Heme oxygenase-1 fused to a TAT peptide transduces and protects pancreatic β-cells

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Abstract

Transplantation of islets is becoming an established method for treating type 1 diabetes. However, viability of islets is greatly affected by necrosis/apoptosis induced by oxidative stress and other insults during isolation and subsequent in vitro culture. Expression of cytoprotective proteins, such as heme oxygenase-1 (HO-1), reduces the deleterious effects of oxidative stress in transplantable islets. We have generated a fusion protein composed of HO-1 and TAT protein transduction domain (TAT/PTD), an 11-aa cell penetrating peptide from the human immunodeficiency virus TAT protein. Transduction of TAT/PTD–HO-1 to insulin-producing cells protects against TNF-α-mediated cytotoxicity. TAT/PTD–HO-1 transduction to islets does not impair islet physiology, as assessed by reversion of chemically induced diabetes in immunodeficient mice. Finally, we report that transduction of HO-1 fusion protein into islets improves islet viability in culture. This approach might have a positive impact on the availability of islets for transplantation.

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Transplantation of pancreatic islets has become a promising approach for treating diabetes. The introduction of a novel glucocorticoid-free immunosuppressive regimen [1] has shown that long-term insulin independence can be achieved following islet transplantation. Despite this success, more than one pancreas preparation per recipient is generally required to observe insulin independence. This limitation is in part due to the quality/viability of pancreatic islets. Once isolated, non-vascularized pancreatic islets are easily damaged in vitro culture by hypoxia and diffusion-limited availability of nutrients, resulting in decreased number and function. Therefore, improvement of islet culture conditions is an important goal in islet transplantation. Such improvement could be provided by direct delivery of protective anti-oxidative stress/anti-apoptotic proteins into islets during culture. This approach has the distinct feature of expressing the desired protein transiently. Temporary expression is an important advantage when the expression of a specific protein is required only for a short period of time. Lipid carriers and protein transduction represent two possible alternatives for protein delivery. Liposome carriers have recently been designed to transport active proteins across cell membranes [2]. Protein transduction is a novel technology by which proteins/peptides can be directly transferred into cells when covalently linked to small peptide domains, known as protein transduction domains (PTDs) [3]. The most common PTDs are part of naturally occurring proteins, among them being homeodomain transcription factors [4], the herpes simplex virus type I protein VP22 [5], and the human immunodeficiency virus trans-activator TAT protein [6–8]. Other peptides, derived from screening peptide-libraries, are capable of transducing different types of cells including pancreatic islets [9]. In particular, PTD-5 was used for the delivery of a peptide inhibiting nuclear factor κ B activation in pancreatic islets [10]. TAT/PTD is one of the most widely studied PTDs. We have previously used TAT/PTD...
fusional protein transduction to antagonize apoptosis/cell death in pancreatic β-cells [11]. The mechanism of internalization of TAT/PTDs is currently unknown, although endocytosis has been reported as having a role in protein transduction [12,13].

This work describes the effects of protein transduction of a recombinant TAT/PTD–heme oxygenase-1 on pancreatic islet viability. Heme oxygenase-1 (HO-1) has been identified as a ubiquitous protein induced in many cell types in response to stress conditions [14]. The induction of HO-1 expression is considered a primary adaptive response of the cellular defense mechanism. HO-1 is the rate-limiting enzyme of heme degradation into its by-products carbon monoxide (CO), iron, and biliverdin. CO has a cytoprotective role in different systems [15–18] and was not toxic to islets and remarkably enhanced the toxicity. Furthermore, transduction with TAT/PTD–HO-1 was not toxic to TC-3 cells [11]. The mechanism of induction has been used to reduce the deleterious effects of cytokine-induced apoptosis and oxidative stress in various cell types and animal models [22–26]. The aim of this study was to generate a TAT/PTD–HO-1 fusion protein that could transduce insulin-producing cells, conferring cytoprotection. We produced and purified recombinant HO-1 protein fused in-frame with TAT/PTD. Transduction of TAT/PTD–HO-1 into pancreatic βTC-3 cells conferred protection against TNF-α cytotoxicity. Furthermore, transduction with TAT/PTD–HO-1 was not toxic to islets and remarkably enhanced the insulin secretion capability of cultured islets.

