RESEARCH PAPER

Therapeutic potential of pterostilbene against pancreatic beta-cell apoptosis mediated through Nrf2

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BACKGROUND AND PURPOSE

Nuclear factor erythroid 2-related factor 2 (Nrf2) is considered to be a 'master regulator' of the antioxidant response as it regulates the expression of several genes including phase II metabolic and antioxidant enzymes and thus plays an important role in preventing oxidative stress-mediated disorders, including diabetes. In this study, for the first time, we investigated the protective properties of a naturally available antioxidant, pterostilbene (PTS), against pancreatic beta-cell apoptosis and the involvement of Nrf2 in its mechanism of action.

EXPERIMENTAL APPROACH

Immunoblotting and quantitative reverse transcriptase (qRT)-PCR analysis were performed to identify PTS-mediated nuclear translocation of Nrf2 protein and the following activation of target gene expression, respectively, in INS-1E cells. In addition, an annexin-V binding assay was carried out to identify the apoptotic status of PTS-treated INS-1E cells, while confirming the anti-apoptotic potential of Nrf2 by qRT-PCR analysis of the expressions of both pro- and anti-apoptotic genes.

KEY RESULTS

PTS induced significant activation of Nrf2, in dose- and time-dependent manner, in streptozotocin-treated INS-1E rat pancreatic beta-cells. Furthermore, PTS increased the expression of target genes downstream of Nrf2, such as heme oxygenase 1 (*HO1*), superoxide dismutase (*SOD*), catalase (*CAT*) and glutathione peroxidase (*GPx*), that confer cellular protection. PTS also up-regulated the expression of anti-apoptotic gene, *Bcl-2*, with a concomitant reduction in pro-apoptotic Bax and caspase-3 expression.

CONCLUSION AND IMPLICATIONS

Collectively, our findings indicate the therapeutic potential of Nrf2 activation by PTS as a promising approach to safeguard pancreatic beta-cells against oxidative damage in diabetes.

Abbreviations

AnnCy3, annexin-Cy3.18; 6-CF, 6-carboxyfluorescein diacetate; CAT, catalase; γ-GCS, γ-glutamylcysteine synthetase; GPx, glutathione peroxidase; HO1, heme oxygenase 1; INS-1E, insulin-secreting rat insulinoma beta-cells; Nrf2, nuclear factor erythroid 2-related factor 2; NQO1, NAD(P)H quinone oxidoreductase-1

Introduction

Several studies have shown that the common features of both type 1 and type 2 diabetes mellitus are a progressive decrease in beta-cell mass and function with a concomitant reduction in insulin secretion, which results in chronic hyperglycaemia that ultimately claims the lives of these diabetic patients (Meier and Bonadonna, 2013). Pancreatic beta-cells, because of their sustained and continuous secretory activity, and lack of endogenous antioxidant potential, are chronically exposed to various kinds of stress, originating from free radicals, misfolded proteins, endoplasmic reticulum hyperactivity and damaged mitochondria (Hartley et al., 2009; Hodish et al., 2010). This accumulated stress causes beta-cell apoptosis, finally leading to dysfunction of the pancreas as a whole.

The coordinated up-regulation of genes coding for detoxifying enzymes, antioxidant enzymes and anti-inflammatory regulators has been shown to be a potential therapeutic strategy against inflammation and oxidative stress-induced pancreatic beta-cell damage (Rodgers et al., 2008; Hernandez-Alvarez et al., 2010; Kume et al., 2013; Xu et al., 2013). The transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2) regulates important cellular defence mechanisms that manage chemical and oxidative stress (Itoh et al., 1999; Kensler et al., 2007); these include intracellular antioxidants, phase II detoxifying enzymes and proteins involved in detoxifying xenobiotics and neutralizing reactive oxygen species (ROS) to promote cell survival and maintain cellular redox homeostasis (Zhang, 2006; Lau et al., 2008). NAD(P)H quinone oxidoreductase-1 (NQO1), heme oxygenase 1 (HO1), glutathione peroxidase (GPx), γ-glutamylcysteine synthetase (γ-GCS), catalase (CAT) and superoxide dismutase (SOD) are among the well-studied target genes of Nrf2 that are up-regulated through the antioxidant response element (ARE) signalling pathway in response to oxidative and chemical stresses (Chan et al., 2001; Cho et al., 2006).

Under basal conditions, Nrf2-dependent transcription is repressed by its negative regulator, Kelch-like ECH-associated protein-1 (Keap1). When cells are exposed to oxidative stress, electrophiles or chemopreventive agents, the Nrf2-Keap1 complex dissociates, leading to the nuclear translocation of Nrf2 and activation of ARE-dependent gene expression to maintain cellular redox homeostasis. In addition, the essential role of Nrf2 in combating oxidative stress induced by a broad spectrum of insults has been clearly demonstrated by increased sensitivity of Nrf2$^{-/-}$ mice to a variety of insults (He et al., 2008). Beyond its antioxidant function, Nrf2 is recognized as a key factor regulating an array of genes that defend cells against the deleterious effects of environmental insults (Zhang, 2006). Because this Nrf2-dependent cellular defence mechanism is present in many organs and tissues, activation of Nrf2 has been implicated in conferring protection against various pathological conditions, including cancer, neurodegenerative diseases, cardiovascular diseases, diabetes, acute and chronic lung injury, autoimmune diseases and inflammation (Theodore et al., 2008). Therefore, elucidating the effect of potential drug molecules on the activity of Nrf2 is crucial for the development of drugs for various therapeutic interventions.

The activation of Nrf2 by natural compounds is a promising approach in the prevention of the hyperglycaemia-induced oxidative stress involved in the pathogenesis of diabetes and its associated complications. Several natural products, including sulforaphane (Jiang et al., 2010), resveratrol (Ungvari et al., 2010), curcuminoids (Yang et al., 2009) and epigallocatechin-3-gallate (Na et al., 2008), have been reported to activate Nrf2 and protect the functions of various cells.

