Inhibition of peptidyl-prolyl cis-trans isomerase B mediates cyclosporin A-induced apoptosis of islet β cells

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Abstract. Cyclosporin A (CsA) is widely used as an immunosuppressor in the context of organ transplantation or autoimmune disorders. Recent studies have revealed the detrimental effects of CsA on insulin resistance and pancreatic β cell failure; however, the molecular mechanisms are unknown. The present study sought to confirm the associations between CsA and β cell failure, and to investigate the roles of proinsulin folding and endoplasmic reticulum (ER) stress in CsA-induced β cell failure. The viability of MIN6 cells treated with CsA was evaluated with MTT assay. Expression levels of insulin, peptidyl-prolyl cis-trans isomerase B (PPIB), cleaved caspase-3, phospho-protein kinase R (PKR)-like endoplasmic reticulum kinase (p-PERK), PKR-like endoplasmic reticulum kinase (PERK), binding immunoglobulin protein (BIP), and C/EBP homologous protein (CHOP) were detected via reducing western blot assay. Non-reducing western blot analysis was performed to examine the expression of misfolded proinsulin peptides. The proliferation of MIN6 cells was not inhibited by CsA at concentrations <1 μmol/l. CsA treatment resulted in the decreased expression of insulin and PPIB; however, it also increased the phosphorylation of PERK, and upregulated the expression of PERK, BIP, CHOP and cleaved caspase-3. The results indicated that CsA could induce pancreatic β cell dysfunction and the potential mechanism underlying this phenomenon may be PPIB-associated proinsulin misfolding, which in turn induces ER stress in β cells.

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Introduction

Cyclosporin A (CsA) is widely used as an immunosuppressor, but clinical study found that after organ transplantation diabetes mellitus associated with the use of immunosuppressants (1,2). New-onset diabetes mellitus (NODM) is an important complication among patients receiving immunosuppressants. Clinical studies have found that CsA induces hyperglycemia and decreased plasma insulin levels in organ transplant patients (3-5). The diabetogenicity of cyclosporin has been attributed to both impaired insulin sensitivity and β cell function (6-8). Animal experiments have confirmed that CsA induces insulin resistance by decreasing the cell surface availability of glucose transporter 4 (GLUT4) (9). Some research suggests that CsA and tacrolimus are Ca^{2+} inhibitors. They likely affect insulin secretion in β cell function, but previous studies have confirmed that CsA maybe induces β cell apoptosis by other mechanisms. Therefore, this research aims to confirm CsA induces β cell apoptosis by specific inhibition of PPIB which could cause the proinsulin misfolded. It is important to know the mechanism of CsA and prevent NODM.

Recent studies have demonstrated that CsA adversely affects pancreatic β cells and activates endoplasmic reticulum (ER) stress and the unfolded protein response (UPR), thus leading to cell death (10). However, the mechanisms through how CsA induces ER stress remain largely unknown, but the ER stress in β cells could be caused by misfolded proinsulin. Pancreatic islet β cells synthesize and secrete insulin and regulate blood glucose concentrations. The post-translational modification of insulin involves two steps. First, insulin mRNA is translated as a single-chain precursor called preproinsulin, and the removal of this precursor's signal peptide during insertion into the ER generates proinsulin (11). Second, proinsulin is cleaved into insulin and C-peptide. There are three intramolecular disulfide bonds in proinsulin and insulin molecules; these bonds are important for insulin bioactivity. Similarly to disulfide bonds in many secretory proteins, these bonds help to stabilize molecules before and after secretion. The proinsulin molecule is folded in the ER; during this process, the three aforementioned evolutionarily conserved disulfide bonds are formed. Properly folded

proinsulin forms dimers and exits the ER, trafficking through the golgi complex into secretory granules, where prohormone convertases (PC1/3 and PC2) (12), in concert with carboxypeptidase E (CPE), process proinsulin into C-peptide and two-chain mature insulin, which is stored in insulin granules (13).

