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Transduction of Tat-Superoxide Dismutase into Insulin-producing MIN6N Cells Reduces Streptozotocin-induced Cytotoxicity

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The reactive oxygen species (ROS) are considered to be an important mediator in pancreatic β cell destruction, thereby triggering the development of insulin-dependent diabetes mellitus. In the present study, HIV-1 Tat-mediated transduction of Cu,Zn-superoxide dismutase (SOD) was investigated to evaluate its protective potential against streptozotocin (STZ)-induced cytotoxicity in insulin-producing MIN6N cells. Tat-SOD fusion protein was successfully delivered into MIN6N cells in a dose-dependent manner and the transduced fusion protein was enzymatically active for 48 h. The STZ induced-cell destruction, superoxide anion radical production, and DNA fragmentation of MIN6N cells were significantly decreased in the cells pretreated with Tat-SOD for 1 h. Furthermore, the transduction of Tat-SOD increased Bcl-2 and heat shock protein 70 (hsp70) expressions in cells exposed to STZ, which might be partly responsible for the effect of Tat-SOD. These results suggest that an increased of free radical scavenging activity by transduction of Tat-SOD enhanced the tolerance of the cell against oxidative stress in STZ-treated MIN6N cells. Therefore, this Tat-SOD transduction technique may provide a new strategy to protect the pancreatic β cell destruction in ROS-mediated diabetes.

Key Words: Diabetes, Tat-mediated transduction, Superoxide dismutase, ROS, Insulin-producing cells

INTRODUCTION

Insulin dependent diabetes mellitus (IDDM) results from the destruction of insulin-producing pancreatic β cells by multiple factors, including autoimmune responses, viruses, toxins, and diets (Bach, 1994). Although the exact cellular mechanism is still obscure, it has been suggested that locally produced reactive oxygen species (ROS) following cytokine stimulation are involved in the pancreatic β cell destruction (Rabinovitch et al, 1996; Sjoholm, 1998). Increased ROS activate redox-sensitive NFκB, which then induces the expressions of cytokines and chemotactic agents involved in the immune response. This cascade results in the cyclic amplification of ROS and eventually leads to β cell death (Ho & Bray, 1999).

The cellular defense against ROS utilizes antioxidant enzymes, such as superoxide dismutase (SOD), catalase, and glutathione peroxidase. In particular, Cu,Zn-SOD plays a pivotal role in this cellular defense mechanism by intercepting and reducing superoxide to hydrogen peroxide, which is readily reduced to water by catalase or glutathione peroxides (Fridovich, 1995). However, pancreatic β cells appear to be exquisitely sensitive to ROS, because of constitutively low expression of these antioxidant enzymes in the pancreas (Lenzen et al, 1996; Tiedge et al, 1997).

A striking example of β cell vulnerability to ROS is the severe damage produced by streptozotocin (STZ), which is a natural toxin widely used to produce experimental diabetes because of its ability to selectively target and destroy pancreatic islet β cells. Its diabetogenic action has been ascribed to the several destructive actions of STZ such as the production of ROS and nitric oxide (Kwon et al, 1994; Szkudelski, 2001), and methylation reactions (Murata et al, 1999).

A number of studies have provided the rationales of increased antioxidant enzyme expression in insulin-producing cells to enhance their resistance to cytotoxic challenges caused by ROS, NO and cytokines. Overexpression of antioxidant enzymes in the pancreas increases the resistance of insulin-producing cells to the cytokine-mediated toxicity through inactivation of ROS (Kubisch et al, 1997; Tiedge et al, 1999). Recently, several small regions of proteins called protein transduction domains (PTD) such as human immunodeficiency virus type 1 (HIV-1) transactivator of transcription (Tat) protein, Drosophila Antennapedia (Antp) protein, and herpes simplex virus VP22 protein have been developed for the delivery of exogenous protein into living cells (Schwarze et al, 2000). Using this protein transduction technology, we recently demonstrated that a genetic in-frame HIV-1 Tat-Cu,Zn superoxide dis-

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ABBREVIATIONS: ROS, reactive oxygen species; NO, nitric oxide; SAR, superoxide anion radicals; Tat, transactivator of transcription; SOD, superoxide dismutase; STZ, streptozotocin; hsp70, heat shock protein70.

mutase (Tat-SOD) fusion protein was efficiently transduced into mammalian cells. Moreover, this transduced Tat-SOD increased the viability of HeLa cells exposed to paraquat, an intracellular superoxide anion generator, suggesting that it could protect cells from the oxidative stress (Kwon et al, 2000; Eum et al, 2002).

