6, PERK, eIF-2α, caspase-9, and PARP in INS-1 cells. To understand the molecular and cellular mechanisms underlying Dex-induced INS-1 cytotoxicity, the effect of Dex treatment at early time points (2, 4 or 8 h) on the expression and/or activity of growth-, stress- and apoptosis-related proteins, including MKP-1, PKB, MKP-1, ERK-1/2, caspases, GRP78, PERK, eIF-2α and ATF-6, in INS-1 cells was investigated by western blot analysis. In INS-1 cells, Dex treatment at 2 h led to a strong upregulation of MKP-1 protein and the MKP-1 upregulation was

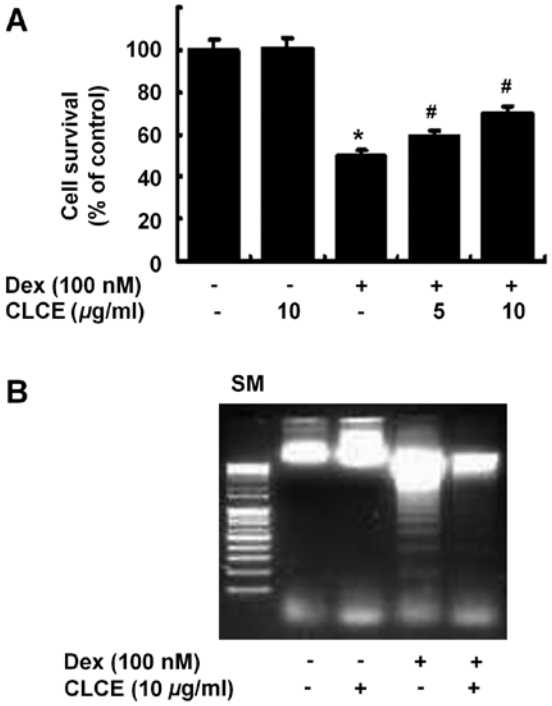

Figure 1. Effects of the *C. lacerata* crude extract (CLCE) on the Dex-induced reduction in survival and apoptosis in INS-1 cells. (A) INS-1 cells were pretreated without or with CLCE (5 or 10 μg/ml) for 1 h and treated without or with Dex (100 nM) in the absence or presence of CLCE for an additional 24 h. The number of surviving INS-1 cells was then counted under a microscope. Data are the means ± SE of three independent experiments. *P<0.05, compared to control value (no Dex). #P<0.05, compared to the value for Dex treatment without CLCE. (B) INS-1 cells were pretreated without or with CLCE (10 μg/ml) for 1 h and treated without or with Dex (100 nM) in the absence or presence of CLCE (10 μg/ml) for an additional 24 h. Extranuclear fragmented DNA from the conditioned cells was then extracted and analyzed on a 1.7% agarose gel. The image is a representative of three independent experiments.

maintained until 8 h (Fig. 2). Dex treatment at 2 h, however, did not affect PKB phosphorylation, but there was a strong and sustained repression of PKB phosphorylation thereafter. Strong and sustained ERK-1/2 dephosphorylation by Dex treatment at the times tested was also noted. However, expression of total PKB and ERK-1/2 remained unchanged by Dex treatment at the times applied. The expression of not only GRP78 and ATF-6 but also the phosphorylation of PERK and eIF-2α, four well-known ER stress markers, were not largely altered by Dex treatment at the times applied. Dex treatment also did not affect the expression of the inactive proform of caspase-9 (procaspase-9) and PARP. Expression of control actin remained constant during Dex treatment at the times applied.

CLCE specifically blocks the Dex-induced PKB dephosphorylation in INS-1 cells. We next determined the effect of CLCE on the Dex-mediated induction of MKP-1 expression and dephosphorylation of PKB and ERK-1/2 in INS-1 cells. For this, INS-1 cells were pretreated without or with CLCE for 1 h and then treated without or with Dex in the absence or presence of CLCE for an additional 4 h, followed by measurement of the expression and/or phosphorylation of MKP-1, PKB