Recently, using a reporter protein complementation imaging assay developed in our laboratory for in vitro and in vivo screening of Nrf2 activators (Ramkumar et al., 2013), we identified pterostilbene (PTS), a naturally methoxylated analogue of resveratrol, as a potential activator of Nrf2. PTS also has the advantage of greater oral bioavailability and higher retention time in vivo when compared to its analogue, resveratrol (Kapetanovic et al., 2011). Furthermore, PTS has also been reported to possess antioxidant properties (McCormack and McFadden, 2013), to lower blood glucose levels in hyperglycaemic rats (Manickam et al., 1997) and improve carbohydrate metabolism (Pari and Satheesh, 2006).

The current study was designed to test the hypothesis that, PTS, a naturally occurring stilbene, can provide protection against streptozotocin (STZ)-induced pancreatic beta-cell apoptosis in INS-1E rat beta-cells by inducing an Nrf2-mediated antioxidant response (Figure 1). This is, to our knowledge, is the first study to elucidate the protective effect of PTS against STZ-induced cytotoxicity in pancreatic beta-cells and reveal the molecular mechanism involved in this effect.

Methods

Cells and culture conditions

INS-1E (insulin-secreting rat insulinoma) beta-cell line was obtained as a kind gift from Dr Pierre Maechler, Department of Cell Physiology and Metabolism, University Medical Center, Geneva, Switzerland. INS-1E is one of the most widely used insulin-secreting cell lines displaying many important characteristics of the pancreatic beta-cell, including a high insulin content, and responsiveness to glucose within the physiological range. The cells were grown as adherent cultures at 37°C (passages 23 to 28) under a humidified 5% CO_2 atmosphere in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (HyClone, Logan, UT, USA), 2 mM glutamine, 1 mM sodium pyruvate, 50 mM 2-mercaptoethanol, 2 mM glutamine, 10 mM HEPES, 100 U·mL^{-1} penicillin and 100 mg·mL^{-1} streptomycin. Cells were washed with sterile PBS, and live cells were detached using 0.25% trypsin in 0.1% EDTA. Cells were then centrifuged at $200 \times g$ for 5 min, resuspended in growth medium and used for further studies.

Cell viability assay

To investigate the cytotoxicity of PTS, cultures of INS-1E cells (1×10^4 cells per well in 96-well plates) grown overnight were exposed to different doses (0–100 μM) of PTS (Cayman Chemical, Ann Arbor, MI, USA) for 24 h and subjected to MTT assay. The EC$_{50}$ value of PTS in INS-1E cells was found to be 25 μM, and in further studies doses lower than this were used. To elucidate the protective role of PTS on STZ-induced
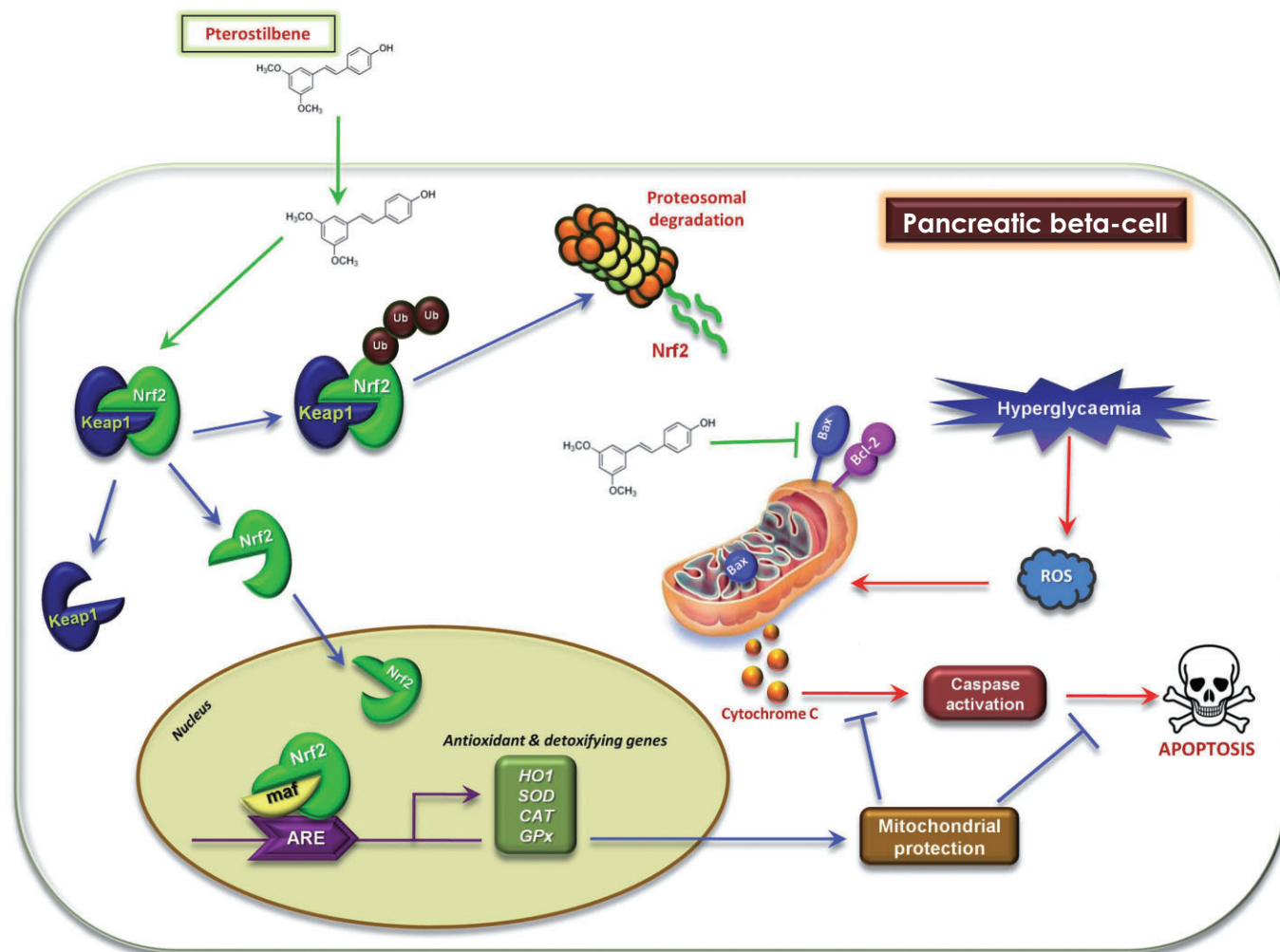
Figure 1

Hypothetical model illustrating the therapeutic potential of pterostilbene against pancreatic beta-cell apoptosis through Nrf2 signalling.

cytotoxicity, the cells were pretreated with various doses of PTS in different time points (0–16 μM at 0–48 h), followed by STZ treatment (10 mM) (Kang *et al.*, 2011) for 1 h. The time-dependent study was carried out with doses of PTS up to 8 μM. The % inhibition of cytotoxicity was calculated as a fraction of control and expressed as a % relative to the cell viability with respect to control.