Materials and methods

Cloning and related techniques

The recombinant TAT/PTD anti-apoptotic protein construct was generated by inserting the DNA coding region of murine heme oxygenase-1 (HO-1) (produced by Robert Oliver University of Miami) in the EcoRI site of the pTAT expression vector (kind gift from Dr. Steven Dowdy from USDC).

Protein generation and purification

The isolation and purification of TAT/PTD–HO-1 and HO-1 (no TAT) were done as previously described [11] with some modifications in the washing steps; briefly, the Ni–NTA column, with bacterial extract, was sequentially washed with 20 mM imidazole and 30 mM imidazole, both in cold PBS. Elution was performed with 100 mM imidazole. Proteins were preserved at −80 °C with 10% glycerol and quantified with Bio Rad protein assay. Purity was assayed by Tris–HCl SDS-PAGE 12.5% gel. Western blot was done with primary mouse Penta His antibody (Quiagen) (1:1000).

Isolation of rat islet of Langerhans

Islets were isolated from Lewis rats (Charles River Labs) by dissociation of the pancreatic tissue using Liberase RI purified enzyme blend (Roche Molecular Biochemical, Indianapolis, IN) at a concentration of 0.16 mg/ml following procedure described previously [11].

Cell line and rat pancreatic islet culture

βTC-3 cells, DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) were cultured in DMEM (high glucose, 4.5 g/L), with 10% fetal bovine serum, and 1% sodium pyruvate (100 mM) at 37 °C and 7.5% CO2. Rat islet cells were cultured in CMRL 1600 supplemented with 10% FBS under the same conditions. When culturing islets for static glucose challenge, an IEq (islet equivalent) = 50 was distributed into 70 ml non-treated culture flasks, one day after islet isolation. Media with TAT/PTD protein were changed on average every 3–4 days. The content of one 70 ml flask was used for each static incubation column. Rat islets were incubated with TAT/PTD–HO-1 the following morning after isolation and kept in culture for 16 days.

Transduction of βTC-3 with labeled TAT–HO-1

TAT–HO-1 was labeled using FluoReporter FITC Protein Labeling Kit (Molecular Probes). TAT–HO-1–FITC (6.7 μM) was added to a tube containing floating βTC-3 in 500 μl of DMEM. Tube with cells was incubated for 3 h at 37 °C and washed extensively with DMEM. Pellet was resuspended in 500 μl DMEM and plated in a glass-bottomed number 0 uncoated γ-irradiated petri dish for confocal microscopy. Forty microliters of a 0.5 mg/ml solution of propidium iodide (PI) was added to cells before analysis to rule out internalization of the TAT-fused protein due to damaged cell membrane. The samples were examined under a LEICA confocal laser scanning microscope (LSM-510) equipped with image analysis software.

βTC-3 cell viability after induced TNF-α cytotoxicity

βTC-3 (106) cells were transduced for 2 h with TAT/PTD–HO-1 (200 nM) before adding 4000 U of mouse (TNF-α) and 10 μg/ml cycloheximide (CHX). After incubation for 16 h cells were assayed with Live/Dead viability Cytotoxicity Kit (Molecular Probes). Pictures were captured by a charge-coupled device camera (Lei-750; Leica) using Video Vixen software and analyzed by Photoshop version 5.5.

Static glucose challenge of islets of Langerhans

Rat islets were subjected to low (40 mg/dl), high (400 mg/dl), and low (40 mg/dl) static glucose challenge as described previously [11].