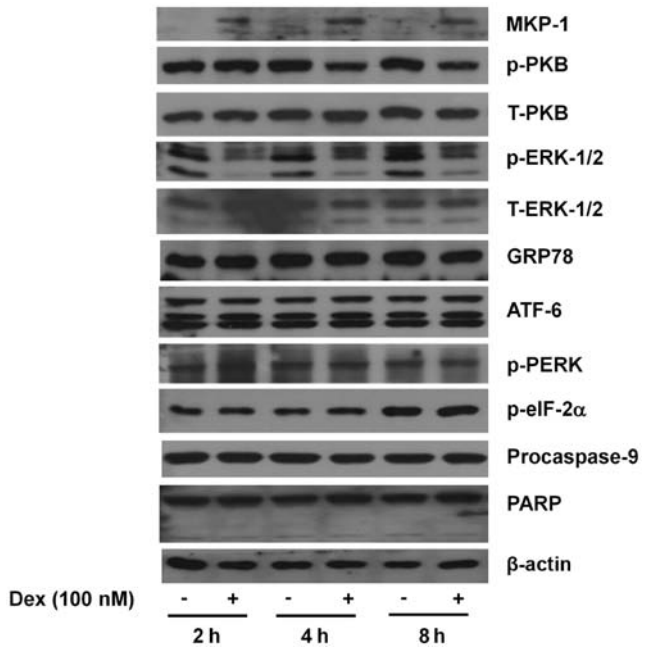

Figure 2. Effects of Dex on the expression and/or activities of MKP-1, PKB, ERK-1/2, GRP78, ATF-6, PERK, eIF-2α, procaspase-9 and PARP in INS-1 cells. INS-1 cells were treated without or with Dex (100 nM) for the indicated times. At each time, whole cell lysates were prepared and analyzed by western blot analysis using the respective antibody. Alternatively, the immunoblotting used for measuring phospho-protein was further stripped and reprobed with the antibody which recognizes total expression levels of the protein. The image is a representative of three independent experiments. p-PKB, phosphorylated PKB; p-ERK-1/2, phosphorylated ERK-1/2; T-ERK-1/2, total ERK-1/2; p-PERK, phosphorylated PERK; p-eIF-2α, phosphorylated eIF-2α.

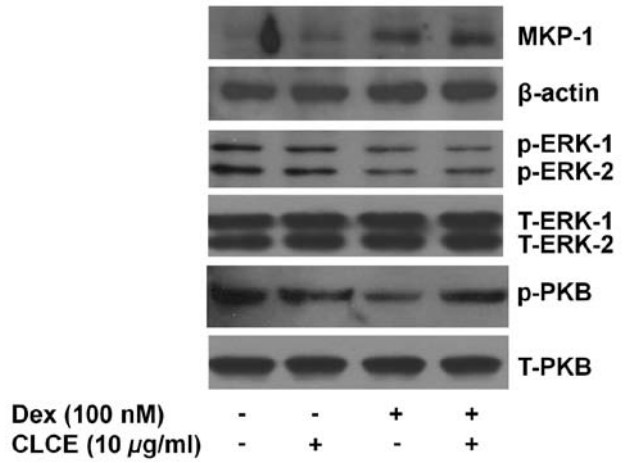

Figure 3. Effects of CLCE on the Dex-induced MKP-1 upregulation and PKB and ERK-1/2 dephosphorylations in INS-1 cells. INS-1 cells were pretreated without or with CLCE (10 μg/ml) for 1 h and treated without or with Dex (100 nM) in the absence or presence of CLCE for additional 4 h. Whole cell lysates were then prepared, and analyzed by western blot analysis using respective antibody. Alternatively, the immunoblot used for measuring phospho-protein was further stripped and reprobed with antibody which recognizes total expression levels of the protein. The image is a representative of three independent experiments. p-ERK-1/2, phosphorylated ERK-1/2; T-ERK-1/2, total ERK-1/2; p-PKB, phosphorylated PKB; T-PKB, total PKB.

and ERK-1/2 in the conditioned cells. CLCE treatment did not interfere with the Dex-induced MKP-1 upregulation and ERK-1/2 dephosphorylation, but attenuated the effect of Dex on PKB dephosphorylation (Fig. 3). Expression of actin, total ERK-1/2 and PKB remained constant following treatment with Dex and/or CLCE.

Protective effect of CLCE on the Dex-induced INS-1 cytotoxicity is attenuated by LY294002. We next investigated the enhanced PKB phosphorylation regarding the protective effect of CLCE on the Dex-induced cytotoxicity to INS-1 cells using LY294002, a pharmacological inhibitor of PI3K/PKB. INS-1 cells were pretreated without or with LY294002 for 1 h and treated without or with Dex in the absence or presence of CLCE for an additional 24 h, following measurement of the number of surviving cells by cell count analysis. As anticipated, single treatment with Dex largely reduced the survival of INS-1 cells (column 5) compared with the control (column 1) (Fig. 4). Of note, treatment with LY294002 alone slightly decreased the survival of INS-1 cells (column 3) and co-treatment with Dex and LY294002 resulted in a more repressive effect on the INS-1 cell survival (column 7) than Dex treatment alone (column 5), suggesting that the necessity of endogenous PI3K/PKB expression and/or activity for the survival of INS-1 cells. Notably, the protective effect of CLCE on the Dex-induced cytotoxicity of INS-1 cells was not evident in the presence of LY294002.