In the present study, we applied the Tat-mediated transduction of Cu,Zn-SOD to insulin-producing MIN6N cells. Our results showed that Tat-SOD could be directly transduced into MIN6N cells across the membrane barrier and it efficiently prevented the STZ-induced cell destruction.

METHODS

Chemicals and cell culture

Cell permeable HIV-1 Tat-SOD was overexpressed in E. coli and purified as previously described (Kwon et al, 2000). A schematic representation of Tat-SOD expression vector system and expressed fusion protein are illustrated in Fig. 1. Ni^{2+}-nitrilotriacetic acid Sepharose superflow was obtained from Qiagen (Valencia, USA), isopropyl-β-D-thiogalactoside (IPTG) from Duchefa (Haarlem, Netherlands), Bcl-2 and hsp70 monoclonal antibodies from Santa Cruz (Santa Cruz, USA), and an enhanced chemiluminescence (ECL) substrate kit was obtained from Pierce (Rockford, USA). The MIN6N8a cell line, insulin-producing cells derived from pancreatic β cells of NOD mice, was kindly provided by the Korean Type Culture Collection (Taejeon, Korea). Fetal bovine serum (FBS), penicillin-streptomycin and trypsin-EDTA were from GibcoBRL (Frederick, USA),

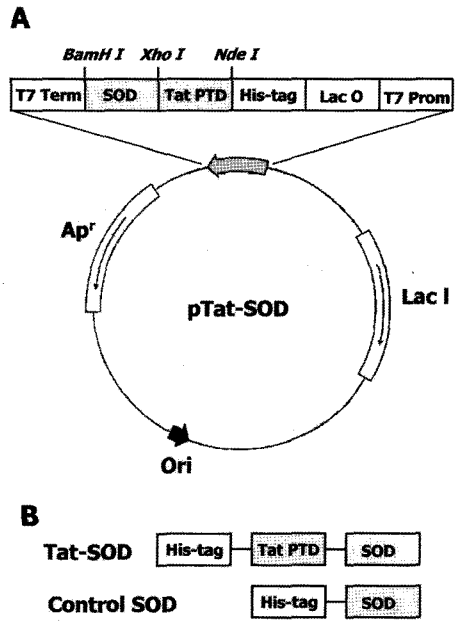

Fig. 1. Schematic representation of Tat-SOD expression vector (pTat-SOD) based on the vector pET15b (A) and expressed Tat-SOD fusion protein (B). The synthetic Tat oligomer was cloned into the Nde , Xho sites, and 486 bp of human Cu,Zn-SOD cDNA was cloned into Xho , BamH sties of pET15b. The expression vector is under the control of the T7 promotor and lacO-operator.

and Dulbecco's modified essential medium (DMEM) was obtained from Bio-Whittaker (Walkersville, USA). MIN6N cells were cultured in DMEM supplemented with 15% FBS and antibiotics (100 μg/ml streptomycin, 100 U/ml penicillin) at 37°C under a humidified condition of 95% air and 5% CO_2.

Transduction of Tat-SOD into MIN6N cells

For transduction of Tat-SOD, MIN6N cells were grown to confluence on a 6 or 12 well plate. The culture medium was then replaced with 1 ml of fresh solution without FBS. After cells were treated with 0.5~3 μM Tat-SOD fusion proteins for 1 h, they were treated with trypsin-EDTA and washed with phosphate-buffered saline. Cells were harvested and extracted for SOD enzyme assay and Western blotting. The dismutation activity of SOD in cell extracts was measured by monitoring the inhibition of ferricytochrome c reduction by the xanthine/xanthine oxidase reaction and Western blot analysis of SOD was performed according to the method described (McCord and Fridovich, 1969; Kwon et al, 2000). The intracellular stability of transduced Tat-SOD fusion protein was estimated as follows: After treated with 3 μM Tat-SOD for 1 h, the cells were washed with fresh culture medium to remove Tat-SOD that was not transduced. Subsequently, the cells were further incubated for 3, 6, 12, 24 and 48 h, and cell extracts were then prepared for SOD enzyme assay and Western blot analysis.