Reversion of hyperglycemia in diabetic immunodeficient mice

Animals. Male Lewis rats (150–170 g body weight) were purchased from Charles River (Wilmington, MA) and used as donors of pancreatic islets. Recipient animals were immunodeficient male CB17–scid mice at an average body weight of 29 g (Jackson Labs). Animals were rendered diabetic by a single intravenous injection of alloxan 90 mg/kg. The islet transplantation procedure and graft function monitoring were performed as previously described [11].

Results and discussion

Generation and purification of recombinant TAT/PTD–HO-1 fusion protein

To generate a TAT/PTD–HO-1 recombinant protein we inserted the coding region of the murine heme oxygenase gene in-frame with the 11-amino-acid PTD of the HIV/TAT protein in the pTAT-HA expression vector.
vector. Bl 21 *Escherichia coli* was used to produce the recombinant TAT/PTD–HO-1 protein. A green coloration of the bacterial culture was consistently observed, suggesting that the recombinant TAT/PTD–HO-1 metabolized the heme from *E. coli* into biliverdin, that accumulated due to the lack of biliverdin reductase in the bacteria (Fig. 1B). The fusion protein has additional six consecutive histidines to allow for purification of the recombinant protein by affinity chromatography. The result of the purification procedure is shown in Fig. 1C. The fusion protein was expressed at relatively high levels (8 mg/L of culture) and was more than 95% pure as assessed by SDS–PAGE. Previous expression of the rat HO-1 in *E. coli* [27] had produced two forms of the protein, the membrane bound 32 kDa native protein and a 30 kDa form, representing the non-membrane bound HO-1 protein, lacking the hydrophobic C-terminal segment responsible for membrane insertion [28]. Both forms were found to catalyze the breakdown of heme to biliverdin [27]. Similarly, once the purified TAT/PTD–HO-1 shows a band of 39 kDa, the expected size of the fusion protein TAT/PTD–HO-1, and a band of approximately 37 kDa, representing the non-membrane bound recombinant protein. Both bands were determined by Western blot analysis using an anti-histidine antibody (data not shown). The specific activity of the purified TAT/PTD–HO-1, assayed as bilirubin formation in the coupled assay with biliverdin reductase, was $24 \times 10^3$ nmols of bilirubin/mg protein/h, slightly larger than that of heme oxygenase-1 from other sources that were purified to homogeneity [29,30].

**TAT/PTD–HO-1 efficiently transduces pancreatic β-cells**

To test the TAT/PTD–HO-1 transduction capability, pancreatic βTC-3 cells were cultured in the presence of TAT/PTD–HO-1 labeled with fluorescein isothiocyanate (FITC). Cells were simultaneously incubated with propidium iodide (PI) to rule out protein internalization due to damaged cell membranes. Evaluation of TAT/PTD transduction by confocal microscopy was performed with live cells in order to avoid potential artifacts caused by cell fixation [31,32]. PI staining was completely negative. The fusion protein was transduced into all pancreatic β-cells (Fig. 2). Of note, βTC-3 cells have a round shape because transduction was performed with detached cells to avoid high background due to stickiness of TAT–HO-1-FITC.

**Transduction of TAT/PTD–HO-1 into islets does not impair insulin secretion capability**

Because bioactive peptides were previously shown to damage target cells [33], it was important to verify that TAT/PTD–HO-1 treatment would not damage islet physiology and insulin secretion. For this reason, we assessed their ability to reverse hyperglycemia in chemically diabetic immunodeficient mice. Freshly isolated...
islets transduced with TAT/PTD–HO-1 protein reversed hyperglycemia as well as control islets (Fig. 3), demonstrating that islet cell performance was not affected by exposure to TAT/PTD fusion protein in vitro for 8 days. After nephrectomy of the kidney with the graft on day 29, hyperglycemia was observed in all animals, confirming that the grafted islets sustained euglycemia. Thus, transduction of TAT/PTD–HO-1 fusion protein did not affect the insulin secretion capabilities of islets.