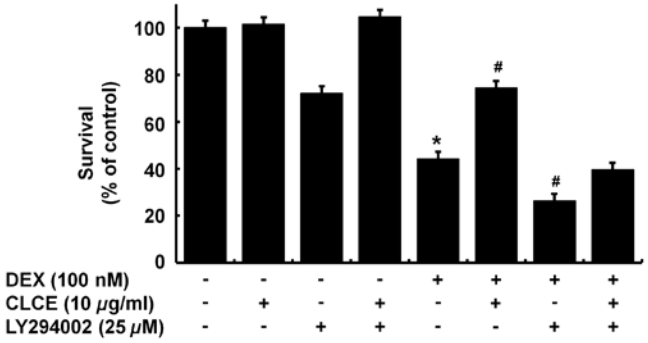

Figure 4. Attenuation of the protective effect of CLCE on the Dex-induced INS-1 cytotoxicity by LY294002 in INS-1 cells. INS-1 cells were pretreated without or with CLCE (10 μg/ml) and/or LY294002 (25 μM), a PI3K/PKB inhibitor for 1 h and treated without or with Dex (100 nM) in the absence or presence of CLCE and/or LY294002 for an additional 24 h. The number of surviving INS-1 cells was then counted under a microscope. Data are the means ± SE of three independent experiments. *P<0.05, compared to control value (no Dex, CLCE or LY294002). #P<0.05, compared to the value for Dex treatment without CLCE or LY294002.

CLCE does not protect INS-1 cells against the cytotoxicity induced by IL-1β, streptozotocin, thapsigargin or tunicamycin. To ascertain specificity, we next investigated the effect of CLCE on the cytotoxicity to INS-1 cells triggered by other insults, such as interleukin-1β (IL-1β, an inflammatory cytokine), streptozotocin (STZ, a diabetogenic durg), thapsigargin (TG, a calcium mobilizing agent and an ER stress inducer), and tunicamycin (TN, a protein N-glycosylation inhibitor and an ER stress inducer). Treatment with IL-1β, STZ, TG or TN strongly reduced the survival of INS-1 cells (Fig. 5A-D). CLCE treatment, however, did not block the IL-1β-, STZ-, TG- or TN-induced cytotoxic effect in INS-1 cells; rather CLCE treatment appeared to slightly enhance the IL-1β- or TN-induced INS-1 cytotoxicity.