Effect of transduced Tat-SOD on the viability of MIN6N cells exposed to STZ

Biological activity of transduced Tat-SOD was assessed by measuring viability of cultured cells exposed to streptozotocin (STZ). Cells were plated into 6 well trays at 70% confluence and were allowed to attach cells per well. After the cells were treated with 3 μM Tat-SOD for 1 h, 1~50 mM STZ was added to the culture medium for 12 h. Cell viability was estimated by colorimetric assay using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide).

Effect of transduced Tat-SOD on expressions of Bcl-2 and hsp70 protein in MIN6N cells exposed to with STZ

MIN6N cells were plated at 5×10^5 cells onto 6 well culture dish. After the cells were treated with 3 μM Tat-SOD for 1 h, 20 mM STZ was added to the culture medium for 1~12 h. The expressions of Bcl-2 and hsp70 were assessed from cell lysates by Western blotting. The nitrocellulose membrane was probed with primary monoclonal antibodies of Bcl-2 (1 : 200) or hsp70 (1 : 200), followed by incubation with goat anti-mouse IgG antibody (1 : 5,000). The bound antibodies were then visualized by ECL kit according to the manufacturer's instruction.

Biochemical analysis and immunofluorescence

The content of superoxide anion radicals (SAR) was determined by monitoring the reduction rate of ferricytochrome c in the reaction mixture, which was measured as the change in absorbance at 550 nm for 10 min at room temperature (McCord and Fridovich, 1969). NO was measured as its stable oxidative metabolite, nitrite, as pre-

viously described (Misko et al, 1993). Briefly, at the end of the incubation, 100 μl of the culture medium was mixed with an equal volume of Griess reagent (0.1% naphthylethylenediamine dihydrochloride and 1% sulfanilamide in 5% phosphoric acid). Absorbance at 550 nm was measured, and the nitrite concentration was determined using a curve calibrated with sodium nitrite standards. Cultured cells were lysed and treated with RNase and proteinase K for analysis of DNA fragmentation (Iwahashi et al, 1996). Briefly, DNA was then extracted with phenol-chloroform, precipitated with isopropanol, washed with ethanol, and air-dried. DNA samples were separated by 1.5% agarose gel electrophoresis. For indirect immunofluorescence, cultured cells were grown on glass cover slips and treated with 3μM Tat-SOD for 1 h. The cells were fixed in 4% paraformaldehyde in 0.5 ml of PBS for 10 min, and then incubated with a monoclonal anti-human Cu,Zn-SOD antibody (hSOD mAb-1.4), followed by incubation with FITC-conjugated anti-mouse IgG antibody (1 : 200) for 1 h. Transduced cells were analyzed by confocal laser microscopy (BioRad, MRC 1024ES).

Statistical analysis

Data were illustrated as means±SEM. Statistical comparisons between two groups were evaluated by the Student's t-test. The difference was considered to be significant when P value is less than 0.05.

RESULTS

To investigate whether Tat-SOD fusion protein could transverse the membrane of MIN6N cells, various concentrations (0.5~3 μM) of fusion proteins were added to the culture media for 1 h, and the levels of protein transduced into the cells were determined by Western blot analysis and SOD enzyme assay. As shown in Fig. 2A, Tat-SOD was successfully delivered into MIN6N cells, whereas the control SOD was not. The transduction of Tat-SOD into cultured cells increased as the medium concentration of fusion protein increased. Similarly, the SOD enzyme activity in the cells rose gradually as the concentration of fusion protein in the medium increased. The intracellular dismutation activity increased approximate 10 fold by 3 μM Tat-SOD treatment. The delivery of Tat-SOD into MIN6N cells was further confirmed by indirect immunofluorescence analysis. As shown in Fig. 2C, almost all cultured cells were found to be transduced with Tat-SOD, whereas immunofluorescence signals were not detected in cells treated with control SOD. The intracellular stability and enzyme activity of Tat-SOD transduced into MIN6N cells were investigated. The transduced Tat-SOD appeared to be de-