**Assessing the biological effects of transduced TAT/PTD–HO-1 fusion protein**

It has been reported that TAT/PTD fusion protein unfolding occurs during the process of internalization into cells, and their refolding assisted by cellular chaperons [34] is subsequently required to regain biological function. Therefore, it is possible for a TAT/PTD fusion protein to efficiently enter cells and lose its biological function if it is not properly refolded inside of the cell.

Pancreatic βTC-3 cell line is highly sensitive to TNF-α toxicity [24,35] and overexpression of HO-1 protects them against TNF-α-mediated cell death [24]. Thus to evaluate whether TAT/PTD–HO-1 was biologically functional upon cell transduction, we investigated the effect of transduced HO-1 fusion protein on βTC-3 cells treated with TNF-α.

Utilizing a viability test combining calcein AM and ethidium homodimer-1 staining, we observed that pancreatic βTC-3 cells transduced with TAT/PTD–HO-1 were more viable than non-transduced cells following TNF-α treatment (Fig. 4). Cells transduced with TAT/PTD–HO-1 fusion protein showed 80 ± 4% (means ± SD) of green (viable) cells compared with 98 ± 0.3% in control cells and only 33 ± 7% in TNF-α/CHX-treated cells. Percentage of viable cells in βTC-3 cells incubated with a HO-1 recombinant protein without TAT/PTD
(33 ± 4%) was similar to that of non-transduced TNF-α/CHX-treated cells (Fig. 4B). The range of protection conferred by TAT/PTD–HO-1 from different experiments varied between 60% and 80%. This indicates that TAT/PTD–HO-1 fusion protein remains biologically active upon cell internalization and that TAT/PTD is absolutely necessary to mediate HO-1 transduction. These results agree with previously reported results showing that βTC-3 cell sensitivity to cytotoxic effect of TNF-α, can be prevented by induction [26] or overexpression of HO-1 [24].

Enhancement of islet culture conditions

Pancreatic islets are susceptible to culture-induced damage, decreasing in number and losing function over time. This is in part due to cell death induced by apoptosis/oxidative stress during isolation and in vitro culture [36,37]. HO-1 has been reported to have anti-apoptotic and anti-oxidative stress effects on different cell types in vitro and in vivo. Therefore, we investigated whether transduction of TAT/PTD–HO-1 had an effect on preserving islets in culture. We observed that TAT/PTD–HO-1 fusion protein protected islets from deterioration. Significant hypoxia can occur during islet culture, depending on the density at which islets are cultured. Signs of hypoxia are: vacuolization, central necrosis, and DNA release that induces adhesion of all islets into large clumps. After 16 days in culture rat control islets showed clear signs of hypoxia. The islets cultured in the presence of TAT/PTD–HO-1 remained with significantly less signs of necrosis and formation of clumps (data not shown). Consistent with a better morphology, islets cultured in the presence of TAT/PTD–HO-1 also had a higher ratio of glucose-induced insulin secretion response (Fig. 5). After 16 days in culture transduced islets had a stimulation ratio (SI) of 6.1 ± 2.3 or 4.3 ± 2.1 for islets incubated with 100 and 200 nM of TAT/PTD–HO-1 fusion protein, respectively, while control islets were totally unresponsive to glucose stimulation (SI 1.1 ± 0.2). Currently we do not know whether in addition to preservation of the β-cell in culture, transduction in the TAT/PTD–HO-1 also enhances performance of β-cells. Experiments using a marginal mass transplantation model [26] will allow us to shed light on this matter.

Conclusions

Our data show that transduction of insulin producing cells with a recombinant HO-1 protein fused to the TAT/PTD confers cytoprotection against TNF-α-cytotoxicity and cell death during culture. Transduction with TAT/PTD–HO-1 protein might be useful to confer transient cytoprotection and therefore enhance the viability of transplantable islets. Prolonged culture of viable islets could help develop immunosuppressive regimes for recipients prior to islet transplantation.

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References


