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Transduction of PEP-1-heme oxygenase-1 into insulin-producing INS-1 cells protects them against cytokine-induced cell death

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ABSTRACT

Pro-inflammatory cytokines play a crucial role in the destruction of pancreatic β-cells, thereby triggering the development of autoimmune diabetes mellitus. We recently developed a cell-permeable fusion protein, PEP-1-heme oxygenase-1 (PEP-1-HO-1) and investigated the anti-inflammatory effects in macrophage cells. In this study, we transduced PEP-1-HO-1 into INS-1 insulinoma cells and examined its protective effect against cytokine-induced cell death. PEP-1-HO-1 was successfully delivered into INS-1 cells in time- and dose-dependent manner and was maintained within the cells for at least 48 h. Pre-treatment with PEP-1-HO-1 increased the survival of INS-1 cells exposed to cytokine mixture (IL-1β, IFN-γ, and TNF-α) in a dose-dependent manner. PEP-1-HO-1 markedly decreased cytokine-induced production of reactive oxygen species (ROS), nitric oxide (NO), and malondialdehyde (MDA). These protective effects of PEP-1-HO-1 against cytokines were correlated with the changes in the levels of signaling mediators of inflammation (iNOS and COX-2) and cell apoptosis/survival (Bcl-2, Bax, caspase-3, PARP, JNK, and Akt). These results showed that the transduced PEP-1-HO-1 efficiently prevented cytokine-induced cell death of INS-1 cells by alleviating oxidative/nitrosative stresses and inflammation. Further, these results suggested that PEP-1-mediated HO-1 transduction may be a potential therapeutic strategy to prevent β-cell destruction in patients with autoimmune diabetes mellitus.

1. Introduction

Pro-inflammatory cytokines, particularly interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ) that are released by infiltrated macrophages and T cells in the pancreas are implicated in the loss of β-cell mass during the progression of type 1 diabetes mellitus [1]. Although cellular mechanisms responsible for cytokine-induced β-cell destruction are not completely understood, increased levels of ROS and NO after cytokine stimulation are thought to play important roles [2–4]. Pro-inflammatory cytokines induce ROS generation by activating NADPH oxidase in β-cells [5]. Further, pro-inflammatory cytokines increase the expression of inducible NO synthase (iNOS) by activating NFκB signaling pathway, followed by NO formation [6]. Recent studies suggest that increased ROS and NO damage the mitochondrial membrane, subsequently inducing its depolarization, and triggering an intrinsic apoptotic pathway in pancreatic β-cells [7,8]. Normally, animal cells use antioxidant enzymes to prevent ROS-induced cellular injury. However, pancreatic β-cells are particularly susceptible to the detrimental effects of ROS because they express low levels of antioxidant enzymes [9]. Studies have shown that overexpression of antioxidant enzyme in β-cells enhances their resistance to cytotoxic challenges induced by cytokines and oxidative stress through the detoxification of ROS [10,11]. Previously, we have shown that exogenous supplementation of Cu,Zn-superoxide dismutase (SOD) in β-cells by using Tat-mediated protein transduction enhanced the tolerance of these cells to oxidative stress and improved the diabetic status of streptozotocin-induced diabetic mice [12].

Heme oxygenase-1 (HO-1) exerts potent cytoprotective effects against oxidative stress and inflammation [13]. HO-1 catalyzes the first step in the degradation of free heme to carbon monoxide (CO),

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ferrous iron, and biliverdin. CO plays an anti-inflammatory role by inhibiting the expression of pro-inflammatory cytokines and enhancing the expression of anti-inflammatory cytokines [14]. CO also suppresses the expression of iNOS and cyclooxygenase-2 (COX-2) in macrophages and inhibits TNF-α-induced apoptosis in endothelial cells [15,16]. Natural antioxidants biliverdin increase cellular resistance to oxidative injury by scavenging ROS and by inhibiting lipid and protein peroxidation. Therefore, HO-1 has been suggested as a target enzyme for inhibiting inflammation, apoptosis, and oxidative damage in inflammatory diseases [17,18]. Upregulation of HO-1 activity by pharmacologic therapy interdicted the diabetic state in NOD mice and enhanced insulin sensitivity in streptozotocin-induced diabetic rats [19,20]. Overexpression of HO-1 also had a similar protective effect against autoimmune diabetes mellitus and prolonged graft survival in NOD mice [21].