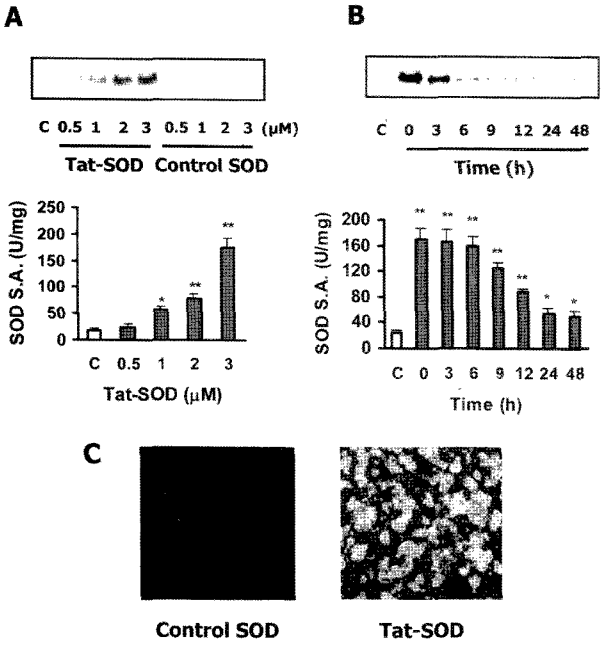

Fig. 2. Transduction of Tat-SOD into cultured MIN6N cells (A). Tat-SOD or control SOD (0.5~3 μM) was added to the culture media for 1 h. Stability of transduced Tat-SOD in MIN6N cells (B). Cells pretreated with 3 μM Tat-SOD were placed in fresh culture medium and further incubated for 3~48 h. The transduction activity was analyzed by Western blot and SOD enzyme assay. Immunofluorescence analysis of transduced Tat-SOD in MIN6N cells (C). MIN6N cells were transduced with 3 μM Tat-SOD for 1 h. Cells were fixed, and incubated with SOD monoclonal antibody/FITC-conjugated anti-mouse IgG antibody, and transduced cells were identified by confocal laser microscopy. Each bar in A and B represents mean±SEM of four experiments. *: P<0.05, **: P<0.01 vs control group.

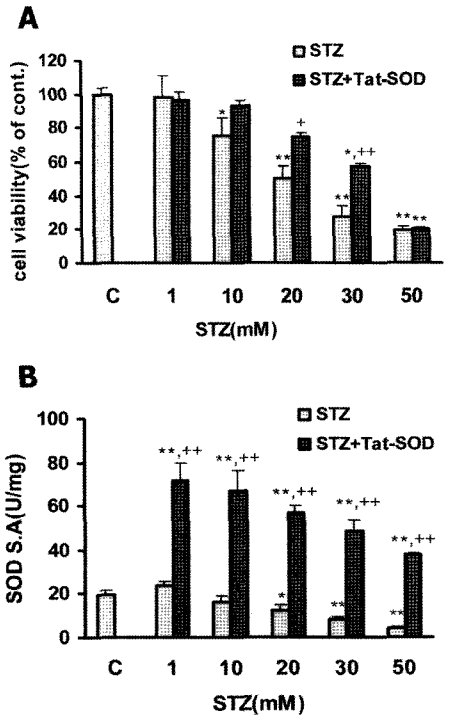

Fig. 3. Effects of Tat-SOD transduction on the viability (A) and intracellular SOD activity (B) of MIN6N cells exposed to STZ. After the cells were treated with 3 μM Tat-SOD for 1 h, 1~50 mM STZ was added to the culture medium for 12 h. Cell viability was estimated by colorimetric assay using MTT. Each bar represents mean±SEM of five experiments. *; P<0.05, **; P<0.01 vs control group. +; P<0.05, ++; P<0.01 vs corresponding STZ-treated group.

graded as a function of incubation time (Fig. 2B). However, significant levels of transduced protein and enzyme activity in MIN6N cells still persisted after 48 h. These data demonstrated that Tat-SOD fusion protein could be efficiently transduced into MIN6N cells and enzymatically active for at least 48 h.