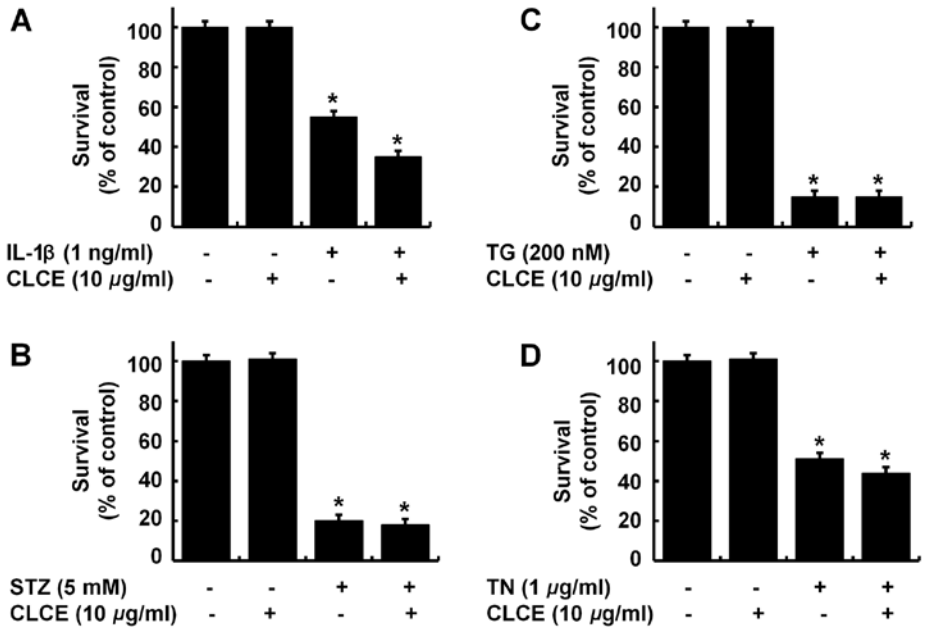

Figure 5. Effects of CLCE on the cytotoxicity to INS-1 cells triggered by other insults. INS-1 cells were pretreated without or with CLCE (10 μg/ml) for 1 h and treated without or with (A) IL-1β (1 ng/ml), (B) STZ (5 mM), (C) TG (200 nM) or (D) TN (1 μg/ml) for additional 24 h. The number of surviving INS-1 cells was then counted under a microscope. Data are the means ± SE of three independent experiments. *P<0.05 compared to control value (no IL-1β, STZ, TG or TN). #P<0.05, compared to the value for each drug treatment without CLCE. IL-1β, interleukin-1β; STZ, streptozotocin; TG, thapsigargin; TN, tunicamycin.

Discussion

Evidence suggests that excessive and/or long-term glucocorticoid therapy reduces β-cell mass in association with inhibition of β-cell survival and/or induction of β-cell apoptosis, which may lead to steroid diabetes. In the present study, we evaluated the effect of a crude extract from *C. lacerata* on the survival and apoptosis of INS-1 insulin-secreting cells in response to Dex exposure. Our data showed that CLCE has a protective effect on Dex-induced INS-1 cytotoxicity through the modulation of the PI3K/PKB pathway.

Through initial experiments, we demonstrated that CLCE largely blocks the Dex-induced decrease in the survival of INS-1 cells (Fig. 1A). Cells undergoing apoptosis have distinct biochemical and morphological characteristics, including nuclear DNA fragmentation (18,19). In our experimental conditions, Dex treatment induced nuclear DNA fragmentation in the INS-1 cells (Fig. 1B), suggesting the Dex-induced apoptosis of INS-1 cells. However, the Dex-induced apoptosis of INS-1 cells was strongly suppressed by CLCE, indicating that CLCE inhibits the Dex-induced apoptotic death of INS-1 cells.

Survival and/or apoptosis of cells, including INS-1, is largely influenced by the expression and/or activities of many intracellular signaling proteins, including PI3K, PKB (also known as Akt) and ERK-1/2. For instance, numerous studies have highlighted the role of PI3K, which catalyzes the production of phosphatidylinositol-3,4,5-trisphosphate, in cell survival pathways (20). The positive relationship between activation of PKB, a known major downstream target of PI3K, and cell survival has also been reported (21). There is further evidence that the activation of PKB signaling is critical for the regulation of β-cell mass and function in response to growth factors, incretins, and nutrients (glucose, amino acids) (22-24), and mediates anti-apoptotic effects induced by glucose, glucagon-like peptide-1, insulin-like growth factor-1, and insulin in β-cells (23-27), as well as protects β-cells against fatty acid-induced apoptosis (28). A role of ERK-1/2 in the quercetin-mediated protection of INS-1 cells from oxidative damage triggered by hydrogen peroxide (H_2O_2) has been previously shown (29). In numerous studies, glucocorticoids are shown to stimulate expression of MKP-1, which results in inhibition of the phosphorylation of numerous substrate proteins, including ERK-1/2 and p38 MAPK, which impair proliferation in human and mouse osteoblast cell lines (30-32). In line with this, a previous study found that Dex induced apoptosis of INS-1 cells by inhibiting phosphorylation of PKB and ERK-1/2 (11). Supporting these previous findings, we herein showed the ability of Dex to inhibit phosphorylation of PKB and ERK-1/2 but to induce expression of MKP-1 (Fig. 2). An important finding in this study is that CLCE blocked Dex-induced PKB dephosphorylation but did not interfere with the Dex-induced ERK-1/2 dephosphorylation and MKP-1 expression in INS-1 cells (Fig. 3), indicating that CLCE has the specific ability to restore (enhance) PKB phosphorylation in Dex-treated INS-1 cells. Of further importance, the present study showed that the protective effect of CLCE on Dex-induced INS-1 cytotoxicity was largely attributed to CLCE's restorative activity of PI3K/PKB, as deduced from the strong attenuation of the protective effect of CLCE by LY294002, a PI3K/PKB inhibitor (Fig. 4). The mechanism by which CLCE restores PKB phosphorylation in Dex-treated INS-1 cells remains uncertain. However, considering no effect of CLCE on the Dex-induced MKP-1 expression in INS-1 cells (Fig. 3), it is unlikely that the restorative effect of CLCE on PKB phosphorylation in the Dex-treated INS-1 cells was the MKP-1-dependent. Recent studies have shown that