On the other hand, several studies have been performed to establish strategies for delivering therapeutic proteins into cells by using protein transduction domains (PTDs), such as TAT, VP22, and PEP-1 [22]. Of these, PEP-1, a recently developed amphipathic 21-residue peptide, is a suitable carrier for delivering exogenous proteins in biologically active forms compared with TAT and VP22 [23]. Recently, we showed that PEP-1-mediated HO-1 transduction prevented lipopolysaccharide (LPS)-stimulated inflammation and suppressed ear edema in mice by inhibiting the overexpression of inflammatory mediators in Raw 264.7 macrophage cells [24].

In this study, we examined whether PEP-1-HO-1 fusion protein could be delivered efficiently into INS-1 cells and whether the transduced PEP-1-HO-1 could protect these cells from cytokine-induced cell death. Further, we examined the possible mechanisms underlying the protective effects of the transduced PEP-1-HO-1 against cytokine stress.

2. Materials and methods

2.1. Materials

PEP-1-HO-1 was overexpressed and purified as described previously [24]. Schematic representation of PEP-1-HO-1 expression vector system and the expressed fusion protein is given in Fig. 1A. Rat IL-1β, TNF-α, and IFN-γ were from R&D Systems (Minneapolis, USA), Enzo (Farmingdale, USA), and Chemicon (Billerica, USA), respectively. Total ROS detection kit was purchased from Enzo. Antibodies against histidine-tag (His-tag), HO-1, iNOS, COX-2, p65, Bax, Bcl-2, cleaved caspase-3, cleaved PARP, pAkt, and pJNK were obtained from Cell Signaling Technology (Danvers, USA). All other chemicals were purchased from Sigma–Aldrich (St. Louise, USA).

2.2. Cell culture and transduction of PEP-1-HO-1 into INS-1 cells

INS-1 rat insulinoma cells were grown in RPMI-1640 medium containing 11.1 mM glucose, 10% fetal bovine serum (FBS), 10 mM HEPES, 2.1 mM L-glutamine, 1 mM sodium pyruvate, 100 μg/ml streptomycin, and 100 U/ml penicillin at 37 °C in humidified atmosphere of 95% air and 5% CO_2. For transduction, INS-1 cells were grown to confluence in a 6-well plate and were starved in culture medium lacking FBS for 6 h. Next, the culture medium was replaced with fresh medium, and the cells were treated with 1–3 μM of PEP-1-HO-1 for 1 h. For time-dependent transduction of PEP-1-HO-1, 2 μM PEP-1-HO-1 was added to the culture medium for 5–120 min. To determine the intracellular stability of the transduced PEP-1-HO-1, cultured cells were treated with 2 μM PEP-1-HO-1 for 1 h. Next, the cells were washed with fresh medium to remove fusion proteins that were not transduced into the cells, and the cells were incubated further for 6–48 h. Next, the cells were harvested, and cell lysates were prepared for western blotting.

2.3. Effects of the transduced PEP-1-HO-1 on cytokine-induced cell death

To observe cytokine-induced cell death, INS-1 cells were seeded in a 48-well plate. On the next day, the cells were starved in culture medium lacking FBS for 6 h. Next, the culture medium was replaced with fresh medium, and the cells were treated with 0.5–25 ng/ml of cytokine mixture (IL-1β, TNF-α, and IFN-γ) for 24 h. To determine the effects of the transduced PEP-1-HO-1 on cytokine-induced cell death, cultured cells were pre-treated with 0.5–3 μM PEP-1-HO-1 for 1 h, followed by treatment with a combination of cytokines (1 ng/ml IL-1β, 5 ng/ml TNF-α, and 5 ng/ml IFN-γ). After incubation for 24 h, the cells were washed with DPBS and were incubated with phenol red-free RPMI-1640 containing 0.5 mg/ml of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) for 3 h at 37 °C. Next, the cells were incubated with 0.5 ml 2-propanol for 30 min. Absorbance of dissolved MTT-derived formazan was measured at 540 nm to assess living cells in the culture. Viability was expressed as the percentage of untreated control cells.