Nuclear and cytosolic fractionation

To study the effect of PTS on Nrf2 translocation, nuclear and cytoplasmic extracts were prepared using a commercially available nuclear extraction kit (Pierce NE-PER®) as per the manufacturer's instructions (Pierce, Rockford, IL, USA). Briefly, cells were homogenized in CER-I buffer using a homogenizer and incubated on ice for 15 min and centrifuged at 10 000 × g for 10 min at 4°C. The supernatant solution was collected (cytoplasmic fraction), and pellets containing nuclei were suspended in NER buffer supplemented with protease inhibitors according to the manufacturer's instructions. After thorough vortexing for 40 min with

1 min break for every 10 min, samples were centrifuged at 16 000 × g for 15 min at 4°C, and supernatant solution was collected (nuclear fraction). After quantification of the protein concentration by Bradford assay, the samples were subjected to Western blot analysis.

Western blotting

The cytoplasmic and nuclear fractions of the cell lysates were probed overnight at 4°C on a rotating platform with the appropriate antibodies, and then immunoprecipitated with Dynabeads (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's protocol. Unbound proteins were removed by washing four times with PBS. The conjugated beads were heat-denatured at 95°C for 10 min, resolved using a 4–12% SDS-PAGE gradient gel (Invitrogen) and electroblotted onto a nitrocellulose membrane (Schleicher & Schuell, Keene, NH, USA) (0.2 μm pore size). Primary and secondary antibodies against Nrf2, β-actin and lamin-B (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used for detecting respective proteins by a standard protocol recommended by the

manufacturers. The enhanced chemiluminescence system (Bio-Rad, Hercules, CA, USA) was used for the detection of the protein bands.

Transfection of small interfering RNA (siRNA)

To confirm whether Nrf2 activation is mediated by PTS, we performed Nrf2 gene silencing studies in INS-1E cells. INS-1E cells were transfected with Nrf2-siRNA (Dharmacon Research, Lafayette, CO, USA) using Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen). The final concentration of the siRNAs used was 20 nmol·L^{-1} (Singh *et al.*, 2013). After the experimental period, the treated cells were subjected to cell viability assays as described previously.

Quantitative reverse transcriptase PCR (qRT-PCR)

The effect of PTS on the Nrf2 signalling pathway was measured by mRNA expression profile of Nrf2 downstream targets, such as HO1, SOD, CAT, GPx, and pro- and anti-apoptotic proteins, such as Bax, Bcl-2 and caspase-3 by qRT-PCR (Applied Biosystems, Foster City, CA, USA) analysis using gene-specific primers. The qRT-PCR primers were designed based upon NCBI human mRNA sequence database (Table 1). The qRT-PCR analysis was performed following the manufacturer's protocol. Briefly, each reaction contained 2 μL of cDNA (0.1 μg of RNA equivalent), 1 μL of each primer (containing 100 pM each of forward and reverse primers), 1 μL of H$_2$O and 5 μL of 2X power SYBRGreen master mix. qRT-PCR was performed as a three-step programme (95°C for 15 s, 60°C for 30 s and 72°C for 30 s for 50 cycles). The qRT-PCR data were normalized to the housekeeping gene β-actin and expressed as relative fold change from untreated control cells.

Measurement of annexin-V binding

Phosphatidylserine (PS) redistribution in the membrane, indicative of early apoptotic events, was measured by the binding of annexin-Cy3.18 (AnnCy3) according to the manufacturer's protocol (Sigma-Aldrich, St. Louis, MO, USA). AnnCy3 binds to PS present in the outer leaflet of the plasma membrane of cells starting the apoptotic process. The same treatment protocol (dose- and time-dependent exposure) as

described previously was followed. After the experimental period, the cells were washed and re-suspended in PBS. Then, 50 μL of each cell suspension was placed on poly-L-lysine-coated slides and left at room temperature for 10 min, allowing the cells to be adsorbed to the slides. Excess liquid was carefully removed and the cells were washed three times with 1X binding buffer. Another 50 μL of the double-label staining solution [AnnCy3 and 6-CF (6-carboxyfluorescein diacetate)] was placed on each circle and incubated for 10 min at room temperature in the dark. After being stained, the cells were again washed and fixed with 1X binding buffer. Fluorescent labels were detected by a fluorescence microscope (Leica Microsystems, Wetzlar, Germany); annexin-V Cy3 staining was visualized with a rhodamine filter (excitation 535 nm, emission 550 nm) and 6-CF staining with a fluorescein filter (excitation 450 nm, emission 490 nm). Images were recorded using a Nikon camera under 400× magnification (Nikon Inc., Tokyo, Japan). Cells stained with Cy3/6-CF were considered to be apoptotic cells and were counted from 10 randomly selected fields containing 40–50 cells and presented as a % of these cells.

Statistical analysis

Results are presented as mean ± SEM of three independent experiments. Statistical analysis was performed by one-way ANOVA, followed by Tukey's test using SPSS version 17.0 software (SPSS Inc., Chicago, IL, USA); $P < 0.05$ was taken to indicate a significant difference between groups.

