Protective Effect of Cyclo(His-Pro) on Streptozotocin-Induced Cytotoxicity and Apoptosis In Vitro

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Cyclo(His-Pro) (CHP) is a naturally occurring, cyclic dipeptide structurally related to thyrotropin-releasing hormone (TRH). CHP was efficiently obtained from soybean meal by hydrolysis with flavourzyme and alcalase. In this study, the effects of CHP on streptozotocin (STZ)-induced β-cell dysfunction and apoptosis were investigated in rat insulinoma cells (RINm5F) secreting insulin. When the RINm5F cells were treated with 2 mM STZ, insulin secretion decreased to approximately 54% that of control cells. However, CHP treatment restored the insulin-secreting activity of RINm5F cells to approximately 71% that of the untreated control cells. Moreover, CHP significantly protected the cells from STZ-mediated cytotoxicity via reduction of nitric oxide (NO) production (2.3-fold) and lipid peroxidation (1.9-fold), which were induced by STZ. Moreover, CHP treatment also attenuated STZ-induced apoptotic events, such as activation of caspase-3, poly(ADP-ribose) polymerase (PARP) cleavage, and DNA fragmentation in RINm5F cells, indicating that CHP could protect the cells from apoptotic cell death induced by oxidative stress of STZ by increasing the expression of an anti-apoptotic protein, Bcl-2. These results suggest that CHP could be a candidate material for a protective and therapeutic agent against STZ-mediated cytotoxicity and apoptosis.

Keywords: Apoptotic events, cyclo(His-Pro), nitric oxide, RINm5F, streptozotocin

Diabetes mellitus is a group of metabolic diseases characterized by high blood sugar levels resulting from defects in insulin secretion or action. More than 90% of diabetic patients suffer from Type II diabetes, which is a progressive disease with adult onset [67]. Type II diabetes, non-insulin-dependent diabetes mellitus (NIDDM), is characterized by insulin resistance, in which the primary insulin target organs (adipose, muscle, and liver tissues) are poorly responsive to insulin action [48] and which may combine with reduced insulin secretion caused by a progressive loss of β-cell function. Type I diabetes, insulin-dependent diabetes mellitus (IDDM), which represents a majority of the diabetes cases in children, is distinguishable by selective destruction via an autoimmune process of the insulin-secreting β-cell in the pancreatic islets of Langerhans, and pancreatic β-cells are thought to be destroyed by apoptotic death [62]. Thus, activation of the pancreatic β-cell survival program might be an important factor in a therapy for Type I and Type II diabetes.

The pancreatic β-cells are vulnerable to oxidative stress, such as reactive oxygen species (ROS) and nitric oxide (NO), which may be involved in β-cell death by apoptosis, possibly due to low level expression of antioxidant enzymes. Antioxidant defense systems to increase production of free radicals are remarkably disordered in the pancreas of diabetic animals [8, 64]. It is well known that superoxide anion (O₂−) is the primary radical formed by the reduction of molecular oxygen, and it may lead to secondary radicals or ROS, such as H₂O₂ and hydroxyl radical (·OH) [1, 15]. Moreover, in diabetic models induced by streptozotocin (STZ), an activated oxygen species, it was proposed that superoxide radical is formed and involved in the death of β-cells [41, 51].

STZ is a β-cell-specific toxin and can be used to chemically induce diabetes in insulinoma cells (RINm5F), rats, and mice to study the effects of antidiabetic agents.
STZ-induced hyperglycemia in rodents or insulinoma cells is a good preliminary screening diabetic (Type I) model that has been used for more than 30 years [7, 22, 30]. The effects of STZ (N-methyl nitro carbamoyl-α-glucosamine) on β-cells are similar to those of IL-1β, and these effects also seem to be mediated by DNA damage through alklylation [6], generation of nitric oxide inducing apoptosis [63], poly(ADP-ribose) polymerase (PARP) activation [36], and caspase-3 activation [68], resulting in cell death via apoptotic phenomena. Accordingly, materials with antiperoxidative and antioxidative properties suppress apoptosis caused by STZ.