2.4. Western blotting

INS-1 cells were washed with DPBS and were treated with lysis buffer (150 mM NaCl, 50 mM Tris–HCl, 1 mM phenylmethanesulfonyl fluoride, 1 mM Na_3VO_4, 1% Nonidet P-40, 0.1% SDS, 0.5% deoxycholic acid, and 1% protease inhibitor cocktail, pH 7.5) on ice. Cytosolic supernatants were obtained by centrifugation at 12,000 × *g* for 20 min at 4 °C. To determine the nuclear level of NFκB p65 subunit, nuclear protein extracts were prepared as described previously [25]. Cell homogenates were resolved by SDS-polyacrylamide gel electrophoresis and were transferred onto a PVDF membrane. The membrane was blocked with 5% non-fat dry milk and was incubated with corresponding primary antibody followed by appropriate horseradish peroxidase-linked secondary antibody. Immunoreactive bands were detected using an enhanced chemiluminescence (ECL) according to the manufacturer's instruction.

2.5. ROS measurement

Intracellular levels of ROS were measured using total ROS detection kit according to the manufacturer's instructions. For detecting ROS, fluorogenic dye-loaded cells were pre-treated with PEP-1-HO-1 and ROS inhibitor for 1 h and 15 min, respectively, and were then incubated with cytokine mixture for 24 h. The cells were then washed twice and fluorescence-positive cells were detected under fluorescence microscope by using a filter set compatible with fluorescein (Ex/Em: 490/525 nm). For each experiment, approximately 100 cells were selected from 4 areas, and mean of fluorescence-positive cells was counted.

2.6. Measurement of nitrite and MDA levels

NO production was determined by measuring the level of its stable oxidative metabolite nitrite, according to a method described previously [26]. Level of MDA in cell extracts was determined using thiobarbituric acid-reacting substance, as described previously [27].

2.7. Statistical analysis

All data in figures are expressed as mean ± SEM. Statistical comparisons between 2 groups were performed using Student's *t*-test. One-way ANOVA with Dunnett's post hoc test was used for

