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ScienceDirect

Journal of Nutritional Biochemistry 44 (2017) 11–21

Journal of Nutritional Biochemistry

RESEARCH ARTICLES

Role of pterostilbene in attenuating immune mediated devastation of pancreatic beta cells *via* Nrf2 signaling cascade

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Received 16 November 2016; received in revised form 1 January 2017; accepted 16 February 2017

Abstract

Nrf2 (nuclear factor erythroid 2-related factor-2) is a transcription factor that regulates oxidative/xenobiotic stress response and also suppress inflammation. Nrf2 signaling is associated with an increased susceptibility to various kinds of stress. Nrf2 has been shown as a promising therapeutic target in various human diseases including diabetes. Our earlier studies showed Pterostilbene (PTS) as a potent Nrf2 activator, and it protects the pancreatic β-cells against oxidative stress. In this study, we investigated PTS confer protection against cytokine-induced β-cell apoptosis and its role on insulin secretion in streptozotocin (STZ)-induced diabetic mice. The Nrf2 activation potential of PTS was assessed by dissociation of the Nrf2–Keap1 complex and by expression of ARE-driven downstream target genes in MIN6 cells. Further, the nuclear Nrf2 translocation and blockage of apoptotic signaling as demonstrated by the reduction of BAX/Bcl-2 ratio, Annexin-V positive cells and caspase-3 activity conferred the cyto-protection of PTS against cytokine-induced cellular damage. In addition, PTS treatment markedly improved glucose homeostasis and abated inflammatory response evidenced by the reduction of proinflammatory cytokines in diabetic mice. The inhibition of β-cell apoptosis by PTS as assessed by BAX/Bcl-2 ratio and caspase-3 activity in the pancreas was associated with the activation of Nrf2 and the expression of its downstream target genes. PTS also inhibited the activation of iNOS and decreased nitric oxide (NO) formation in the pancreas of diabetic animals. The results obtained from both *in vitro* and *in vivo* experiments showed that PTS improves β-cell function and survival against cytokine stress and also prevents STZ-induced diabetes.

Keywords: Cytokine cocktail; Diabetes; MIN6; Nrf2; Streptozotocin

1. Introduction

Diabetes is a chronic metabolic disorder mainly characterized by hyperglycemia, resulting from defects in insulin secretion, insulin action or both. Type 1 diabetes mellitus (T1D) is mainly caused by inflammatory infiltrates in pancreatic islets leading to selective destruction of insulin producing β-cells [1]. Numerous studies have been reported that autoreactive T-cells and infiltrating macrophages that release proinflammatory cytokines such as interleukine-1 beta (IL-1β), tumor necrosis factor alpha (TNFα) and interferon gamma (IFNγ) are the major contributors for functional disorders of the pancreatic β-cells [2]. In addition to inflammatory stress, excessive generation of free radicals also contributed in the functional impairment of β-cells [3]. Since pancreatic islets are highly vulnerable to various stresses due to low levels of antioxidant potential, it is conceivable to postulate that blockage of proinflammatory cytokine production and free radicals generation at any of the checkpoint of β-cell death pathway is a crucial line of attack for the protection.

The mechanisms leading to β-cell loss are diverse such as increased production of cytokines and free radicals which in turn increase iNOS expression and NO release, disruption of mitochondrial membrane potential, imbalance in Bcl-2 family proteins, caspase-3 activation and finally leads to death of cells [4,5]. Hence, tempering of these molecular aspects is a primary approach for the protection of β-cells by small molecules, which enhance its function and survival. Over the past decade, several strategies have been reported to combat against various insults mainly including "mechanism based antioxidants approach" that can perform multiple functions such as suppression of inflammation, oxidative stress and regulating cell-signaling cascade that contribute to apoptosis.