PPIase family proteins are isomerases that shuffle disulfide bonds and allow proinsulin molecules to potentially reach their native conformation. Peptidyl-prolyl cis-trans isomerase b (PPIB) is a member of the cyclophilin-type PPIase family and contributes to protein folding (14-16). If proinsulin is not properly folded within the ER, ER stress-induced cell apoptosis occurs. CsA is not only an immunosuppressant but also a known inhibitor of PPIB (17,18). PPIB may be the key player in CsA-induced β cell death and may be involved in the molecular mechanism through which unfolded proinsulin activates ER stress.

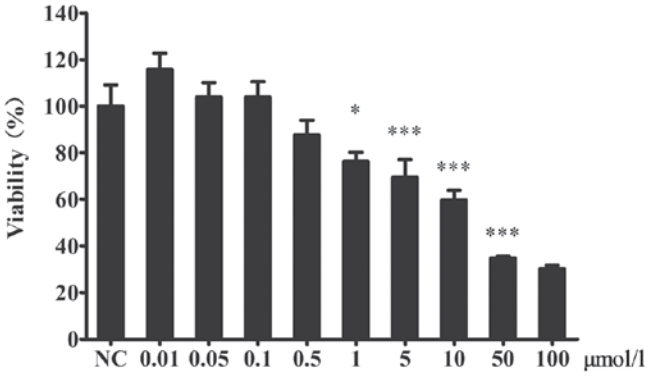

Figure 1. Effect of CsA on the viability of MIN6 cells. Cells were treated with different concentrations of CsA for 24 h, and cell viability was determined via MTT assays. *P<0.05 and ***P<0.001 vs. NC group. NC, normal control; CsA, cyclosporin A.

Materials and methods

Reagents and antibodies. CsA and palmitic acid (PA) were purchased from Sigma (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). PA/Bull Serum Albumin (BSA) conjugates were prepared as described previously. Briefly, a 100 mmol/l solution of PA in 0.1 N NaOH was incubated at 70˚C for 30 min, and fatty acid soaps were then complexed with 5% BSA in PBS at a 19:1 molar ratio of fatty acids to BSA. CsA was dissolved in PBS to obtain a 10 mmol/l stock solution. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was obtained from Biosharp (Sigma-Aldrich; Merck KGaA). Primary antibodies, including anti-insulin (1:1,000; cat. no. 8138), anti-caspase-3 (1:2,000; cat. no. 9662), anti-phospho-PKR-like endoplasmic reticulum kinase (p-PERK; 1:1,000 cat. no. 3179), anti-PKR-like endoplasmic reticulum kinase (PERK; 1:2,000; cat. no. 3192), anti-binding immunoglobulin protein (BIP; 1:3,000; cat. no. 3183), and anti-C/EBP homologous protein (CHOP; 1:3,000; cat. no. 2895) and anti-β-actin antibodies (1:5,000; cat. no. 3700), and secondary antibodies anti-rabbit IgG (1:2,000; cat. no. 7074) and anti-mouse IgG (1:2,000; cat. no. 7076) were obtained from Cell Signaling Technology (Beverly, MA, USA). Anti-C-peptide (cat. no. ab14181; 1:1,000) and anti-PPIB primary antibodies (cat. no. ab16045; 1:1,000) were obtained from Abcam (Cambridge, UK).

Cell culture. MIN6 mouse insulinoma cells were obtained from the Key Laboratory of Human Functional Genomics of Jiangsu Province, Nanjing Medical University (Nanjing, China), and cultured in Dulbecco's modified Eagle's medium (DMEM), a high-glucose culture medium, supplemented with 15% fetal bovine serum (both Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 50 μmol/l 2-mercaptoethanol (Sigma-Aldrich; Merck KGaA), 100 U/ml penicillin and 100 U/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.). These cells were incubated in a humidified atmosphere of 5% CO_2 and 95% air at 37˚C.