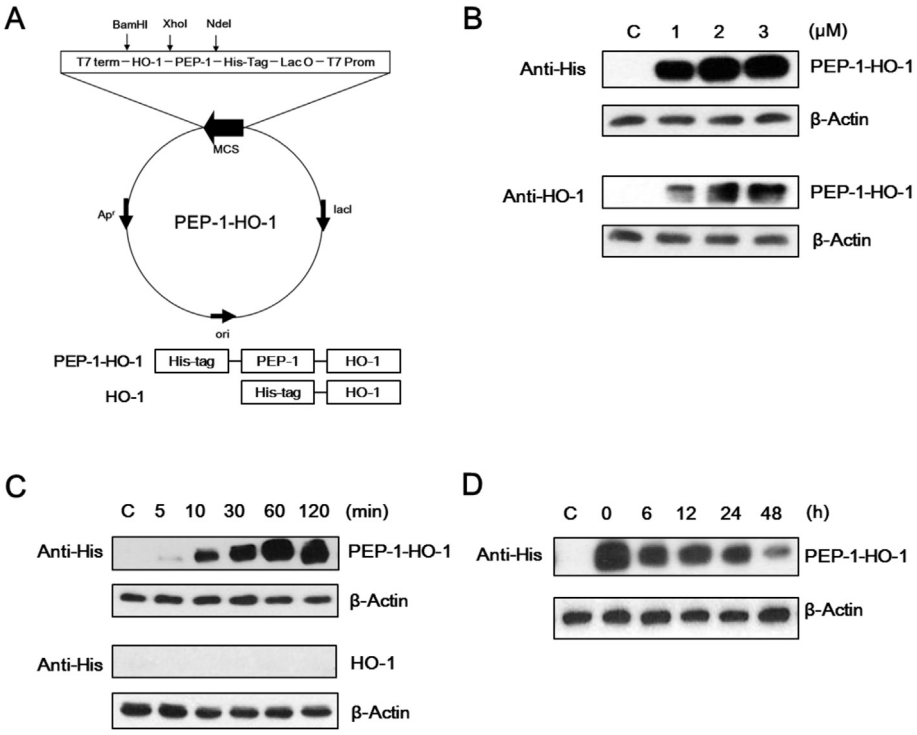

Fig. 1. Transduction of PEP-1-HO-1 into INS-1 cells. PEP-1-HO-1 expression vector system was constructed using plasmid pET-15b. Sequences of the expressed PEP-1-HO-1 and control HO-1 are illustrated at the bottom (A). For dose-dependent transduction of PEP-1-HO-1, INS-1 cells were treated with 1–3 μM of PEP-1-HO-1 for 1 h, and the level of transduced PEP-1-HO-1 was determined using western blotting with specific antibodies against His-tag and HO-1 (B). For time-dependent transduction of PEP-1-HO-1, INS-1 cells were treated with 2 μM PEP-1-HO-1 or control HO-1 for 5–120 min (C). For measuring intracellular stability of the transduced PEP-1-HO-1, INS-1 cells pre-treated with 2 μM PEP-1-HO-1 were incubated in a fresh medium for 6–48 h. The transduced PEP-1-HO-1 in these cells was determined by western blotting (D).

multiple comparisons. Statistical significance was considered at $p < 0.05$.

3. Results and discussion

3.1. Transduction of PEP-1-HO-1 into INS-1 cells

PTD-mediated protein transduction technology has been proven as a highly effective tool for exogenous delivery of biological macromolecules into mammalian cells [12,22,23]. Recently, we reported that transduced PEP-1-HO-1 exerted a potent anti-inflammatory effect in LPS-stimulated Raw 264.7 cells and mouse edema model [24]. In this study, we applied PEP-1-HO-1 cell-permeable fusion protein to pancreatic β-cells under cytokine stress. Fig. 1A shows the schematic representation of PEP-1-HO-1 expression vector based on pET-15b plasmid containing sequences for 6 histidine residues, PEP-1, and HO-1. In addition, the pET-15b plasmid lacking the PEP-1 sequence was constructed for expressing control HO-1. To assess whether PEP-1-HO-1 could traverse the membrane of INS-1 cells, cultured cells were incubated with 1–3 μM PEP-1-HO-1 for 1 h or with 2 μM PEP-1-HO-1 for 0–120 min. To confirm the presence of the transduced PEP-1-HO-1 in the cells, we analyzed cell lysates by western blotting with 2 specific antibodies against His-tag and HO-1, respectively. We observed that PEP-1-HO-1 was successfully transduced into INS-1 cells in dose- and time-dependent manner (Fig. 1B and C), which was similar to that observed in Raw 264.7 cells [24]. However, control HO-1 lacking the PEP-1 domain could not be delivered into these cells. Although the transduced PEP-HO-1 gradually disappeared with an increase in incubation time, some of the transduced protein remained in INS-1 cells for 48 h after the transduction (Fig. 1D). These results indicated that PEP-1-HO-1 could be efficiently transduced and could be stably maintained in INS-1 cells.