Results

Effect of PTS on STZ-induced cytotoxicity in INS-1E cells

The initial evaluation of PTS-induced cytotoxicity in INS-1E cells found significant toxicity only at doses higher than 16 μM (Figure 2A), and hence, we limited the experimental dosage up to 16 μM for further studies. Furthermore, to identify the effect of PTS on STZ-induced cytotoxicity, we performed cell viability assays in PTS-pretreated (0–16 μM) cells for 24 h, followed by STZ (10 mM) treatment for 1 h (Figure 2B). However, PTS-pretreated cells showed increased

Table 1

Real-time PCR primer sequences for validated targets

| Gene name	Short name	Forward primer 5'–3'	Reverse primer 5'–3'
Heme oxygenase 1	HO1	CTCTGAAGTTTAGGCCATTG	AGTTGCTGTAGGGCTTTATG
Catalase	CAT	TCATGACATTTAATCAGGCA	GTGTCAGGATAGGCAAAAAG
Superoxide dismutase	SOD	GAAGGTGTGGGGAAGCATTA	ACATTGCCCAAGTCTCCAAC
Glutathione peroxidase	GPX	TTCCCGTGCAACCAGTTTG	TTCACCTCGCACTTCTCGAA
B-cell lymphoma 2	Bcl2	GCTGAGGCAGAAGGGTTATG	GCCCCCTTGAAAAAGTTCAT
Bcl-2-associated X protein	Bax	AGGGTTTCATCCAGGATCGAGCAG	ATCTTCTTCCAGATGGTGAGCGAG
Caspase-3	CASP3	TTTGTTTGTGTGCTTCTGAGCC	ATTCTGTTGCCACCTTTCGG
β-Actin	ACT	GGCGGACTATGACTTAGTTG	AAACAACAATGTGCAATCAA
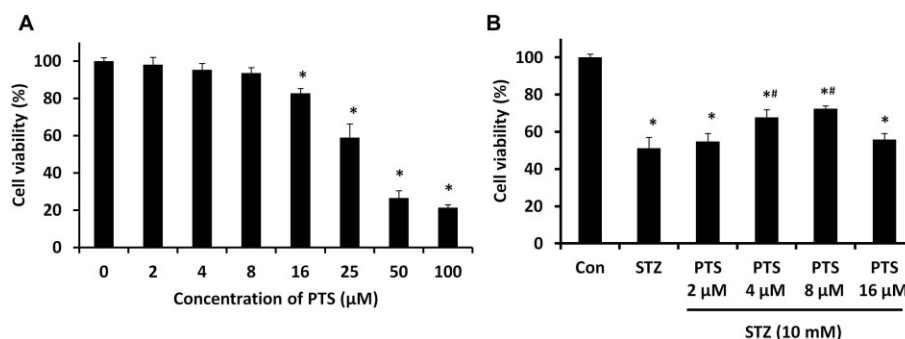
Figure 2

Dose-dependent protective effect of pterostilbene in INS-1E cells assessed by MTT assay. (A) Pterostilbene treatment (0–100 μM for 24 h) showed profound cell viability up to 16 μM. (B) Pterostilbene pretreatment (0–16 μM) resulted in a significant dose-dependent protection against STZ toxicity (1 h). Data are expressed as mean ± SEM of three separate experiments. Statistical analysis was performed by one-way ANOVA, followed by Tukey's test. *Significant compared with untreated control; #Significant compared with control + STZ group; $P < 0.05$.
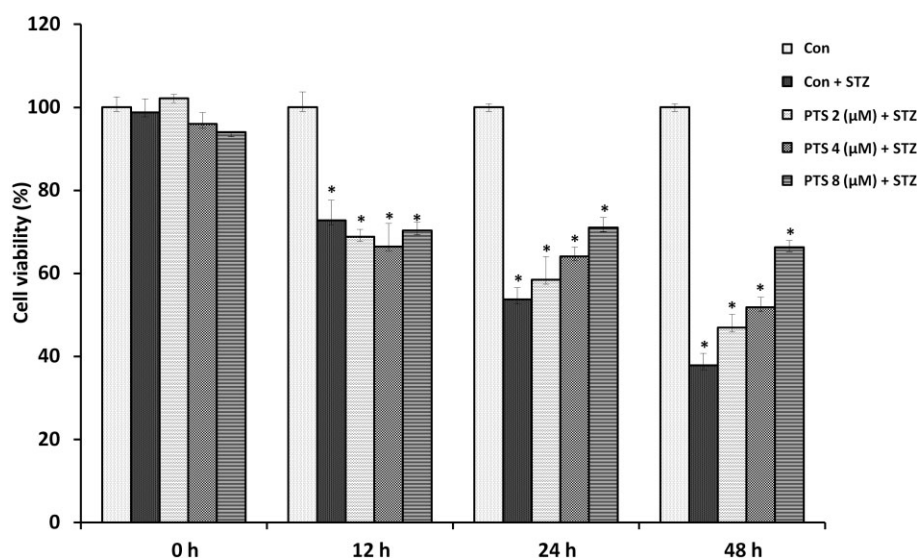

Figure 3

Time-dependent protective effect of pterostilbene in INS-1E cells assessed by MTT assay. Pterostilbene pretreatment (0–8 μM) for 0–48 h of STZ-treated cells (1 h) resulted in a significant time-dependent protection up to 24 h. Data are expressed as mean ± SEM of three separate experiments. *Significant compared with untreated control; $P < 0.05$.

viability of 67 ± 3.4% and 72 ± 2.7% at 4 and 8 μM concentrations, respectively, compared to cells treated with STZ (10 mM) for 1 h. This indicates profound protective property of PTS against STZ-induced cytotoxicity in INS-1E cells. Increasing the PTS concentration to 16 μM showed a slight reduction in cell viability.

Based on these results, in the time-dependent experiments (0–48 h), PTS doses were restricted to below 8 μM. We found that 8 μM PTS had little effect on the viability of the cells for up to 24 h, but was found to be slightly cytotoxic when incubated for 48 h (Figure 3). Therefore, further studies were restricted to 24 h, for concentrations of PTS up to 8 μM. PTS showed a dose- and time-dependent protective effect against STZ-induced toxicity in INS-1E cells.

Effect of PTS on nuclear translocation of Nrf2 in INS-1E cells

Nrf2 activators induce dissociation of Nrf2/Keap1 complex in the cytoplasm, which allows Nrf2 to translocate into the nucleus to mediate activation of cellular protective gene expression. To identify the PTS-induced translocation of Nrf2, its concentrations were measured in the cytoplasmic and nuclear extracts of PTS-pretreated INS-1E cells. PTS induced a dose-dependent increase in Nrf2 protein in the nuclear extracts with an associated decrease in cytoplasmic extracts of untreated (Figure 4A,B) and STZ-treated cells (Figure 5A,B). These results provide strong evidence that PTS induces Nrf2 activation and its nuclear translocation in INS-1E cells.