Many researchers are trying to find ways to use these β-cells to help control or prevent diabetes. The RINm5F cell line is particularly sensitive to damage by NO and free radicals because of its low levels of free radical scavenging enzymes [33, 58]. The RINm5F cell line (ATCC CRL-11605) [11], which is derived from a rat insulinoma, is a well-established and a commonly used insulin-secreting cell line for studies of pancreatic islet cell biology [4]. In addition, pancreatic β-cells are sensitive to a number of proapoptotic stimuli [19]. For these reasons, the RINm5F cell line was selected to examine the protective effect of cyclo(His-Pro) on STZ-induced cytotoxicity and apoptosis.

Cyclo(His-Pro) (CHP), which has numerous biological activities, is a naturally occurring cyclic dipeptide consisting of histidyl and proline and is a metabolite of thyrotrophin-releasing hormone (TRH), but it also is produced directly via apoptosis of MTT solution (5 mg/ml in PBS) was added to each well, and the CHP (0.05, 0.1, 0.25, 0.5, and 1 mg/ml) for 24 h. Thereafter, 20 l of medium was deproteinized by adding 200 l of dimethyl sulfoxide (DMSO) was added to 100 l of methanol (HPLC grade) and allowing the mixture to stand at 4°C for 15 min. The sample was centrifuged (12,000 g) in an Eppendorf centrifuge for 3 min, and the supernatants were filtered with a 0.22-µm membrane filter. The CHP content for each hydrolysate was analyzed by high-performance liquid chromatography (HPLC, Varian 230; Varian Inc., Palo Alto, CA, USA) using a Hamilton PRP-1 RP 10 µm (250 mm×4.1 mm ID; Hamilton Co.) column and a Hamilton PRP 10 µm (25 mm×1.3 mm ID) pre-column. The mobile phase was a mixture of acetonitrile and 0.75 g/l 1-heptanesulfonic acid in 0.004 M aqueous TFA [10:90 (v/v)], adjusted to pH 2.4 with NaOH before use. The flow rate was 0.5 ml/min. The sample (20 µl) was injected and monitored spectrophotometrically at 220 nm. The pure (synthetic) cyclo(His-Pro) was purchased from the Sigma Chemical Co. (St. Louis, MO, USA) and used for the CHP analysis as a standard material. After centrifugation at 3,000 ×g for 20 min, the supernatant was dried under a spray dryer. The sterile extract was stored at –70°C for the next experiments.

Cell Culture

A rat insulinoma cell line (RINm5F) was purchased from American Type Culture Collection. RIN, clone 5F (RINm5F), is an insulinoma cell line derived from an NEDH rat islet cell tumor [34]. Cells were grown at 37°C under a humidified, 5% CO₂ atmosphere in RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 units/ml of penicillin, and 100 µg/ml of streptomycin, and the medium was changed every three days. Cells from passages 23–28 were used. The resulting cells were plated in 96-well plates or petri dishes.

Cell Proliferation

Proliferation of RINm5F cells after treatment with CHP was determined by assaying for the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan, as described previously [23]. Briefly, cells were seeded in 96-well plates (2×10⁴ cells per well in 200 µl of medium) and left to adhere to the plastic plates overnight before being exposed to CHP (dissolved in RPMI-1640 medium without FBS). After 24 h of incubation, the cells were washed and placed in culture medium with different concentrations of crude CHP (0.05, 0.1, 0.25, 0.5, and 1 mg/ml) for 24 h. Thereafter, 20 µl of MTT solution (5 mg/ml in PBS) was added to each well, and the cells were incubated at 37°C for 4 h. Thereafter, the medium was removed, and 100 µl of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. Absorbance was measured at 570 nm with a microplate reader (Molecular Devices, Spectra MAX 250). The proliferation rate of cells was calculated by the following formula: (mean value of treated group/control group)×100%.
Cytotoxicity Assay
RINm5F cells were seeded in 96-well plates (2×10^4 cells per well in 200 µl of medium). After 24 h of incubation, STZ (2 mM) was added to each well and the cells were incubated for 30 min at 37°C. After 30 min, the medium containing STZ was removed and replaced with standard RPMI-1640 containing CHP (0.05, 0.1, 0.25, 0.5, or 1 mg/ml), and the cells were incubated for 24 h. After incubation, MTT solution (5 mg/ml in PBS) was added to each well, and the cells were incubated at 37°C for an additional 4 h. Theretofre, the medium was removed, and DMSO (100 µl) was added to dissolve the formazan crystals. Absorbance was measured at 570 nm with a microplate reader (Molecular Devices, Spectra MAX 250).