inhibition of PKB phosphorylation is linked to the action of protein phosphatase-2A (PP-2A) (33) or the dephosphorylation of PIP3 molecules by the lipid phosphatase PTEN (34). Moreover, there are several lines of evidence that Dex treatment increases the activity of PP-2B, also called calcineurin (35), and treatment with the calcineurin inhibitor, FK506 or deltamethrin, attenuates Dex-induced apoptosis in INS-1 cells (36). It will be important to ascertain, in future experiments, whether CLCE modulates the expression (activity) of PP-2A, PP-2B and/or PTEN in Dex-treated INS-1 cells and whether treatment with pharmacological inhibitor or siRNA of PP-2A, PP-2B and/or PTEN alters the restorative effect of CLCE on PKB phosphorylation and/or the protective effect of CLCE on the apoptosis of INS-1 cells induced by Dex.

Induction of apoptosis is also closely linked to activation of caspases, a group of essential proteases required for the execution of cell death by apoptotic stimuli (37). In resting cells, caspases are synthesized as zymogens (inactive precursors), but upon exposure to apoptotic stimuli, they become processed via partial proteolytic cleavage and are activated in cells. Active caspase cleaves many cellular proteins, including PARP, protein kinase C-δ, and other vital proteins, leading to induction/execution of apoptosis (38,39). The Dex-mediated apoptotic cell death through the activation of the mitochondrial apoptotic pathway was previously revealed (5,6,40). A recent study also demonstrated that in INS-1 cells Dex induced apoptosis through the activation of caspase-3, downregulation of Bcl-2, dephosphorylation of BAD and mitochondrial depolarization (36), which further points to involvement of the mitochondrial-mediated activation of the caspase pathway in Dex-induced apoptosis in INS-1 cells. However, the present study, based on the fact that Dex treatment did not trigger activation of the caspase pathway in INS-1 cells (Fig. 2), excludes the possibility of involvement of the caspase pathway in the Dex-induced apoptosis of INS-1 cells. Differential effects of the caspase pathway on the Dex-induced apoptosis of INS-1 cells in the previous and present studies may be due to the different experimental conditions used (the treatment time of Dex: 48 vs. 2-8 h). Pancreatic β-cells have a well-developed ER and express high amounts of chaperones and protein disulfide isomerases to meet the high demand for translation of many proteins. There is strong evidence that excessive or severe ER stress in β-cells is linked to the development of diabetes (41) and also diabetes is a disease of ER stress (42). In general, cells undergoing ER stress display many cellular alterations, including upregulation of molecular chaperones (GRP78) (43), reduction of p90ATF-6 (44), and phosphorylation of eIF-2α (45). Thus, assuming that Dex treatment for 2 or 8 h does not change the expression and/or activities of ER stress markers, GRP78, ATF-6, PERK and eIF-2α, in INS-1 cells herein (Fig. 2), it is unlikely that Dex induces ER stress in INS-1 cells and that ER stress plays a role in the Dex-induced INS-1 cytotoxicity.

Evidence suggests that mushrooms have anti-diabetogenic properties (46,47). It has also been suggested that polysaccharides are the best known and most potent mushroom-derived substances with antidiabetes and antitumor activities (48,49). However, a recent study concerning ternatin, a highly methylated cyclic heptapeptide isolated from the mushroom *Coriolus versicolor*, was found to inhibit hyperglycemia (50) suggesting the anti-diabetogenic activity by mushroom peptides. In the present study, we know through phenol-sulfuric acid and bicinchoninic acid methods that the major components in CLCE are exopolysaccharides (EPS) and proteins, and the amount of EPS and proteins is ~50:50% (data not shown). It is therefore conceivable that the protective effect of CLCE herein is closely associated with the components of EPS and proteins (or peptides) in CLCE. Not only further fractionation of each component (EPS, proteins, and/or EPS-protein complex) in CLCE but also further biochemical characterization of the action mechanism by which each component mediates the protective effect of CLCE on Dex-induced INS-1 cytotoxicity warrant future study.