A

Pterostilbene (μM)

Con 2 4 8 16

Lamin B

Nuclear Nrf2

Increase in nuclear Nrf2

β-Actin

Cytosolic Nrf2

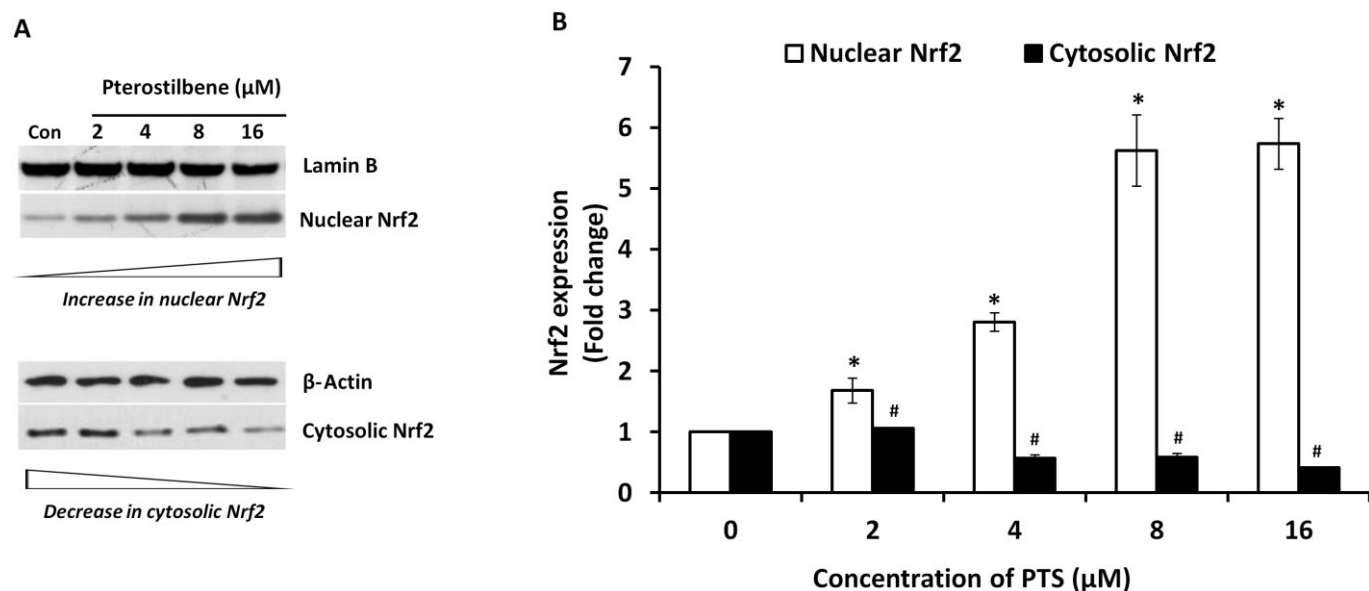*Decrease in cytosolic Nrf2*

B

Figure 4

(A, B) Effect of pterostilbene on nuclear translocation of Nrf2 in INS-1E cells. Nuclear and cytoplasmic fractions were prepared using a commercially available nuclear extraction kit (Pierce NE-PER) as per manufacturer's instructions from control and pterostilbene-treated INS-1E cells. Immunoblot analysis of the soluble cell lysates showed effective Nrf2 activation. Data are expressed as fold-change over control and presented as mean ± SEM of three separate experiments. *Significant compared with untreated control; #Significant compared with respective control; $P < 0.05$.

A

STZ (10 mM)

Con   PTS (4 μM)   PTS (8 μM)