Assay for Nitric Oxide
Nitric oxide concentration in the cell culture was measured by a microplate assay method, as previously described [65]. Briefly, cells were seeded in 96-well plates (2×10^4 cells per well in 200 µl of medium) and treated for 30 min at 37°C with 2 mM STZ. After 30 min of incubation, the medium was removed, CHP (0.05, 0.1, and 0.15 mg/ml) was added, and the plate was incubated at 37°C for 24 h. Theretofre, 100 µl aliquots were removed from the conditioned medium and incubated with 100 µl of Griess reagent [1% sulfanilamide/0.1% N-(1-naphtyl)-ethylenediamine dihydrochloride/2.5% H3PO₄] at room temperature for 10 min. The absorbance at 540 nm was determined using a microplate reader. The nitric oxide content was determined using sodium nitrate as a standard [21].

Measurement of Lipid Peroxidation
Lipid peroxidation was measured by a lipid peroxide test kit (Bioassay Systems, Hayward, CA, USA). The RINm5F cells (5×10^4) were resuspended in 200 µl of ice-cold 1×PBS and sonicated at 40 volt for 20 s. Then the samples were processed following the manufacturer’s instructions. In the final step, 10% trichloroacetic acid (TCA)-treated supernatant and thiobarbituric acid (TBA) were mixed (1:1) and heated at 100°C for 1 h. The mixture was cooled to room temperature and centrifuged at 800 × g for 5 min. The absorbance of the supernatant was measured at 532 nm using a microplate reader. The levels of MDA were expressed as nmol/cell.

Detection of DNA Fragmentation
Cell pellets (2×10^4) were resuspended in 500 µl of lysis buffer (0.1 M NaCl, 0.01 M EDTA, 0.3 M Tris-HCl, pH 7.5, and 0.2 M sucrose) at room temperature for 15 min; 25 µl of 10% SDS was added and the cells were incubated at 65°C for 30 min. Theretofre, 120 µl of 5 M potassium acetate was added, and the mixture was incubated on ice for 1 h. After incubation, cell lysates were clarified by centrifugation at 16,000 × g for 10 min at 4°C. DNA was extracted twice with phenol/chloroform (1:1), precipitated with ethanol, and resuspended in TE-RNase solution (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 10 µg/ml RNase). The DNA was analyzed after separation by 1.5% agarose gel electrophoresis.

Statistical Analysis
The results were analyzed for statistical significance by one-way analysis of variance (ANOVA) test using the Statistical Package of the Social Science (SPSS) program. All data are expressed as mean ± SD values. In all analyses, a P value of <0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Comparison of Biological Activities Between CHP-Rich Crude Extract and Pure CHP
Preparation of cyclo(His-Pro) was performed as described in Materials and Methods. As a result, the HPLC analysis of crude CHP used in this study showed the same pattern as pure CHP (Sigma, St. Louis, MO, USA) and the hydrolysate contained CHP (8.1 mg/g dry weight of hydrolysate) with other minor constituents (data not shown). It was reported that the concentrations of CHP in several common foods were 1.46 µg/g in dried shrimp and 1.18 µg/g in fish sauce [18]. Subsequently, high levels of CHP were found in several common nutritional supplements and it was reported that the dietary intake of CHP-rich supplements in healthy volunteers can increase the levels of CHP in plasma far above the baseline values (829 pg/ml) [17]. Thus, this evidence in the literature clearly demonstrated that dietary CHP can be absorbed from the gastrointestinal tract. Accordingly, biological activities between CHP-rich crude extract and pure CHP were compared in RINm5F cells. As analyzed using the ChemiImager (Alpha Innotech Corporation, San Leandro, CA, USA).
preliminary experiments, we first evaluated the CHP-rich crude extract with reference to the pure CHP product in in vitro experiments, because the use of crude CHP has a higher impact from the practical point of view. It was found that most biological activities between the two products had no significant difference (data not shown). Therefore, subsequent experiments were conducted using various concentrations of crude CHP, which corresponded to effective concentrations of pure CHP.