Nuclear factor erythroid 2-related factor 2 (Nrf2) is a master regulator of the redox homeostasis, which functions by the induction of Phase II detoxifying and antioxidant enzymes. Under normal physiological conditions, Nrf2 is associated with its negative regulator, Kelch-like ECH-associated protein-1 (Keap1), whereas in response to stress, this complex dissociates and Nrf2 translocate to the nucleus where it binds with antioxidant responsive element (ARE) and

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http://dx.doi.org/10.1016/j.jnutbio.2017.02.015
0955-2863/

triggers a set of antioxidant and detoxifying genes including, Heme Oxygenase 1 (HO1), Superoxide dismutase (SOD), NAD(P)H Quinone Oxidoreductase 1 (NQO1) and Glutathione S-transferase (GST) [6]. Recently, many natural and synthetic Nrf2 activators such as sulforaphane, curcumin, aromatic malononitriles and others fortify the defensive mechanism against inflammatory responses [7]. Hence, the activation of Nrf2 is a promising approach to protect the β-cells against proinflammatory cytokine action and free radicals.

Pterostilbene (PTS), a natural analogue of resveratrol, has been shown to possess powerful antioxidant and antiinflammatory properties in both *in vitro* and *in vivo* models [8,9]. This compound has been identified as a potent Nrf2 activator [10] and protects β-cells against oxidative stress [9]. Since the activation of Nrf2 by small molecules, suppresses inflammatory response by triggering its downstream targets, the current study was designed to investigate the protective roles of pterostilbene, a potent Nrf2 activator against proinflammatory cytokine-induced cell death in insulin-secreting MIN6 cells and STZ-induced diabetic mice.

2. Materials and methods

2.1. Culturing of MIN6

MIN6 cells (Insulinoma cell line) were procured from the National Centre For Cell Science (NCCS), Pune, India. The cells were maintained in DMEM (Dulbecco's modified Eagle's medium) with 10% FBS (fetal bovine serum), 100U/ml penicillin and 100μg/ml streptomycin at 37 °C with 5% CO_2. All experiments were carried out at passages 16–21 at a confluence rate of 70–80%.

2.2. Measurement of cytokines-mediated cytotoxicity

MIN6 cells (3×10^4cells/well) were seeded in a flat-bottom 96-well microtiter plate and exposed to cytokine cocktail (50-U/mL IL-1β, 1000-U/mL TNFα and 750-U/mL IFNγ) (R&D Systems, Canada) for different time points, 12, 24, 36 and 48 h, and the cell viability was assessed using MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay. Briefly, after cytokine exposure, the media was replaced with MTT (5 mg/mL) and incubated at 37 °C for 3 h in CO_2 incubator. Later, media were aspirated carefully, the formazan crystals were dissolved in DMSO and the absorbance was read at 540 nm using a multiwell plate reader (Tecan, Switzerland). Since 50% of cytotoxicity was observed at 36 h of cytokine cocktail exposure, all further experiments were carried out with same protocol.

2.3. Effect of PTS on cell viability against cytokine stress

In order to study the effect of PTS on cytokine stress, MIN6 cells were plated in a 96-well microplates and pretreated PTS at different concentrations (0–16 μM) for 8 h at 37 °C, followed by cytokine cocktail exposure for 36 h. Cell viability was measured using MTT assay. For each set of conditions, the experiments were performed in triplicates.

2.4. Nuclear and cytosolic fractionation

To assess the Nrf2 activation potential, nuclear and cytoplasmic extracts were separated using Nuclear Extraction Kit (Abcam, UK), according to the manufacturer's instructions. Briefly, cells were trypsinized, centrifuged for 5 min at 1000 rpm; the pellet was resuspended in cytosolic-extraction buffer and vortexed vigorously, and the lysate was centrifuged for 2 min at 12,000 rpm at 4 °C; cytoplasmic protein fraction was collected. To the nuclear pellet, extraction buffer with protease inhibitor cocktail and DTT was added and incubated on ice for 15 min. The lysate was centrifuged, and the supernatant containing the solubilized nuclear proteins were collected and used for immunoblotting.