To determine whether the transduced fusion protein was functionally active in MIN6N cells, we examined the effect of Tat-SOD transduction on STZ-induced cytotoxicity. As shows in Fig. 3A, STZ induced MIN6N cell destruction in a dose-dependent manner: Approximately half of the cells were destroyed after 12 h exposure to 20 mM STZ. However, the cell viability was considerably increased by pretreating the cells with 3 μM Tat-SOD for 1 h: Tat-SOD transduction significantly increased the cell viability by 50 and 110% in the 20 and 30 mM STZ-treated groups, respectively. Consistent with this observation, the SOD enzyme assay showed 3~10 fold increment in dismutation activity in Tat-SOD treated-cells compared to control cells (Fig. 3B).

To determine whether these cytoprotective effects of Tat-SOD were directly correlated with the intracellular levels of ROS and NO, we examined the effects of Tat-SOD transduction on the levels of superoxide anion radicals (SAR) and NO. As expected, STZ dose-dependently increased the intracellular levels of SAR and NO. Transduction with Tat-SOD markedly suppressed STZ-evoked SAR to a level similar to the basal level (Fig. 4A). However, the increase of NO level was not affected by Tat-SOD treatment (Fig. 4B). STZ is known to induce DNA fragmentation via the generation of ROS or NO, as well as direct alkylation of DNA. As shown in Fig. 5, a distinct DNA fragmentation was detected after 9 h with 20 mM STZ in culture medium, and extensive fragmentation was observed after 12 h. Such a deleterious action of STZ on DNA was markedly attenuated by pretreatment of the cells with Tat-SOD for 1 h (Fig. 5). These results indicate that the transduced Tat-SOD protects the cells from oxidative stress by removing the SAR.

Next, we assessed the effects of Tat-SOD transduction on the expressions of Bcl-2 and heat shock protein 70 (hsp70), which are known as potent antiapoptotic proteins against several cytotoxic stimuli. When 20 mM STZ was added to culture medium, Bcl-2 expression markedly decreased at 1 h, slowly returned to the basal level at 9

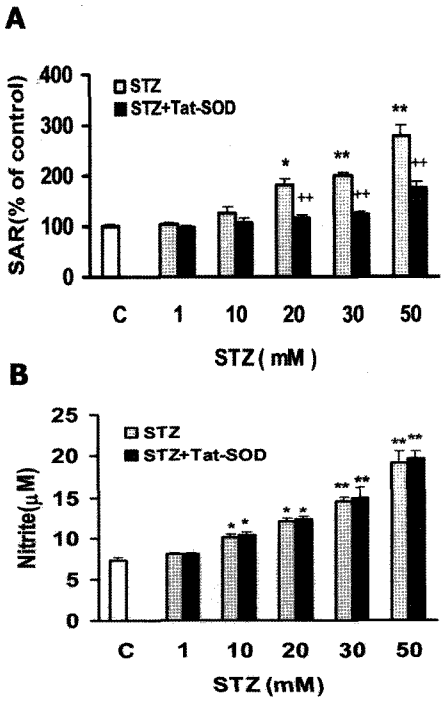

Fig. 4. Effects of Tat-SOD transduction on the levels of superoxide anion radical (SAR) (A) and nitrite (B) in MIN6N cells exposed to STZ. After the cells were treated with 3μM Tat-SOD for 1 h, 1~50 mM STZ was added to the culture medium for 12 h. Each bar represents mean±SEM of five experiments. *; $P<0.05$, **; $P<0.01$ vs control group. ++; $P<0.01$ vs corresponding STZ-treated group.

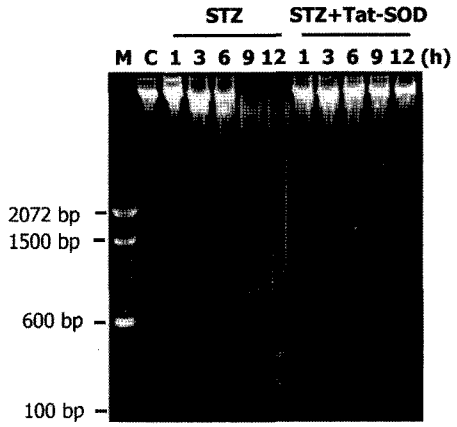

Fig. 5. Effects of Tat-SOD transduction on STZ-induced DNA fragmentation in MIN6N cells. After the cells were treated with 3 μM Tat-SOD for 1 h, 20 mM STZ was added to the culture medium for 1~12 h. The DNA samples were separated by 1.5% agarose gel electrophoresis and stained with ethidium bromide. M; DNA molecular size marker (100 bp DNA ladder), C; control.