**Effects of CHP on Proliferation and Viability of RINm5F Cells**

To investigate whether or not the survival of cells was affected by CHP, RINm5F cells were incubated with CHP (0.05–1 mg/ml) for 24 h, with or without STZ, and the cell viability was determined by MTT assay. As shown in Fig. 1, when the cells were treated with CHP alone, cell proliferation was not largely influenced by CHP concentrations, even though the cell viability increased to 8% at 0.1 mg/ml of CHP. This suggests that CHP did not show significant cytotoxicity. To induce a Type I diabetes-like model for RINm5F cells, STZ was used in this experiment. The half maximally effective concentration ($EC_{50}$) of STZ, at which 50% of the cells lose their viability in the MTT cytotoxicity assay, was determined to be 2 mM (data not shown), and all subsequent studies were performed using this concentration of STZ. Accordingly, RINm5F cells were first cultured for 24 h, treated with 2 mM STZ for 30 min, and incubated with CHP at doses of 0.05, 0.1, 0.25, 0.5, or 1 mg/ml for 24 h. After incubation for an additional 24 h with CHP, cell viability was determined by MTT assay. As shown in Fig. 2, treating RINm5F cells with a single dose of 2 mM STZ decreased viable cells to approximately 52.4% that of the control cells. When the cells were treated with 0.1 mg/ml of CHP, viability was restored to approximately 76.2% that of the control cells, suggesting that CHP significantly protected the cells from STZ-induced cytotoxicity through blocking or restoring a step of the STZ-action mechanism. STZ is a $\beta$-glucopyranose derivative of N-methyl-N-nitrourea (MNU) and a diabetogenic caused by the selective destruction of insulin-producing $\beta$-cells [31]. It was suggested that the $\beta$-cell-selective toxicity of STZ is related to the glucose moiety in its chemical structure, which enables STZ to enter the $\beta$-cells via the low affinity glucose transporter GLUT2 in the plasma membrane [47]. It is known that the toxic action of STZ is related to the DNA alkylating activity of its MNU moiety [39]. However, although it is generally accepted that the cytotoxicity produced by STZ depends on DNA alkylation, evidence indicates that free radicals play an essential role in the DNA damage and cytotoxicity mechanisms of STZ [2, 40]. In the present study, it was demonstrated that death of the RINm5F pancreatic $\beta$-cells induced by STZ is suppressed by CHP treatment. Accordingly, on the basis of this study, the following experiments were conducted at CHP concentrations in the range of 0.05–0.15 mg/ml.

**Effect of CHP on Insulin Secretion of RINm5F Cells**

The RINm5F cell line is a clone derived from the RIN-m rat islet cell line, which produces and secretes insulin [11]. To test whether or not CHP or STZ affects insulin secretion, we studied the effect of CHP on insulin levels in the culture medium after treatment of RINm5F cells. When the RINm5F cells were treated with STZ, the insulin secreting function decreased to approximately 54.6% that of the control cells (Table 1). However, the insulin secreting function was slightly enhanced with the addition of 0.05 mg/ml of CHP (approximately 60% that of the control cells). In response to increasing CHP concentrations (0.1 and 0.15 mg/ml), the secreting function improved remarkably up to approximately 71% that of the untreated control cells.
in a CHP dose-dependent manner. The insulin level of the CHP-treated group was 17% higher than that of the STZ-treated group, indicating that the insulin secreting function of RINm5F cells is directly proportional to cell viability (Fig. 2). Several researchers investigated insulinotropic effects on β-cell lines of compounds derived from natural sources. Alcoholic extract of Gymnema sylvestre increased insulin release in vitro through permeabilization of the β-cell plasma membrane [45], resulting in a hypoglycemic effect by repair/regeneration of the β-cell. However, that material did not influence cell proliferation [12]. In contrast, exopolysaccharide from a mycelial culture of Laetiporus sulphureus obviously enhanced β-cell proliferation and insulin secretion [20]. Similarly, cell proliferation and insulin secretion effects were reported for the plant extract of Scoparia dulcis [30]. It was suggested that the possible mechanisms underlying the insulin releasing actions of natural compounds may enhance insulin secretion by binding to sulfonylurea receptors on the β-cell, with subsequent closure of K⁺-ATP channels, membrane depolarization, and Ca²⁺ influx [13].