Cell viability assay. Cells were seeded in 96-well plates at a density of 10^3 cells per well and continuously exposed to CsA, PA or a combination of both drugs. Twenty-four hours later, the cells were treated with MTT (5 mg/ml) at 37˚C for 4 h. Culture medium containing MTT was discarded, and DMSO was added to each well to dissolve the precipitate. Absorbance values were measured at a spectral wavelength of 570 nm using a microplate reader after the plates were incubated with vibration at 37˚C for15 min.

Western blot analysis. After treatment, MIN6 cells ($5x10^6$ cells) were washed with PBS three times and lysed in RIPA lysis buffer (25 mmol/l HEPES, 1.5% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.5 M NaCl, 5 mmol/l EDTA, 50 mM NaF, 0.1 mmol/l sodium vanadate, and 1 mmol/l phenylmethylsulfonyl fluoride, pH 7.8) on ice for 30 min. The cell lysate was centrifuged at 12,000 rpm for 30 min at 4˚C, and the protein concentration in the supernatant was determined via a BCA assay. Reducing loading buffer or non-reducing loading buffer (lacked DTT and 2-mercaptoethanol; both Beyotime Institute of Biotechnology, Shanghai, China) was added to the supernatant, which was subsequently boiled for 5 min and then electrophoresed on a 12.5 or 10% SDS-PAGE gel. Proteins were transferred to a PVDF membrane, and non-specific binding sites were blocked with 5% nonfat dry milk in PBST (PBS containing 0.05% Tween-20) for 2 h. The membrane was then incubated with primary antibodies: Insulin, C-peptide, PPIB, caspase-3, p-PERK, PERK, BIP, CHOP overnight at 4˚C and then with peroxidase-conjugated secondary antibodies at room temperature for 2 h. A sequential enhanced chemiluminescence reagent was used to detect bands.

Statistical analysis. SPSS 22.0 software (IBM Corp., Armonk, NY, USA) was used to assess study data via one-way analysis of variance followed by Dunnett's t-test or an LSD test. All experiments were performed three times, and the results are presented as means ± standard deviation. Differences were regarded as significant if P<0.05.

Results

The effect of CsA on the viability of MIN6 cells. The viability of MIN6 cells was decreased by CsA. There were no significant differences between the CsA and control groups at CsA concentrations lower than 1 μmol/l (Fig. 1).