In addition to glucocorticoids, there are other diabetogenic factors that lead to type 1 and/or 2 diabetes. For instance, the inflammatory cytokines, including IL-1β, are well-known inducers of type 1 diabetes, and high levels of IL-1β are detected in newly diagnosed type 1 diabetic patients (51,52). Numerous studies have also shown that STZ, which is an antibiotic originally isolated from *Streptomyces achromogenes* and an N-acetyl-glucosamine analog, is cytotoxic to pancreatic β-cells after being transported through glucose transporter 2 (53) and is used to generate type 1 diabetes model animals (54). Recently, the stimulation of islet cell and/or β-cell apoptosis by classic ER stress inducers, such as TG, a pharmacological inhibitor of SERCA pumps that depletes ER calcium levels, or TN, an inhibitor of protein N-glycosylation, has been reported (55-57). In the present study, treatment of INS-1 cells with IL-1β, STZ, TG or TN strongly reduced the survival of INS-1 cells (Fig. 5A-D), suggesting that they all have a cytotoxic effect on INS-1 cells. However, we demonstrated that CLCE did not protect INS-1 cells from IL-1β-, STZ-, TG- or TN-induced cytotoxicity. These results strongly suggest that CLCE may have a unique ability to protect INS-1 cells against the cytotoxicity induced by Dex.

In conclusion, we demonstrated for the first time that CLCE has a protective effect on Dex-induced INS-1 cytotoxicity, and the effect is mediated through the modulation of the PI3K/PKB pathway. The findings presented herein may shed light on the possibility of applying CLCE, as a single and/or combinatorial regimen with other anti-diabetes therapies, for the prevention and/or treatment of steroid diabetes in which reduction of β-cell survival and induction of β-cell apoptosis play pathogenic roles.

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References

1. Hoogwerf B and Danese RD: Drug selection and the management of corticosteroid-related diabetes mellitus. Rheum Dis Clin North Am 25: 489-505, 1999.
2. Schacke H, Docke WD and Asadullah K: Mechanisms involved in the side effects of glucocorticoids. Pharmacol Ther 96: 23-43, 2002.
3. Lambillotte C, Gilon P and Henquin JC: Direct glucocorticoid inhibition of insulin secretion: an in vitro study of dexamethasone effects in mouse islets. J Clin Invest 99: 414-423, 1997.