### Effects of CHP on STZ-Induced Nitric Oxide Formation and Lipid Peroxidation

Nitric oxide attack results in mitochondrial malfunction and chromosomal DNA damage on pancreatic β-cells [37]. A previous study suggested that some of the diabetogenic properties of STZ were related not to its alkylating ability but to its potential to act as a nitric oxide donor [63]. Accordingly, we believed that the protective effect of CHP may be a scavenging effect against NO production by STZ in RINm5F cells. Owing to the short half-life of nitric oxide radicals, the concentration of nitrite, the final product of the nitric oxide radical, was measured using Griess reagents. After incubation of RINm5F cells with 2 mM STZ for 30 min, RINm5F cells were treated with the optimized concentrations of CHP (0.05–0.15 mg/ml) for 24 h. As shown in Fig. 3, a single treatment of RINm5F cells with 2 mM STZ significantly increased nitrite production, 3.3 times that of the control cells. However, treatment with CHP alone did not show nitrite production (data not shown). With increasing CHP concentrations (0.05, 0.1, and 0.15 mg/ml), nitrite productions were decreased to 30%, 57%, and 58%, respectively, that of STZ-treated cells. At 0.15 mg/ml of CHP, inhibition of nitrite formation was similar to that of the control cells, indicating that the protective effect of CHP was related to a scavenging effect against NO produced by STZ, and its action correlated well with increased cell viability (Fig. 2). The cytotoxicity of NO is attributed to its ability to react with iron-containing enzymes of the respiratory cycle and to interfere with the synthesis of DNA [59]. Additionally, nitric oxide reacts with and depletes glutathione, which increases the susceptibility of β-cells to oxidative stress [60]. The involvement of nitric oxide in the pathogenesis of Type I diabetes has been demonstrated using NO synthase inhibitors, which delay the onset and reduce the incidence of diabetes in the nonobese diabetic (NOD) mouse model [57]. It also was reported that cytokine-induced NO formation in β-cells inhibits insulin secretion, decreases cellular ATP levels, and increases cyclic GMP levels, DNA damage, and cell death [6, 13, 14]. Accordingly, nitric oxide production by STZ could affect cell viability, insulin secretion, and apoptosis, whereas the data clearly demonstrate that cellular damage caused by nitric oxide was successfully prevented through the scavenging effect of CHP treatment against NO.

Lipid peroxidation refers to the oxidative degradation of lipids. It is the process whereby free radicals steal electrons from the lipids in cell membranes, resulting in cell damage. It most often affects polyunsaturated fatty acids, and this process is followed by a free radical chain reaction mechanism.

### Table 1. Effect of cyclo(His-Pro) on insulin secretion in STZ-treated RINm5F cells.

<table>
<thead>
<tr>
<th>Insulin level (ng/ml)</th>
<th>Control</th>
<th>STZ</th>
<th>STZ+0.05 mg/ml CHP</th>
<th>STZ+0.1 mg/ml CHP</th>
<th>STZ+0.15 mg/ml CHP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Values are the means ± SD.</td>
<td>57.99±1.02</td>
<td>31.67±0.92</td>
<td>34.83±0.58</td>
<td>41.32±4.08*</td>
<td>41.61±2.53*</td>
</tr>
</tbody>
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*P<0.05, compared with that in the STZ group.

![Fig. 3. Effect of cyclo(His-Pro) on nitrite generation in STZ-treated RINm5F cells.](image-url)

Values are the mean ± SD of triple determinations.
As shown in Fig. 4, there was a marked increase in the level of thiobarbituric acid reactive substances (TBARS) in STZ-treated cells compared with that of control cells, whereas the level of TBARS from CHP-treated RINm5F cells significantly decreased with increasing CHP concentrations (0.05, 0.1, and 0.15 mg/ml). Although the lipid peroxidation level caused by CHP (0.15 mg/ml) was 5-fold higher than that of the control cells, it significantly decreased STZ-induced lipid peroxidation by approximately 50% that of STZ-treated cells, suggesting that STZ also produced oxidative radicals, by which lipid peroxidation occurred and CHP might possess antioxidant activity to remove free radicals. Associated with changes in lipid peroxidation, the diabetic pancreas showed decreased activity of key antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT), and the level of a nonenzymatic antioxidant, GSH, which play an important role in scavenging the toxic intermediates during incomplete oxidation [30].