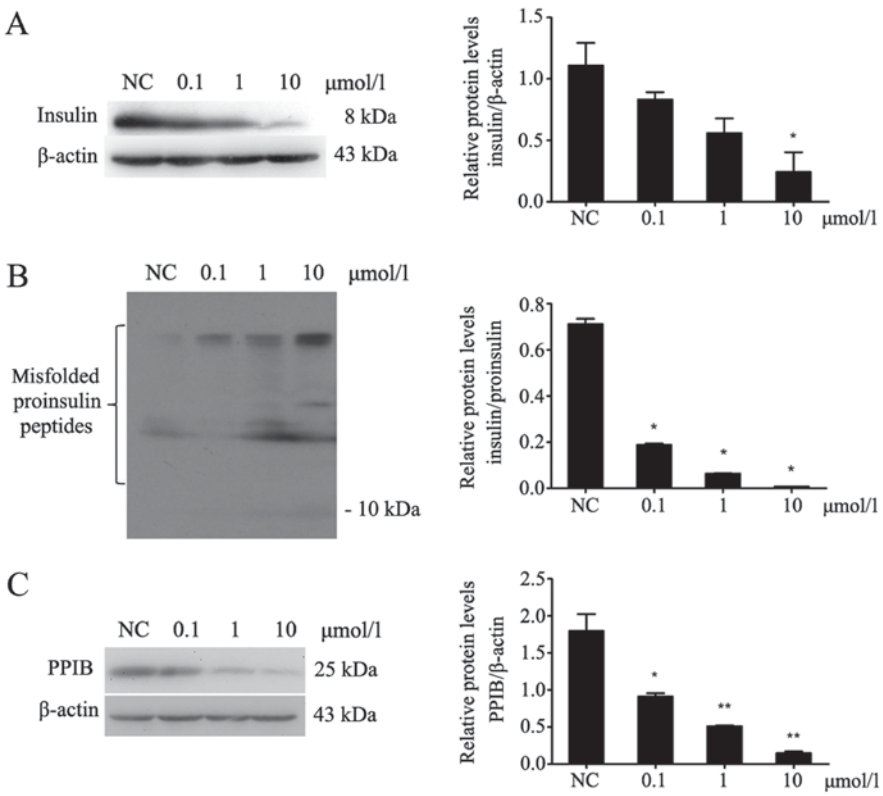

Figure 2. CsA inhibits the expression of insulin, proinsulin and PPIB in MIN6 cells. (A) Following MIN6 cell treatment with different concentrations of CsA for 24 h, insulin expression was determined via a reducing western blot assay. (B) In addition, the levels of misfolded proinsulin peptides were determined via a non-reducing western blot assay. (C) The expression of PPIB also decreased with increasing treatment. *P<0.05 and **P<0.01 vs. NC group. NC, normal control; CsA, cyclosporin A; PPIB, peptidyl-prolyl cis-trans isomerase B.

CsA inhibits the expression of insulin and PPIB and increases the misfolding of proinsulin peptides in MIN6 cells. After MIN6 cells were treated with different concentrations of CsA for 24 h (Fig. 2), the protein expression of PPIB was decreased (P<0.05; Fig. 2C). Additionally, insulin expression in MIN6 cells was decreased (P<0.05; Fig. 2A), whereas the expression of misfolded proinsulin peptides was increased (P<0.05; Fig. 2B).

CsA and PA upregulate ER stress and the expression of apoptosis-related proteins. Cells were treated with CsA, PA or a combination of both drugs for 24 h (Fig. 3). Cleaved caspase-3, p-PERK, PERK, BIP and CHOP were upregulated after the cells were exposed to CsA or PA (P<0.05; Fig. 3A and C). Thus, CsA and PA induced ER stress-related apoptosis in MIN6 cells. The expression of PPIB and insulin was inhibited by treatment with these two drugs. Additionally, both CsA and PA caused the upregulation of misfolded proinsulin peptides but the downregulation of insulin expression (P<0.05; Fig. 3B).

Discussion

Cyclophilins are a family of proteins present in vertebrates and other organisms. They bind to CsA, an immunosuppressant that is commonly used to suppress rejection after organ transplantation (19-20). The molecular mechanisms of CsA-induced β cell apoptosis probably involve the downregulation of cyclophilins.

This study in our research, MIN6 cells were treated with different concentrations of CsA (0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50 and 100 μmol/l). There were no significant differences between the control and 1 μmol/l CsA group. The results reveal after treatment with CsA concentrations lower than 1 μmol/l. There is a limitation of the current study, we should be confirmed the apoptosis in some ways such as flow cytometry, but we have already detected the expression of cleaved caspase-3. MIN6 cells were treated with CsA for 24 h has obviously changes in apoptosis. So we chose this time point (other time points not show). We used chose three concentrations of CsA (0.1, 1 and 10 μmol/l) to study the resultant effects on proinsulin folding.