4. Jeong IK, Oh SH, Kim BJ, *et al*: The effects of dexamethasone on insulin release and biosynthesis are dependent on the dose and duration of treatment. Diabetes Res Clin Pract 51: 163-171, 2001.
5. Hegardt C, Andersson G and Oredsson SM: Spermine prevents cytochrome c release in glucocorticoid-induced apoptosis in mouse thymocytes. Cell Biol Int 27: 115-121, 2003
6. Tonomura N, McLaughlin K, Grimm L, *et al*: Glucocorticoid-induced apoptosis of thymocytes: requirement of proteasome-dependent mitochondrial activity. J Immunol 170: 2469-2478, 2003.
7. Renner K, Amberger A, Konwalinka G, *et al*: Changes of mitochondrial respiration, mitochondrial content and cell size after induction of apoptosis in leukemia cells. Biochim Biophys Acta 1642: 115-123, 2003.
8. Roma LP, Bosqueiro JR, Cunha DA, *et al*: Protection of insulin-producing cells against toxicity of dexamethasone by catalase overexpression. Free Radic Biol Med 47: 1386-1393, 2009.
9. Amsterdam A, Tajima K and Sasson R: Cell-specific regulation of apoptosis by glucocorticoids: implication to their anti-inflammatory action. Biochem Pharmacol 64: 843-850, 2002.
10. Kovalovsky D, Refojo D, Holsboer F, *et al*: Molecular mechanisms and Th1/Th2 pathways in corticosteroid regulation of cytokine production. J Neuroimmunol 109: 23-29, 2000.
11. Avram D, Ranta F, Hennige AM, *et al*: IGF-1 protects against dexamethasone-induced cell death in insulin secreting INS-1 cells independent of AKT/PKB phosphorylation. Cell Physiol Biochem 21: 455-462, 2008.
12. Abraham SM, Lawrence T, Kleiman A, *et al*: Antiinflammatory effects of dexamethasone are partly dependent on induction of dual specificity phosphatase 1. J Exp Med 203: 1883-1889, 2006.
13. Wu W, Pew T, Zou M, *et al*: Glucocorticoid receptor-induced MAPK phosphatase-1 (MPK-1) expression inhibits paclitaxel-associated MAPK activation and contributes to breast cancer cell survival. J Biol Chem 280: 4117-4124, 2005.
14. Miller AL, Webb MS, Copik AJ, *et al*: p38 mitogen-activated protein kinase (MAPK) is a key mediator in glucocorticoid-induced apoptosis of lymphoid cells: correlation between p38 MAPK activation and site-specific phosphorylation of the human glucocorticoid receptor at serine 211. Mol Endocrinol 19: 1569-1583, 2005.
15. Nicoletti-Carvalho JE, Lellis-Santos C, Yamanaka TS, *et al*: MKP-1 mediates glucocorticoid-induced ERK1/2 dephosphorylation and reduction in pancreatic beta-cell proliferation in islets from early lactating mothers. Am J Physiol Endocrinol Metab 299: 1006-1015, 2010.
16. Paterson RR: Ganoderma - a therapeutic fungal biofactory. Phytochemistry 67: 1985-2001, 2006.
17. Lee JW, Gwak KS, Park JY, *et al*: Biological pretreatment of softwood *Pinus densiflora* by three white rot fungi. J Microbiol 45: 485-491, 2007.
18. Wyllie AH, Kerr JF and Currie AR: Cell death: the significance of apoptosis. Int Rev Cytol 68: 251-306, 1980.
19. Allen RT, Hunter WJ III and Agrawal DK: Morphological and biochemical characterization and analysis of apoptosis. J Pharmacol Toxicol Methods 37: 215-228, 1997.
20. Cantley LC: The phosphoinositide 3-kinase pathway. Science 296: 1655-1657, 2002.
21. Song G, Ouyang G and Bao S: The activation of Akt/PKB signaling pathway and cell survival. J Cell Mol Med 9: 59-71, 2005.
22. Elghazi L, Balcazar N and Bernal-Mizrachi E: Emerging role of protein kinase B/Akt signaling in pancreatic beta-cell mass and function. Int J Biochem Cell Biol 38: 157-163, 2006.
23. Brubaker PL and Drucker DJ: Glucagon-like peptides regulate cell proliferation and apoptosis in the pancreas, gut, and central nervous system. Endocrinology 145: 2653-2659, 2004.
24. Dickson LM and Rhodes CJ: Pancreatic beta-cell growth and survival in the onset of type 2 diabetes: a role for protein kinase B in the Akt? Am J Physiol Endocrinol Metab 287: 192-198, 2004.
25. Dickson LM, Lingohr MK, McCuaig J, *et al*: Differential activation of protein kinase B and p70(S6)K by glucose and insulin-like growth factor 1 in pancreatic beta-cells (INS-1). J Biol Chem 276: 21110-21120, 2001.
26. Srinivasan S, Bernal-Mizrachi E, Ohsugi M, *et al*: Glucose promotes pancreatic islet beta-cell survival through a PI 3-kinase/Akt-signaling pathway. Am J Physiol Endocrinol Metab 283: 784-793, 2002.
27. Wang Q, Li L, Xu E, *et al*: Glucagon-like peptide-1 regulates proliferation and apoptosis via activation of protein kinase B in pancreatic INS-1 beta cells. Diabetologia 47: 478-487, 2004.
28. Wrede CE, Dickson LM, Lingohr MK, *et al*: Protein kinase B/Akt prevents fatty acid-induced apoptosis in pancreatic beta-cells (INS-1). J Biol Chem 277: 49676-49684, 2002.
29. Youl E, Bardy G, Maqous R, *et al*: Quercetin potentiates insulin secretion and protects INS-1 pancreatic beta-cells against oxidative damage via the ERK1/2 pathway. Br J Pharmacol 161: 799-814, 2010.
30. Kassel O, Sancono A, Krätzschmar J, *et al*: Glucocorticoids inhibit MAP kinase via increased expression and decreased degradation of MKP-1. EMBO J 20: 7108-7116, 2001.
31. Lasa M, Abraham SM, Boucheron C, *et al*: Dexamethasone causes sustained expression of mitogen-activated protein kinase (MAPK) phosphatase 1 and phosphatase-mediated inhibition of MAPK p38. Mol Cell Biol 22: 7802-7811, 2002.
32. Engelbrecht Y, de Wet H, Horsch K, *et al*: Glucocorticoids induce rapid up-regulation of mitogen-activated protein kinase phosphatase-1 and dephosphorylation of extracellular signal-regulated kinase and impair proliferation in human and mouse osteoblast cell lines. Endocrinology 144: 412-422, 2003.
33. Gao T, Furnari F, Newton AC, *et al*: PHLPP: A phosphatase that directly dephosphorylates Akt, promotes apoptosis, and suppresses tumor growth. Mol Cell 18: 13-24, 2005.
34. Woodgett JR: Recent advances in the protein kinase B signaling pathway. Curr Opin Cell Biol 17: 150-157, 2005.
35. Tumlin JA, Lea JP, Swanson CE, *et al*: Aldosterone and dexamethasone stimulate calcineurin activity through a transcription-independent mechanism involving steroid receptor-associated heat shock proteins. J Clin Invest 99: 1217-1223, 1997.
36. Ranta F, Avram D, Berchtold S, *et al*: Dexamethasone induces cell death in insulin-secreting cells, an effect reversed by exendin-4. Diabetes 55: 1380-1390, 2006.
37. Fearnhead HO, Dinsdale D, Cohen GM, *et al*: An interleukin-1 beta-converting enzyme-like protease is a common mediator of apoptosis in thymocytes. FEBS Lett 375: 283-288, 1995.
38. Emoto Y, Manome Y, Meinhardt G, *et al*: Proteolytic activation of protein kinase C delta by an ICE-like protease in apoptotic cells. EMBO J 14: 6148-6156, 1995.
39. Lazebnik YA, Kaufmann SH, Desnoyers S, *et al*: Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. Nature 371: 346-347, 1994.
40. Almawi WY, Melemedjian OK and Jaoude MM: On the link between Bcl-2 family proteins and glucocorticoid-induced apoptosis. J Leukoc Biol 76: 7-14, 2004.
41. Fonseca SG, Burcin M, Gromada J, *et al*: Endoplasmic reticulum stress in beta-cells and development of diabetes. Curr Opin Pharmacol 9: 763-770, 2009.
42. Thomas SE, Dalton LE, Daly ML, *et al*: Diabetes as a disease of endoplasmic reticulum stress. Diabetes Metab Res Rev 26: 611-621, 2010.
43. Lee AS: The glucose-regulated proteins: stress induction and clinical applications. Trends Biochem Sci 26: 504-510, 2001.
44. Haze K, Yoshida H, Yanagi T, *et al*: Mammalian transcription factor ATF6 is synthesized as a transmembrane protein and activated by proteolysis in response to endoplasmic reticulum stress. Mol Biol Cell 10: 3787-3799, 1999.
45. De Haro C, Méndez R and Santoyo J: The eIF-2alpha kinases and the control of protein synthesis. FASEB J 10: 1378-1387, 1996.
46. Sun JE, Ao ZH, Lu ZM, *et al*: Antihyperglycemic and antilipidperoxidative effects of dry mater of culture broth of *Inonotus obliquus* in submerged culture on normal and alloxan-diabetes mice. J Ethnopharmacol 118: 7-13, 2008.
47. Jeong SC, Jeong YT, Yang BK, *et al*: White button mushroom (*Agaricus bisporus*) lowers blood glucose and cholesterol levels in diabetic and hypercholesterolemic rats. Nutr Res 30: 49-56, 2010.
48. Mizuno T: Development of antitumor polysaccharides from mushroom fungi. Food Food Ingred J Jpn 167: 69-85, 1996.
49. Wasser SP and Weis AL: Medicinal properties of substances occurring in higher basidiomycetes mushrooms: current perspectives. Int J Med Mushrooms 1: 31-62, 1999.
50. Kobayashi M, Kawashima H, Takemori K, *et al*: Ternatin, a cyclic peptide isolated from mushroom, and its derivative suppress hyperglycemia and hepatic fatty acid synthesis in spontaneously diabetic KK-A(y) mice. Biochem Biophys Res Commun 427: 299-304, 2012.