As a result, these data indicate that CHP treatment also might be effectively protecting RINm5F cells from STZ-induced reactive oxygen species and subsequent lipid peroxidation-related damage.

Effects of CHP on DNA Fragmentation, Bcl-2/Bax Expression, Caspase-3 Activity, and PARP Cleavage

It was reported that DNA fragmentation is a characteristic pattern of apoptotic cell death in various cell types, including β-cell lines [26, 27, 61]. Treatment of RINm5F cells with STZ caused cell death by apoptosis, which leads to activation of DNA endonuclease, resulting in DNA fragmentation. To determine the effects of STZ and CHP on apoptosis, RINm5F cells were cultured to near confluence, treated with 2 mM STZ for 30 min, and exposed to CHP for 24 h. Genomic DNA was prepared from STZ-treated RINm5F cells that were incubated in the absence (Fig. 5, lane 3) or presence (Fig. 5, lanes 4–6) of various concentrations of CHP. Apoptosis is usually accompanied by characteristic degradation of DNA to oligonucleosomal fragments, which appear as a “ladder” after fractionation on agarose gel, as can be seen in Fig. 5. Apoptotic cell death, as demonstrated by the DNA ladder phenomenon, increased with a raise in STZ concentration (data not shown). However, STZ-induced DNA ladder formation was prominently attenuated by CHP treatment, and with increasing CHP concentration, DNA ladders were significantly reduced in the gel. Accordingly, this result correlates well with the restoration of cell viability (Fig. 2), suppression of NO generation (Fig. 3), and reduction of lipid peroxidation (Fig. 4) induced by CHP treatment of STZ-treated RINm5F cells.

It was reported that cytokines induce both necrosis and apoptosis via a common Bcl-2 inhibitable pathway in RINm5F cells, and the anti-apoptotic protein Bcl-2 is located primarily in the outer mitochondrial membrane, where it may inhibit the insertion or oligomerization of Bax protein, a pro-apoptotic molecule [50]. To examine the relationship between STZ-induced apoptosis and Bcl-2/Bax proteins, Western blot was carried out for proteins extracted from STZ- and CHP-treated RINm5F cells (Fig. 6). In STZ-treated cells, Bcl-2 expression decreased by 50% compared with that of the control cells, whereas Bax protein was expressed at approximately 1.5-fold that of the control cells, indicating that STZ-induced apoptosis could occur through the Bcl-2/Bax protein-involved pathway. With increasing CHP concentration, the Bcl-2/Bax ratio progressively increased from 0.33 (only STZ) to 1.01 (0.05 mg/ml of CHP), 1.61 (0.1 mg/ml of CHP), and 1.52 (0.15 mg/ml of CHP). These data show that CHP can protect the RINm5F cells from apoptosis by increasing the expression of an anti-apoptotic protein, Bcl-2. That is, Bcl-2, a member of the anti-apoptotic Bcl-2 family, was
up-regulated, whereas Bax, a pro-apoptotic molecule, was down-regulated in CHP-treated cells.

Caspases, cysteine-dependent aspartate-directed proteases, are a family of cysteine proteases that play a central role in the execution of apoptosis, or programmed cell death. Caspase-3, the main executioner caspase, can be activated by caspase-8 and caspase-9 [16]. Once activated, caspase-3 cleaves many substrate proteins including PARP and structural proteins to generate the characteristic apoptotic morphology [25, 27, 28]. Based on the ability of caspase-3 to trigger apoptosis, we examined the involvement of caspase-3 by Western blot analysis. As can be seen in Fig. 7A, activation of caspase-3 is determined by cleavage of pro-caspase-3 (35 kDa), which generates 19 kDa proteins. The loaded protein quantities were normalized with β-actin.