Lamin B

Nuclear Nrf2

Increase in nuclear Nrf2

β-Actin

Cytosolic Nrf2

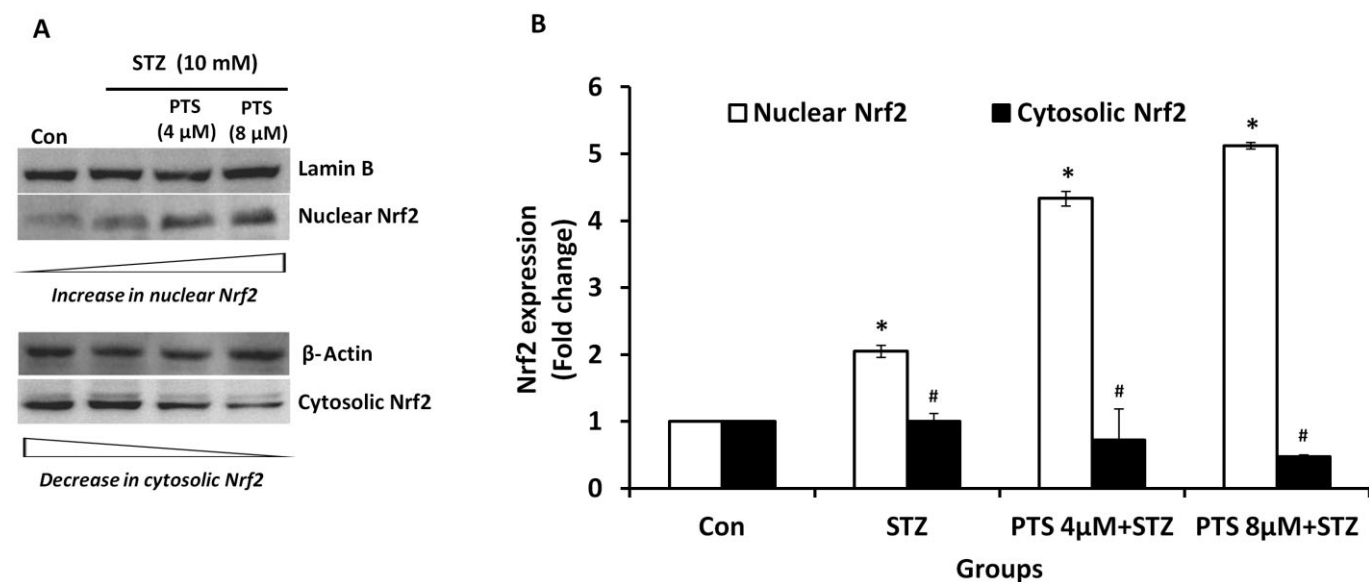*Decrease in cytosolic Nrf2*

B

Figure 5

(A, B) Effect of pterostilbene on nuclear translocation of Nrf2 in STZ-treated INS-1E cells. Nuclear and cytoplasmic fractions were prepared using commercially available nuclear extraction kit (Pierce NE-PER) as per manufacturer's instructions from control and pterostilbene-pretreated INS-1E cells. Immunoblot analysis of the soluble cell lysates showed effective Nrf2 activation in STZ-treated cells. Data are expressed as fold-change over control and presented as mean ± SEM of three separate experiments. *Significant compared with untreated control; #Significant compared with respective control; $P < 0.05$.
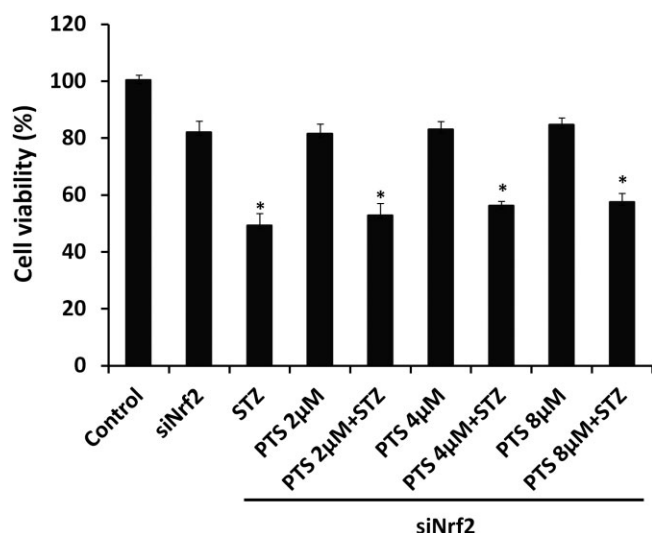
Figure 6

Effect of pterostilbene-mediated protective effect in cells down-regulated for Nrf2 expression by siRNA. INS-1E cells transfected with Nrf2-siRNA were treated with different concentrations of pterostilbene and then STZ and cell viability assay was performed. Data are expressed as mean ± SEM of three separate experiments. *Significant compared with respective control; $P < 0.05$.

Effect of PTS-mediated protective effect in cells down-regulated for Nrf2 expression by siRNA

To confirm whether the protective effect of PTS was mediated through Nrf2 activation, Nrf2 was silenced in INS-1E cells using Nrf2-siRNA and the cells were then treated with PTS and/or STZ. In these Nrf2 silenced cells, PTS pretreatment did not show any significant improvement in cell viability against STZ-induced toxicity, whereas its protective effect was not affected in non-silenced cells (Figure 6).

Effect of PTS on Nrf2 downstream target gene expression

PTS pretreatment significantly increased the expression of HO1, SOD, CAT and GPx compared with the STZ-treated group (Figure 7A–D). These results suggest that PTS enhances the expression of antioxidant genes triggered by Nrf2, thereby protecting beta-cells against STZ-induced toxicity.

Anti-apoptotic effect of PTS on STZ-treated INS-1E cells

To study the anti-apoptotic potential of PTS against STZ toxicity, we performed annexin-V staining in INS-1E cells, which specifically identifies early apoptosis. A large number of apoptotic cells were detected in the STZ-treated groups and PTS pretreatment significantly reduced the number of apoptotic cells in this group. Similar to the untreated cells (control) about 5% of apoptotic cells with no significant morphological changes were observed in the PTS-pretreated groups (Figure 8). Furthermore, analysis of the mRNA expression
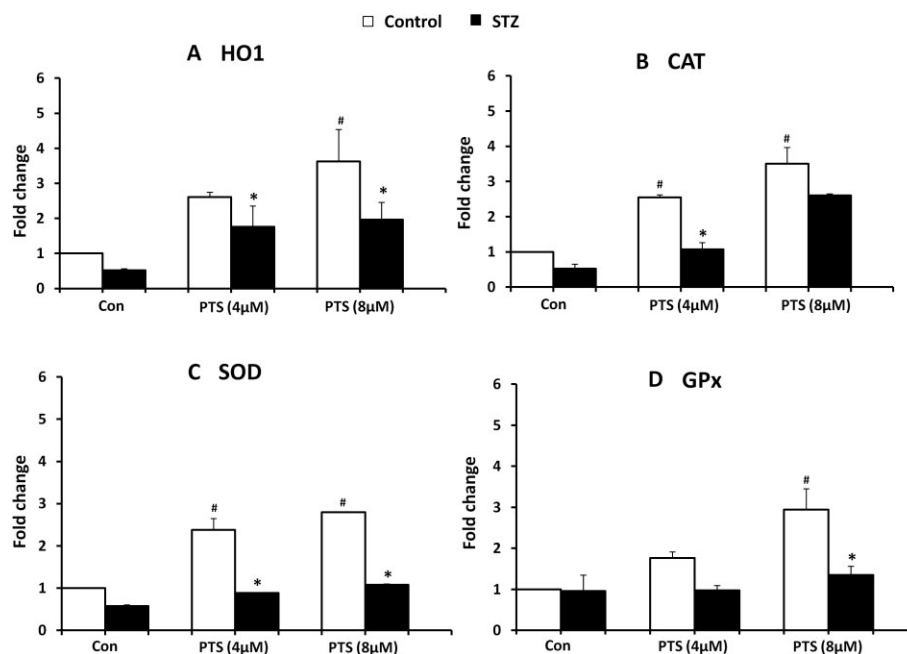