3.2. Effects of the transduced PEP-1-HO-1 on cytokine-induced cell death

Cytoprotective effect of the transduced PEP-1-HO-1 was assessed by measuring the viability of cytokine-exposed INS-1 cells. Infiltrated macrophages and T cells in the pancreas release some pro-inflammatory cytokines in clusters, these cytokines might work together, ultimately resulting in the destruction of β-cells in patients with autoimmune diabetes mellitus [1]. Therefore, we used cytokine mixture of IL-1β, TNF-α, and IFN-γ to induce cell destruction. Exposure of INS-1 cells to various concentrations of cytokine mixture for 24 h significantly decreased the viability of these cells in a dose-dependent manner (Fig. 2A). To elucidate the effects of the transduced PEP-1-HO-1 on cytokine-induced β-cell death, INS-1 cells were treated with a combination of 1 ng/ml IL-1β, 5 ng/ml TNF-α, and 5 ng/ml IFN-γ. As shown in Fig. 2B, cell survival rate of cytokine-treated INS-1 cells was approximately 50% lower than that of untreated control cells. Pre-treatment of INS-1 cells with 0.5–3 μM PEP-1-HO-1 for 1 h, followed by exposure to cytokines for 24 h, gradually increased their viability in a dose-dependent manner. Viability of INS-1 cells pre-treated with 3 μM PEP-1-HO-1 was almost the same as that of control cells. However, control HO-1 did not affect the survival of INS-1 cells. ROS and NO are crucial mediators of cytokine-induced dysfunction and destruction in pancreatic β cells. As shown in Fig. 2C, cytokine-induced β-cell death was significantly suppressed by N-acetyl-L-cysteine (NAC), an antioxidant drug, thus confirming the involvement of ROS in the cytotoxic effects of cytokines. To assess the involvement of ROS and NO in inducing the protective effects of the

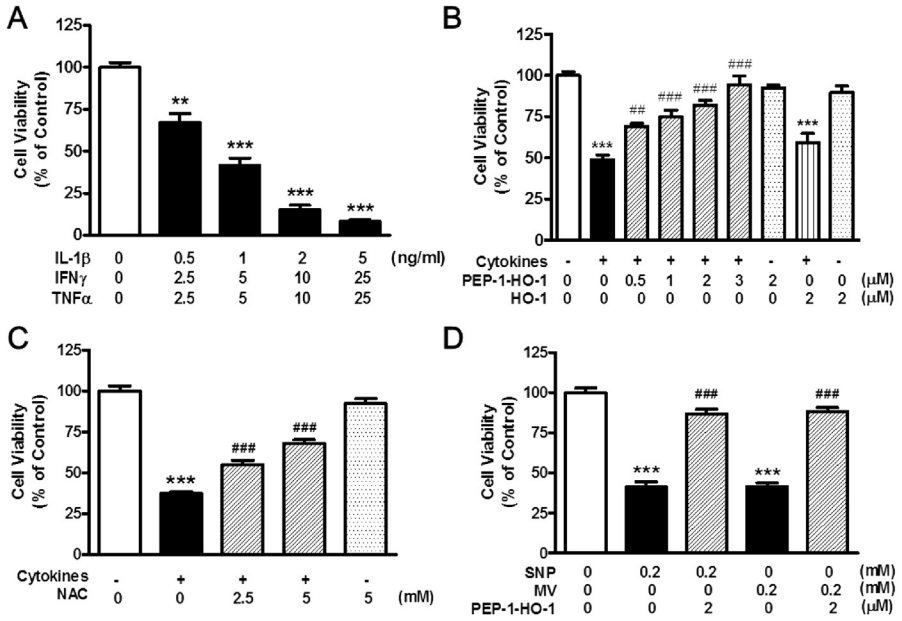

Fig. 2. Effects of the transduced PEP-1-HO-1 on the viability of INS-1 cells treated with cytokines, SNP, or MV. Dose-dependent effect of cytokine mixture on cell viability (A). Cultured cells were treated with various concentrations of cytokine mixture (IL-1β, TNF-α, and IFN-γ) for 24 h, and cell viability was measured using MTT assay. Effects of PEP-1-HO-1 and NAC on cytokine-induced cell death (B and C). Cultured cells pre-treated with 0.5–3 μM PEP-1-HO-1 or 2.5–5 mM NAC were incubated with a combination of cytokines (1 ng/ml IL-1β, 5 ng/ml TNF-α, and 5 ng/ml IFN-γ) for 24 h. Effects of PEP-1-HO-1 on SNP- and MV-induced cell death (D). Cultured cells pre-treated with 2 μM PEP-1-HO-1 were incubated with 0.2 mM SNP or MV for 12 h. Bars represent mean ± SEM of data obtained from 6 to 8 independent experiments. $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with untreated control group, and $\#\#p < 0.01$ and $\#\#\#p < 0.001$ compared with cytokine-, SNP-, or MV-treated group.