51. Netea MG, Hancu N, Blok WL, *et al*: Interleukin 1 beta, tumour necrosis factor-alpha and interleukin 1 receptor antagonist in newly diagnosed insulin-dependent diabetes mellitus: comparison to long-standing diabetes and healthy individuals. Cytokine 9: 284-287, 1997.
52. Kaizer EC, Glaser CL, Chaussabel D, *et al*: Gene expression in peripheral blood mononuclear cells from children with diabetes. J Clin Endocrinol Metab 92: 3705-3711, 2007.
53. Schnedl WJ, Ferber S, Johnson JH, *et al*: STZ transport and cytotoxicity. Specific enhancement in GLUT2-expressing cells. Diabetes 43: 1326-1333, 1994.
54. Deeds MC, Anderson JM, Armstrong AS, *et al*: Single dose streptozotocin-induced diabetes: considerations for study design in islet transplantation models. Lab Anim 45: 131-140, 2011.
55. Duprez J and Jonas JC: Role of activating transcription factor 3 in low glucose- and thapsigargin-induced apoptosis in cultured mouse islets. Biochem Biophys Res Commun 415: 294-299, 2011.
56. Rosengren V, Johansson H, Lehtiö J, *et al*: Thapsigargin down-regulates protein levels of GRP78/BiP in INS-1E cells. J Cell Biochem 113: 1635-1644, 2012.
57. Peng L, Men X, Zhang W, *et al*: Dynamin-related protein 1 is implicated in endoplasmic reticulum stress-induced pancreatic β-cell apoptosis. Int J Mol Med 28: 161-169, 2011.