Figure 7

Effect of pterostilbene treatment on Nrf2-induced gene expression in INS-1E cells in control and STZ-treated cells. Transcript levels of HO1 (A), CAT (B), SOD (C) and GPx (D) were measured using qRT-PCR analysis. Data are expressed as mean ± SEM of three separate experiments. *Significant compared with respective control; #Significant compared with control + STZ group; $P < 0.05$.

profile of pro- and anti-apoptotic genes indicated that compared with STZ-treated cells, PTS pretreatment dose-dependently increased Bcl-2 expression (Figure 9A), with a concomitant decrease in Bax (Figure 9B) and caspase-3 expression (Figure 9C).
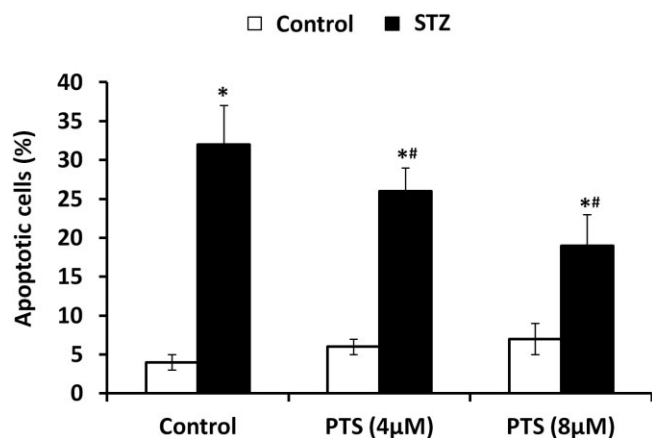

Figure 8

Effect of pterostilbene on apoptotic status of INS-1E cells exposed to STZ as measured by annexin-V binding assay. Apoptotic cells (Cy3/6-CF) were counted and presented as a percentage of total number of cells. Data are expressed as mean ± SEM of three separate experiments. *Significant compared with respective control; #Significant compared with untreated control; $P < 0.05$.

Discussion

Nrf2 activation is a promising approach for the enhancement of a cytoprotective defence mechanism to prevent oxidative stress-related cellular damages, therefore, it is not surprising that attention has been focused on activation of the Nrf2 pathway for protection against many cellular disorders. Both genetic and biochemical studies have indicated that the Nrf2 system acts as a defence mechanism against chemical toxicity, diabetic complications, cancer, and in the pathogenicity of chronic diseases, such as autoimmune, neurodegenerative and inflammatory conditions, many of which involve oxidative stress (Robertson et al., 2003; Sun et al., 2009).

In diabetes, oxidative stress mediates excessive generation of free radicals, leading to pancreatic beta-cell damage (Robertson et al., 2003). Pancreatic beta-cells are considered exceptionally vulnerable to the cytotoxic actions of ROS due to their inherent lack of expression of various antioxidant enzymes (Lenzen et al., 1996). Experimental studies have shown that ROS generation was elevated in cytokine-stimulated (Rabinovitch et al., 1996) and STZ-treated pancreatic beta-cells (Szkudelski, 2001), whereas overexpression of antioxidant enzymes in transgenic animals protects beta-cells from such insults (Azevedo-Martins et al., 2003). In the present study, INS-1E rat pancreatic beta-cell line was used because it secretes insulin in response to physiological concentrations of glucose (Hohmeier et al., 2000).

In the present study, for the first time, we investigated the therapeutic potential of PTS, a natural analogue of resveratrol, against STZ-induced apoptosis in beta-cells thereby iden-
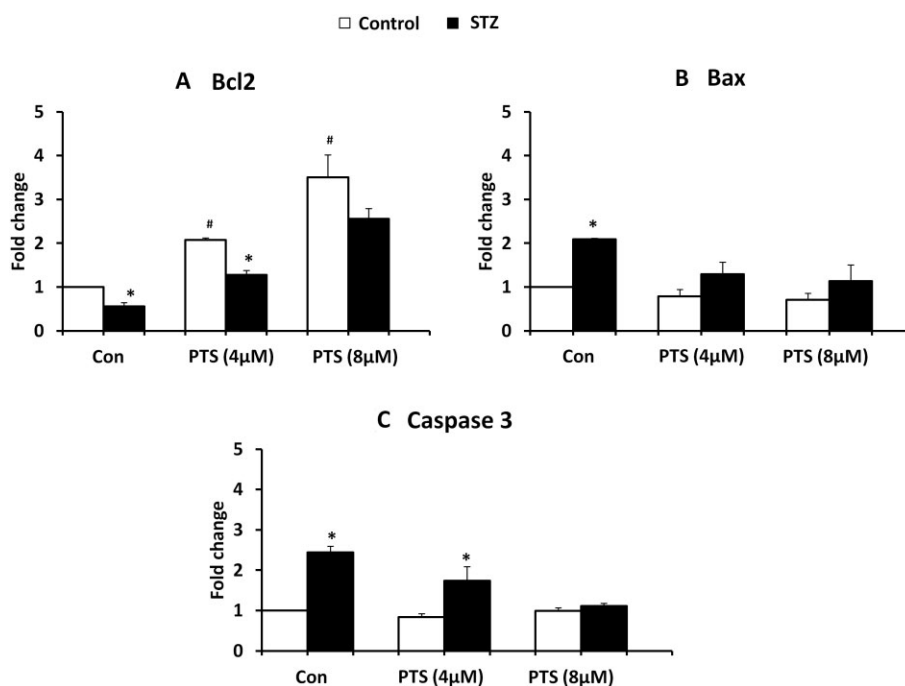

Figure 9

Effect of pterostilbene treatment on apoptotic gene expression in INS-1E cells exposed to STZ. Transcript levels of Bcl-2 (A), Bax (B) and caspase-3 (C) were measured using qRT-PCR analysis. Data are shown as mean ± SEM of three separate experiments. *Significant compared with respective control; #significant compared with untreated control; $P < 0.05$.

tifying that it exerts a protective effect through a mechanism involving Nrf2. In contrast, in other studies PTS was found to induce concentration-dependent apoptosis in cells, such as breast cancer and lung cancer cells (Schneider *et al.*, 2010; Moon *et al.*, 2013). The major reason for this discrepancy is probably due to more specific effects of PTS against certain cellular targets in different types of cells, as well as the different concentrations of PTS used. In fact, PTS has also been shown to possess potent, concentration-dependent, antioxidant and cancer chemopreventive effects (Rimando *et al.*, 2002; Mikstacka *et al.*, 2010).