transduced PEP-1-HO-1, we tested the effect of PEP-1-HO-1 on β-cell death induced by methyl viologen (MV) or sodium nitroprusside (SNP), which are used to produce intracellular ROS and NO, respectively. Only approximately 40% of the total cells remained viable after treatment with 0.2 mM MV or SNP for 12 h. However, pre-treatment with 2 μM PEP-1-HO-1 significantly increased the survival rate of INS-1 cells treated with MV or SNP (Fig. 2D).

3.3. Effects of the transduced PEP-1-HO-1 on cytokine-induced increase in ROS, NO, and MDA levels

Cytokine-induced ROS and NO result in macromolecule damages such as lipid peroxidation, enzyme inactivation and DNA fragmentation, and then ultimately induce β-cell death by triggering apoptotic pathways [2,4,7,28]. To determine whether the protective effects of PEP-1-HO-1 against cytokines were directly associated with the suppression of cytokine-induced ROS and NO production, we measured the levels of ROS, NO, and MDA in cells treated with cytokines and PEP-1-HO-1 alone or in combination. Intensity of ROS-induced fluorescence increased to 3.6 fold in cells treated with cytokines mixture (Fig. 3A). However, this increase was significantly suppressed in cells pre-treated with 2 μM PEP-1-HO-1 or 5 mM NAC. Similarly, cytokine-induced NO production was inhibited by the transduced PEP-1-HO-1 in a dose-dependent manner (Fig. 3B). However, NO level was not influenced by treatment with HO-1. Cellular MDA level, one of the common biomarker of oxidative stress, was also significantly increased by treatment with cytokines. However, the elevated MDA level returned to the basal level after the transduction of PEP-1-HO-1 (Fig. 3C). These data indicated that the transduced PEP-1-HO-1 protected β-cells against the deleterious effects of cytokines by lowering the elevated levels of ROS and NO. It has been suggested that peroxynitrite formed from ROS and NO is highly cytotoxic and is involved in cytokine-induced destruction of β-cells [29]. Although we did not quantify the intracellular level of peroxynitrite, it is likely that the transduced PEP-1-HO-1 may reduce peroxynitrite formation by neutralizing cytokine-induced ROS and NO, thus attenuate cytokine toxicity.

3.4. Effects of the transduced PEP-1-HO-1 on the levels of pro-inflammatory mediators and cell apoptosis-/survival-related proteins

Pro-inflammatory cytokines activate NFκB that controls the expression of many inflammation-related genes and induces local inflammation, ultimately resulting in β-cell dysfunction and destruction [1,6,30]. In this study, we confirmed that pro-inflammatory cytokines induced the expression of pro-inflammatory mediators iNOS and COX-2 in INS-1 cells (Fig. 4A). Cytokine-induced expression of iNOS and COX-2 was remarkably suppressed by the transduction of 2 μM PEP-1-HO-1, which was consistent our previous observation in LPS-stimulated Raw 264.7 cells [24]. The inhibitory effect of the transduced PEP-1-HO-1 on the expression of iNOS and COX-2 may be attributed to the suppression of cytokine-induced nuclear translocation of NFκB p65 subunit. The level of p65 in nuclear extract was remarkably high in cytokine-treated cells. However, this increase was suppressed by pre-treatment of 2 μM PEP-1-HO-1. These results indicated that transduced PEP-1-HO-1 play well an anti-inflammatory role such as native HO-1 enzyme in cytokine-exposed β-cells and suggested that PEP-1-HO-1 may have a therapeutic potential for treating autoimmune diabetes mellitus.