In order to reveal the molecular mechanism of CsA induced MIN6 apoptosis, proinsulin folding and UPR were detected. Reducing and non-reducing SDS-PAGE assays were used to examine the expression of misfolded proinsulin peptides. The reducing SDS-PAGE buffer contained mercaptoethanol, which reduces disulfide bonds and breaks down the complex structure of proinsulin. Meanwhile the non-reducing SDS-PAGE-based western blot assay does not break misfolded peptides into monomers (21,22). Proinsulin has two forms in the ER: the correctly folded monomer and misfolded proinsulin peptides. Misfolded proinsulin peptides produce insoluble polymers of various molecular weights (23). Only correctly folded monomers can be cleaved into mature insulin (24). Proinsulin peptides of different molecular weights can be detected via a non-reducing western blot assay. Our results demonstrated that CsA decreased the expression of insulin and increased the

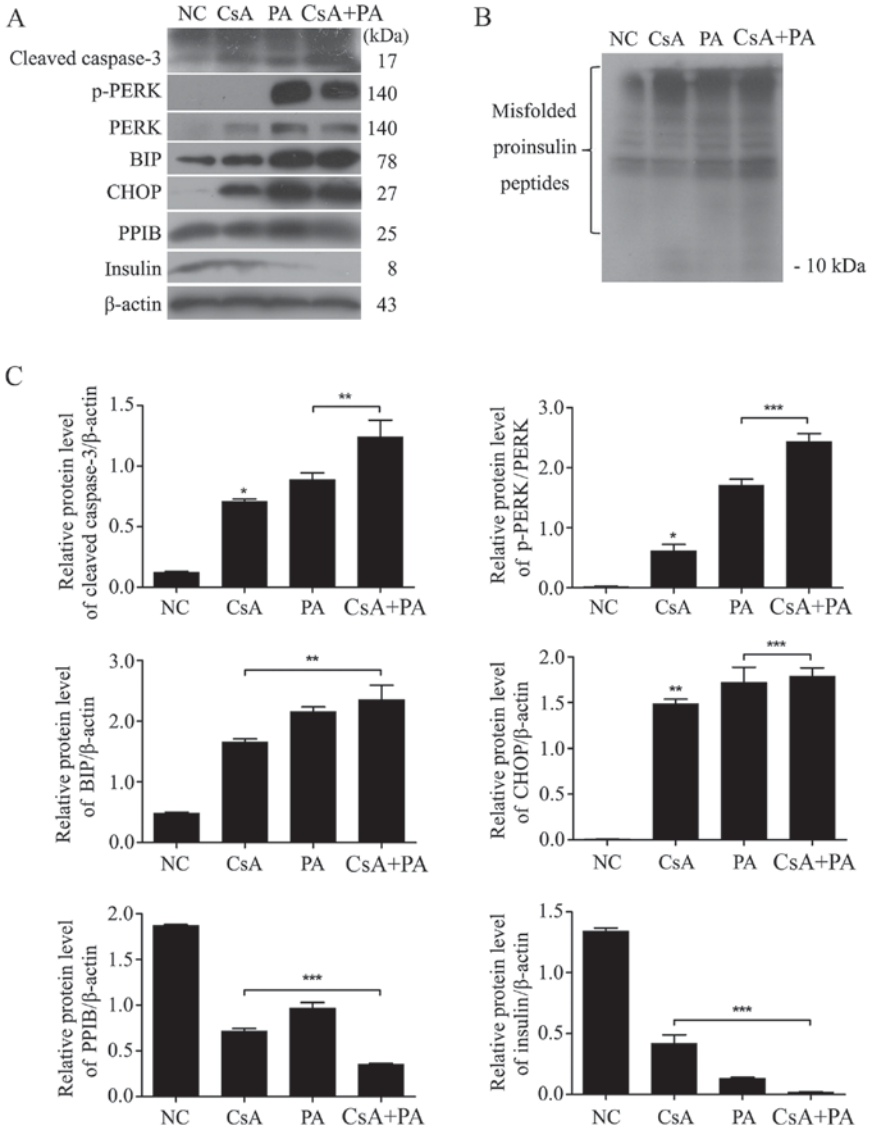

Figure 3. CsA and PA upregulate ER stress and the expression of apoptosis-associated proteins. (A) MIN6 cells were treated with 1 μmol/l CsA, 0.5 mmol/l PA or a combination of the two for 24 h. The expression of ER stress-induced cell apoptosis-associated proteins (cleaved caspase-3, p-PERK, PERK, BIP and CHOP) and insulin was then determined via a western blot assay. (B) Misfolded proinsulin peptides were detected via a non-reducing western blot assay. (C) The relative protein levels of cleaved caspase-3, p-PERK, BIP and CHOP were upregulated, and those of PPIB and insulin were downregulated. *P<0.05, **P<0.01 and ***P<0.001 vs. NC, or as indicated. NC, normal control; CsA, cyclosporin A; PA, palmitic acid; ER, endoplasmic reticulum; p-, phosphorylated; PERK, protein kinase R-like endoplasmic reticulum kinase; BIP, binding immunoglobulin protein; CHOP, C/EBP homologous protein; PPIB, peptidyl-prolyl cis-trans isomerase B.

expression of misfolded proinsulin peptides. These findings suggest that in cells treated with CsA, insulin mRNA can be translated into proinsulin; however, this proinsulin could not become insulin in the ER. CsA also down regulated the expression of PPIB. PPIB could potential be a key protein involved in proinsulin folding. We revealed the relationship between proinsulin and PPIB, but further studies are needed to investigate the potential direct association between them.