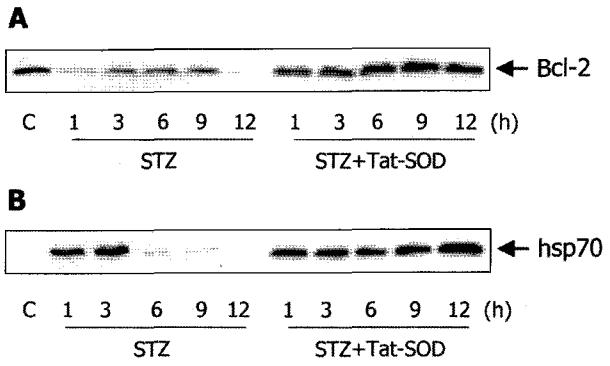

Fig. 6. Effects of Tat-SOD transduction on the expressions of Bcl-2 (A) and hsp70 (B) in MIN6N cells exposed to STZ. After the cells were treated with 3 μM Tat-SOD for 1 h, 20 mM STZ was added to the culture medium for 1~12 h. The expressions of Bcl-2 and hsp70 were assessed from cell lysates by Western blotting. The nitrocellulose membrane was probed with primary monoclonal antibodies of Bcl-2 or hsp70, followed by incubation with goat anti-mouse IgG antibody. The bound antibodies were then visualized by enhanced chemiluminescence method.

h, and decreased again at 12 h (Fig. 6A). However, in the Tat-SOD transduced cells, the Bcl-2 expression remained high and was not affected by STZ. In the case of hsp70, STZ induced a transient increase in its expression up to 3 h, after which it rapidly declined (Fig. 6B). However, in the cells pretreated with Tat-SOD the level of hsp70 expression remained high, independent of STZ treatment.

DISCUSSION

In an effort to replenish the Cu,Zn-SOD activity in cells of various human disorders related to oxidative stress, we have recently taken a genetic approach of the Tat-SOD transduction into mammalian cells, and showed that Cu,Zn-SOD fused with HIV-1 Tat protein could be directly delivered into HeLa cells across the membrane barrier, and the transduced Tat-SOD could play its defensive role in the cells under oxidative stress (Kwon et al, 2000; Eum et al, 2002). In this study, we applied this protein transduction technology to insulin-producing MIN6N cells to enhance cellular defense potentiality.

We found that Tat-SOD fusion protein could be transduced into MIN6N cells and the intracellular SOD activity increased as a function of the fusion protein concentration in the medium (Fig. 2A), the intracellular SOD activity being increased 10 fold by treatment with 3 μM Tat-SOD. This was in contrast to the other recent reports that intracellular SOD activity increased 2~3 fold by adenovirus-mediated gene transfer in human islet and INS-1 cells, and about 2 fold by SOD overexpression by stable transfection in an insulin-producing RINm5F cell line (Lortz et al, 2000; Moriscot et al, 2000). Moreover, the SOD fluorescence signal was found to be uniformly distributed in MIN6N cells treated with Tat-SOD (Fig. 2C), suggesting that the transduced fusion proteins were distributed in both cytoplasm and nucleus. It has recently been suggested that the cluster of nine basic amino acids of Tat protein (Tat 49-57) is enough to serve as a nuclear-localization signal (Park et al, 2002). Although an apparent degradation of transduced Tat-SOD was observed, intracellular fusion protein was still detectable 48 h after the transduction (Fig. 2B). These results suggest that the protein transduction with Tat-SOD could be a more powerful approach to replenish antioxidant enzyme activity in insulin-producing cells than viral-mediated gene transgenesis.