Although pro-inflammatory cytokines may induce β-cell destruction by both apoptosis and necrosis, apoptosis is probably the more dominant form of β-cell death [1]. The intrinsic pathway of apoptosis is activated by the release of mitochondrial cytochrome *c*, which is regulated by the balance between pro-apoptotic and anti-apoptotic Bcl-2 family proteins. The common execution phase of apoptosis involves proteolytic cleavage of caspase-3 by other caspase. The activated caspase-3 then cleaves nuclear protein

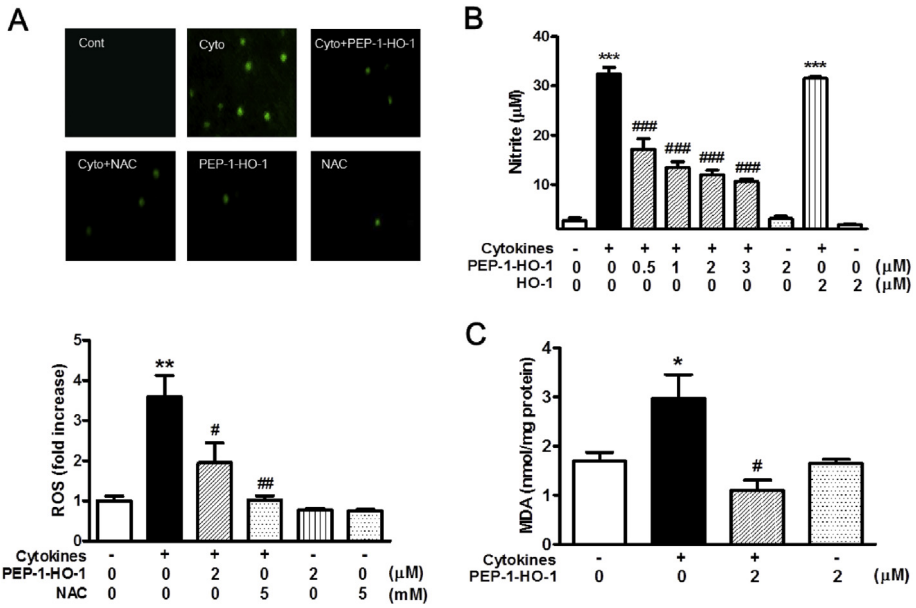

Fig. 3. Effects of the transduced PEP-1-HO-1 on cytokine-induced elevated levels of ROS, nitrite, and MDA. Cultured cells pre-treated with PEP-1-HO-1 or NAC were incubated with a combination of cytokines (1 ng/ml IL-1β, 5 ng/ml TNF-α, and 5 ng/ml IFN-γ) for 24 h. Levels of ROS, nitrite, and MDA were measured as described in Materials and methods. Bars represent mean ± SEM of data obtained from 4 independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared with untreated control group, and $\#p < 0.05$, $\#\#p < 0.01$, and $\#\#\#p < 0.001$ compared with cytokine-treated group.

PARP and eventually induces apoptosis. To determine detailed mechanisms by which PEP-1-HO-1 exerted cytoprotective effects against cytokines, we examined the effect of the transduced PEP-1-HO-1 on the levels of apoptosis-related proteins. Exposure of cells to cytokines increased the expression level of pro-apoptotic Bax by approximately 4.5 fold and induced the cleavage of caspase-3 and PARP. These elevated levels of pro-apoptotic proteins were markedly decreased by the transduced PEP-1-HO-1 (Fig. 4B). On the contrary, the level of anti-apoptotic Bcl-2 protein decreased in cells exposed to cytokines. This decrease in Bcl-2 expression was attenuated by pre-treatment of 2 μM PEP-1-HO-1. These data indicating that the cytoprotective effects of the transduced PEP-1-HO-1 involved suppression of the intrinsic apoptotic pathway. The Bcl-2 family proteins and caspase-3 have been reported to be involved in cytokine-induced apoptosis of pancreatic β-cells [7,31,32].