Proinsulin misfolding maybe the initial factor causing β cell failure. If nonfunctional peptides accumulate in the ER, ER stress-induced cell apoptosis occurs (25). To study the effect of misfolded peptides in the ER. PA was used as a positive control for ER stress (26,27). Certain ER stress-related proteins were detected in response to PA treatment. CsA and PA can both increase misfolded peptides, cleaved caspase-3, p-PERK, PERK, BIP and CHOP. We

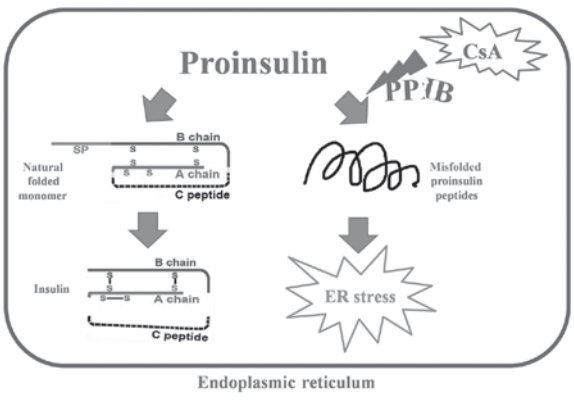

Figure 4. CsA induces β cell dysfunction, and downregulates the expression of PPIB. A schematic of the potential mechanism underlying how PPIB related proinsulin misfolding can induce ER stress in β cells. CsA, cyclosporin A; PPIB, peptidyl-prolyl cis-trans isomerase B; ER, endoplasmic reticulum.

was suggested to detect the mRNA levels at same time to make a strong conclusion, and it will be accepted in further research. These findings indicate that ER stress is activated in response to an accumulation of unfolded or misfolded proteins in the lumen of the ER (28-30). Our data also revealed that ER stress was induced by CsA and PA, both of which had similar effects on proinsulin folding and ER stress (Fig. 4).

The purpose of this paper is to prove that the proinsulin-fold disorder and endoplasmic reticulum stress occur at the same time. A reasonable possible mechanism between proinsulin and endoplasmic reticulum stress was mentioned in this manuscript. Further research is needed to confirm the potential mechanism. In conclusion, CsA induces β cell dysfunction and downregulates the expression of PPIB. The reasonable underlying mechanism of this phenomenon may be that PPIB-related proinsulin misfolding induces ER stress in β cells.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

GC, JW and CL designed research. XW, DZ, XM and QW performed the experiments. CF analyzed the data, and XW and CF wrote the manuscript. All authors discussed the results and reviewed the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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