The level of activated caspase-3 in STZ-treated cells significantly increased by approximately 21-fold when compared with that of the control cells, whereas this elevation of caspase-3 activity was inhibited by approximately 2.8-fold by treatment with CHP at a concentration of 0.15 mg/ml, indicating that CHP could protect the cells from apoptosis through inhibition of caspase-3 activation. This also was demonstrated by previous data, such as the scavenging effect against NO, reduction of lipid peroxidation, and increase of the Bcl-2/Bax ratio by CHP. Furthermore, poly(ADP-ribose) polymerase, which is an enzyme implicated in DNA damage and repair mechanisms and the substrate

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**Fig. 6.** Western blot analyses of Bcl-2 (A) and Bax (B) in RINm5F cells after exposure to STZ and CHP. Values are the mean ± SD of triple determinations.

**Fig. 7.** Inhibitory effects of cyclo(His-Pro) on the activation of caspase-3 (A) and PARP (B) cleavage in STZ-treated RINm5F cells. Values are the mean ± SD of triple determinations.
protein of caspase-3, is cleaved by caspase-3 during the apoptotic process. Cleavage of PARP from its native 116 kDa to 85 kDa was an important hallmark of apoptosis. As can be seen in Fig. 7B, immunoblot analysis of protein extracts using an anti-PARP antibody revealed the appearance of a 85 kDa band, whereas the intensity of a 116 kDa band concomitantly decreased in STZ-treated cells. The level of activated PARP in STZ-treated cells significantly increased by approximately 83-fold compared with that of the control cells, and this elevation of PARP activity was maximally inhibited by approximately 3-fold upon treatment with CHP (0.1 mg/ml). In the present study, short-term exposure of RINm5F cells to STZ induced cell death mediated by activation of the apoptotic enzyme caspase-3 in vitro. In general, the apoptosis cascade is triggered by various stimuli, such as DNA damage, cell cycle perturbation, metabolic imbalance, and oxidative stress. Activation of caspase-3 has been used as a marker of apoptosis in the RINm5F cells [32]. Consistent with these reports, our results showed that caspase-3 plays an important role in STZ-induced apoptosis. However, we could not exclude any additional effect of CHP in its protective mechanism for STZ-induced cell death. The cyclic dipeptide has a marked neuroprotective activity in multiple rodent models of traumatic brain injury, and attenuates both necrotic and apoptotic neuronal cell death in vitro [9]. Moreover, CHP, a cyclic form of L-histidyl-proline, is structurally capable of chelating zinc and stimulating intestinal zinc absorption and flux in muscle tissue [49]. Zinc is an essential trace element that plays various biological roles in the control of diabetes, including the regulation of glucose transport, insulin synthesis, and insulin secretion [5]. Zinc is a cofactor in many enzymes and proteins involved in antioxidant defense, electron transport, DNA repair, and p53 protein expression, and the ability to synthesis and secrete insulin and to use glucose are impaired in the zinc-deficient state [56]. Accordingly, it is possible that CHP might partially restore the insulin-secreting ability of RINm5F cells by increasing zinc uptake from the medium.

In conclusion, we have demonstrated the potent inhibitory effect of CHP on STZ-induced β-cell destruction using an insulinoma cell line, RINm5F. Cellular damage caused by nitric oxide or oxidative stress was successfully restored by a scavenging effect of CHP against nitric oxide, resulting in normal viability and insulin secreting function. Attenuation of the apoptotic death by CHP treatment correlates well with restoration of cell viability, suppression of NO generation, and reduction of lipid peroxidation in STZ-treated RINm5F cells. These data showed that CHP exerts protective and therapeutic effects on STZ-induced cell death through an increase of the Bcl-2/Bax ratio and the inhibition of caspase-3 activation and PARP cleavage. It is likely that CHP could be a candidate material for an antidiabetic supplement if the effectiveness is proven by future in vivo tests. These results will provide valuable information for elucidating the mechanisms involved in autoimmune β-cell death. To elucidate the underlying molecular mechanism for the antidiabetic action of CHP, further studies will be performed on STZ-induced Type I diabetes in rats and genetically diabetic ob/ob mice using proteomics and DNA chip technology.

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Abbreviations

CHP, Cyclo (His-Pro); STZ, Streptozotocin; NO, Nitric oxide; IDDM, Insulin-Dependent Diabetes Mellitus; PARP, poly(ADP-ribose) polymerase

References