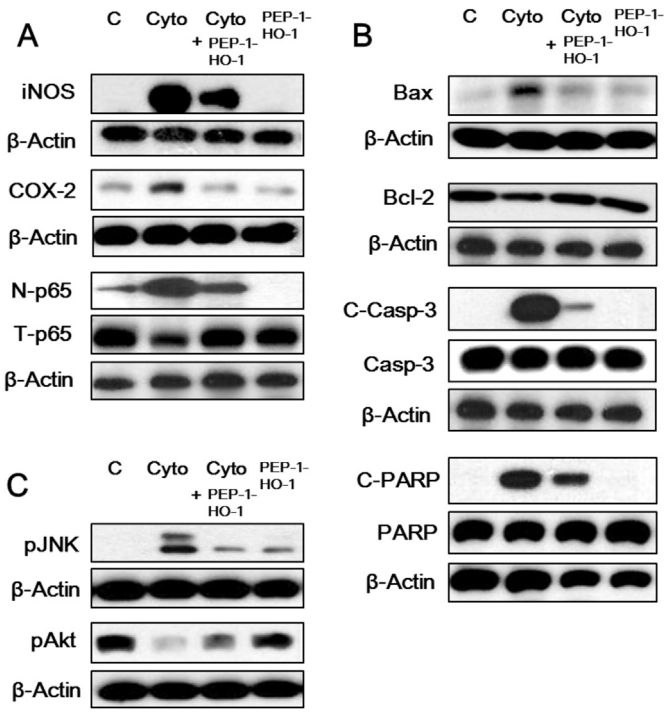

Fig. 4. Effects of the transduced PEP-1-HO-1 on the expression levels of pro-inflammatory mediators and apoptosis-/survival-related proteins. Cultured cells pre-treated with PEP-1-HO-1 were incubated with a combination of cytokines (1 ng/ml IL-1β, 5 ng/ml TNF-α, and 5 ng/ml IFN-γ) for 24 h. Levels of pro-inflammatory mediators (A), pro-apoptotic/anti-apoptotic proteins (B), and pJNK/pAkt (C) in the cell lysates were assessed by western blotting. N-p65; nuclear p65, T-p65; total p65, C-Casp-3; cleaved caspase-3, C-PARP; cleaved PARP.

JNK and Akt have also been implicated in cytokine-induced apoptosis of pancreatic β-cells. Studies have shown that inhibition of the JNK or stimulation of the Akt pathway protects β-cells from cytokine-induced apoptosis [33,34]. In this study, phosphorylation of JNK was remarkably increased and that of Akt was remarkably suppressed in cytokine-exposed INS-1 cells (Fig. 4C). However, these cytokine-induced changes in JNK and Akt activation were remarkably reversed by pre-treatment of 2 μM PEP-1-HO-1. Recent study showed that NO contributed to cytokine-induced apoptosis of INS-1 cells by simultaneously enhancing JNK activation and inhibiting Akt activation [3]. In this study, we observed that PEP-1-HO-1 significantly inhibited cytokine-induced NO production in INS-1 cells (Fig. 2B). Therefore, it is possible that PEP-1-HO-1 influenced JNK and Akt activation by inhibiting cytokine-induced NO production.

In conclusion, we showed that HO-1 was efficiently delivered into INS-1 cells by PEP-1-mediated transduction and that the transduced PEP-1-HO-1 exerted a protective effect against cytokine-induced damage by inhibiting ROS and NO accumulation and subsequently suppressing expression of pro-inflammatory mediators and pro-apoptotic proteins. Thus, PEP-1-mediated HO-1 transduction may be an efficient tool to prevent β-cell destruction in patients with autoimmune diabetes mellitus. However, for the practical application of PEP-1-HO-1 in antidiabetic therapy, further studies need to be performed to examine its transduction

efficiency and to evaluate its cytoprotective functions and side effects by using different β-cells and animal models of autoimmune diabetes mellitus.

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Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

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