The biological effectiveness of transduced Tat-SOD in the cell was estimated by measuring the viability of STZ-treated MIN6N cells. STZ was chosen, since it has widely been used to induce experimental diabetes, because of its ability to selectively target and destroy pancreatic β cells. This cytotoxic action has been associated with an increase of intracellular ROS and NO (Kwon et al, 1994; Szkudelski, 2001). Our data confirmed that the elevated production of ROS and NO in STZ-treated cells induced DNA fragmentation typical of apoptosis, ultimately leading to cell destruction (Fig. 3~5). However, these changes were prevented by Tat-SOD transduction, suggesting that the transduced fusion proteins protected MIN6N cells from STZ effect most likely by removing SAR (Fig. 3A and Fig. 4A). The effect of STZ on intracellular NO level was not affected by Tat-SOD transduction, suggesting that NO was not directly involved in the cytoprotective action of Tat-SOD. It should be noted that NO has been identified as an important mediator of β cell destruction in autoimmune diabetes (Sjoholm, 1998; Mandrup-Poulsen, 2001). Furthermore, it has recently been suggested that peroxynitrite, a highly reactive nitrogen species formed from the reaction of NO and SAR, is highly cytotoxic and critically involved in the development of autoimmune diabetes in NOD mice and destruction of human pancreatic islet β cells (Suarez-Pinzon et al, 1997; Lakey et al, 2001). Therefore, although we did not measure intracellular peroxynitrite concentration, it is possible that transduced Tat-SOD indirectly reduced peroxynitrite formation by lowering intracellular SAR, thereby attenuating STZ toxicity. Intracellular action of STZ results in DNA fragmentation in pancreatic β cells. This STZ-induced DNA damage was obviously prevented by Tat-SOD transduction (Fig. 5). Several studies have shown that ROS and NO are involved in STZ-induced DNA fragmentation (Kroncke et al, 1995; Szukudelski, 2001), and they may also synergistically contribute to STZ-induced DNA fragmentation through forming highly toxic peroxynitrite. In the present study, we showed that increased ROS scavenging by Tat-SOD transduction attenuated the STZ-induced DNA damage.

Although exact mechanism has not been elucidated in this study, we observed that the Tat-SOD transduction enhanced the expression of potent antiapoptotic proteins, Bcl-2 and hsp70 in STZ-treated MIN6N cells. As shown in Fig. 6, the Bcl-2 and hsp70 expressions were markedly increased by Tat-SOD transduction, and the expression level was sustained high for at least 12 h in STZ-treated cells. Bcl-2, a well-characterized antiapoptotic protein, has been shown to prevent many forms of apoptosis, including oxidative stress-induced programmed cell death (Antonsson and Martinou, 2000; Chen et al, 2003). Overexpression of Bcl-2 in insulin-producing cell lines at least partially prevents cytokinin-induced apoptotic cell death (Iwahashi et al, 1996; Barbu et al, 2002). SOD expression in other cell system is enhanced and its activity is increased in the presence of Bcl-2, possibly leading to reduction of intracellular ROS (Ellerby et al, 1996; Esposti et al, 1999). Conversely, ROS induces down-regulation of Bcl-2 protein expression in parallel to decrease of bcl-2 mRNA level in neuronal cells (Pugazhenthi et al, 2003). Based on these earlier observations, Tat-SOD might have enhanced Bcl-2 expression by scavenging STZ-evoked ROS in MIN6N cells. In contrast to the Bcl-2 expression, the hsp70 expression was markedly induced by STZ, although the expression rapidly declined after 6 h (Fig. 6B). It is well known that hsp70, a potent antiapoptotic intracellular chaperone, is readily induced by several stresses, such as heat, oxidative stress, or anticancer drugs (Garrido et al, 2001), and up-regulation of hsp70 has been shown to increase resistance against ROS, NO and STZ in rat pancreatic islet cells (Bellmann et al, 1995). Although the mechanism remains unknown, transduction of Tat-SOD resulted in a sustained increase of hsp70 expression in STZ-treated MIN6N cells (Fig. 6B), which might be partly responsible for the protective effect of Tat-SOD. Further studies are required to establish this notion.

In summary, we demonstrated in this study that Cu,Zn-SOD, one of the key antioxidant enzymes, can be efficiently delivered into insulin-producing MIN6N cells by HIV-1 Tat-mediated protein transduction in vitro. The transduced Tat-SOD is enzymatically and biologically active, and protects the STZ-induced cell destruction by scavenging ROS. Therefore, this Tat-SOD transduction may provide a new strategy to protect the pancreatic β cell

destruction in ROS-mediated diabetes.

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