Compared to resveratrol, PTS appears to be better absorbed following oral ingestion and is more potent as an antioxidant. The antioxidant activity of resveratrol and its analogues depends significantly on the position of the hydroxyl groups. Structure–activity relationship analysis indicated that structural determinants are necessary for the antioxidant activity of resveratrol and its analogues; the hydroxyl group is essential in the 4′ position, as well as a trans-configuration of the ethylene functional group in the stilbene skeleton (Stivala *et al.*, 2001; Hasiah *et al.*, 2011). PTS, due to its two methoxy groups, exhibits increased hydrophobicity and diffuses into a cell more effectively than resveratrol (Kapetanovic *et al.*, 2011).

Although resveratrol remains to be a model Nrf2 activator, it suffers from low bioavailability and metabolic structural instability, and hence studies with PTS, which is reported to have greater oral bioavailability and higher retention time *in vivo* conditions (Kapetanovic *et al.*, 2011), will have a greater impact in translational applications. Hence, we hypothesize that PTS mediates a protective effects on pancreatic beta-cells against STZ-induced oxidative insult through a mechanism involving Nrf2.

STZ, an *N*-{methylnitrocarbamoyl}-D-glucosamine, is particularly toxic to the insulin-producing beta-cells of the pancreas. Pancreatic islets and insulinoma cells are vulnerable to serious damage induced by cytotoxic NO and/or oxidative stress generated by STZ, due to their low-expression levels of antioxidant enzymes (Spinas, 1999). Although the generally accepted primary mode of genotoxicity and cytotoxicity of STZ is DNA alkylation, the drug may also exert its action through the formation of free radicals (Koo and Vaziri, 2003). Research evidence indicates that STZ-mediated hydroxyl radicals and generation of ROS may be crucial effectors in beta-cell damage (Gille *et al.*, 2002). In the present study, the pancreatic beta-cells were directly exposed to STZ at a dose of 10 mM for 1 h to induce apoptosis. The dosage was chosen based on the LD$_{50}$ of the compound, which was supported by previous findings (Kang *et al.*, 2011).

Our data showed a significant dose- and time-dependent protective effect induced by PTS against STZ-induced toxicity in INS-1E cells. This protective effect was possibly due to increased Nrf2 activation and this was clearly supported by our immunoblot analysis of Nrf2 translocation from cytosol to nucleus and activation of antioxidant enzymes. These findings also corroborate those from earlier reports indicating that resveratrol elicited translocation of Nrf2 to the nucleus, leading to its transcriptional activation by increasing the phosphorylation status of Nrf2 (Hsieh *et al.*, 2006). Furthermore, the cytoprotective effects of PTS against STZ toxicity as a result of increased Nrf2 activation are clearly

demonstrated by its failure to protect Nrf2-silenced INS-1E cells.

Further evidence that PTS-mediated activation and subsequent nuclear translocation of Nrf2 exerted a cytoprotective effect against STZ toxicity in INS-1E beta-cells was obtained through the up-regulation of Nrf2-dependent antioxidant enzymes, such as HO1, SOD, CAT and GPx. The Nrf2 pathway conferred protection against tissue injury by orchestrating antioxidant (SOD, CAT, GPx) and detoxification (HO1, NQO1, GCS) responses in chronic kidney diseases (Kim and Vaziri, 2010). Earlier findings suggest that Nrf2 activation by curcuminoids induced phase 2 enzymes, especially HO1 in pancreatic beta-cells (Pugazhenthi *et al.*, 2007). Recently, Song *et al.* reported that Nrf2 activation by either sulforaphane treatment or genetic overexpression induces phase 2 enzymes, resulting in resistance to cytokine- or STZ-induced pancreatic beta-cell damage (Song *et al.*, 2009). The improved expression of antioxidant genes, such as *SOD*, *CAT* and *GPx*, may alleviate oxidative damage by scavenging free radicals, thereby protecting pancreatic beta-cells.

The annexin-V binding assay indicated a large number of early apoptotic cells (stained with Ann-Cy3) in STZ-treated cells, whereas PTS pretreatment showed a significant reduction in apoptosis. Furthermore, PTS pretreatment was found to induce a profound dose-dependent increase in the expression of anti-apoptotic gene, *Bcl-2*, with a concomitant decrease in pro-apoptotic Bax and effector caspase-3. Collectively, these results indicate the importance of the Nrf2-mediated antioxidant response triggered by PTS.

It is noteworthy that Nrf2 concomitantly up-regulates *Bcl-2* gene expression along with a battery of cytoprotective genes encoding detoxifying enzymes, antioxidant proteins and drug transporters (Niture and Jaiswal, 2012). Therefore, it is reasonable to assume that the coordinated activation of cytoprotective genes and anti-apoptotic Bcl-2 caused by PTS-induced Nrf2 activation contributed to the reduced apoptosis and enhanced cell survival. Furthermore, PTS pretreatment also decreased the expression of Bax, which correlates with the inhibition of caspase-dependent apoptosis. Our conclusion is, therefore, further confirmed by the observed anti-apoptotic potential conferred by PTS in INS-1E cells against STZ toxicity.

Conclusion

The promising and accumulating lines of evidence for this naturally occurring stilbene compel current research to identify its multifaceted role against several disorders. Interestingly, our data revealed that PTS confers a profound cytoprotective effect through Nrf2 activation against STZ-induced toxicity in pancreatic beta-cells. In addition, the antioxidant and anti-apoptotic properties of PTS also provide evidence for its cellular protective functions. A recent clinical trial revealed that PTS controls cholesterol and reduces blood pressure in adults as well as improving the markers for oxidative stress in patients with dyslipidaemia. Further insights into PTS's molecular mechanism of action are essential in order to conclude whether this naturally occurring compound could be used as an anti-diabetic drug, particularly against beta-cell apoptosis. Collectively, our findings indicate the therapeutic

potential of Nrf2 activation by PTS as a promising approach to safeguard pancreatic beta-cells against oxidative damage in diabetes.

Conflict of interest

None.

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