2.5. Western blotting

Total protein was extracted using RIPA buffer and quantified with Bradford method using BSA as a standard. Proteins were denatured with sample buffer, separated on a SDS-PAGE and electroblotted onto nitrocellulose membrane. The membrane was then exposed with blocking solution, followed by overnight incubation at 4 °C with respective antibodies against AKT, pAKT, β-actin (Santa Cruz, CA, USA), Nrf2, pNrf2, Caspase-3, GAPDH and Lamin-B (abcam, MA, USA). After incubation, the membrane was washed (TBST) and probed with an HRP-conjugated antirabbit or antimouse antibodies. By using an enhanced chemiluminescence (ECL) kit (Biorad, PA, USA), the signals were detected and captured using documentation system (GBOX, Syngene, UK).

2.6. ARE-luciferase reporter gene assay

hNQO1-ARE-Luc and GST1-ARE-Luc reporter gene constructs (a generous gift from Donna D. Zhang – College of Pharmacy, University of Arizona, Tucson, AZ, USA) were used for cell-based reporter assay [10]. MIN6 cells were transfected with ARE-luciferase vector (500 ng) using Lipofectamine 2000 reagent. After 6 h, the media were changed and pretreated with PTS (4 and 8 μM) for 8 h followed by cytokine cocktail exposure for 36 h. At the end of the treatment, cells were lysed using cell lysis reagent (Promega, US), and luciferase activity was measured using a luminometer (Promega, US). Luciferase activities were expressed as fold induction relative to control. Results were represented after normalizing with protein and scrambled control, can potentially be considered as reporter gene activation and showed as mean values of three independent experiments.

2.7. Nrf2 downstream target gene expression by qPCR analysis

Total mRNA was isolated from the control and treated cells using RNA isolation kit (Qiagen, Germany), and an equal quantity of RNA was converted to cDNA using cDNA conversion kit (Qiagen, Germany), as per the manufacturer's instructions. The resulted cDNA was used for the expression studies using Realtime PCR system (Biorad cfx connect systems). The sequences for the primers were listed in the Table 1.

2.8. Flow cytometric quantification of apoptosis using annexin-V and PI staining

The percentage of apoptotic population was measured using Annexin-V-FITC Apoptosis Detection kit (BD Biosciences, CA, USA). Annexin-V and PI dual staining were carried out to determine the protective effect PTS against cytokine-induced apoptosis. Briefly, after the treatment, cells were trypsinized and pelleted by centrifugation at 1500 rpm for 3 min. The pellet was resuspended in staining solution, which includes binding buffer, Annexin-V-FITC and PI and incubated in dark for 15 min. Further, cells were analyzed using flow cytometer (BD Biosciences, CA, USA).

2.9. Flow cytometric assessment of cell cycle using PI staining

MIN6 cells were pretreated with PTS for 8 h, followed by cytokine exposure for 36 h. After the treatment, the cells were washed and fixed with 70% ethanol for 24 h at -20 °C, followed by centrifugation at 1500 rpm for 5 min, and the pellet was washed twice with PBS. Finally, the fixed cells were incubated in dark for 30 min at 37 °C with PI solution [(RNaseA (1 mg/ml); Triton X-100 (5 μL); PI (50 μg/ml) in 10 mL PBS)]. After incubation, stained cells were analyzed using flow cytometer (BD Biosciences, San Jose, CA, USA).

2.10. Measurement of NO production by Griess reagent system

MIN6 cells were treated with PTS followed by cytokines exposure as described earlier. After treatment, the supernatant was collected, and nitrite concentration was determined by using the Griess Reagent (Promega, Wisconsin, USA). Briefly, 50-μL sample/standard was added to a 96-well plate (n=3), followed by addition of sulfanilamide and incubated for 10 min at room temperature protected from light. After incubation, 50 μL of N-1-napthylethylenediamine dihydrochloride solution for 10 min at room temperature, the absorbance was measured at 550 nm in a microplate reader (Infinite 1000, Tecan, Switzerland).

2.11. Animals and diet

Animal experiments were performed using 6-week-old male Swiss Albino Mice (20± 5 g) and maintained under a standard 12-h light/dark cycle. The mice were provided with *ad libitum* access to water and pellet diet (Hindustan Lever Ltd., Bangalore, India). The current study protocol was approved by the Institutional Animal Ethical Committee and abided to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) of King Institute of Preventive Medicine and Research Center (KIPMER), Chennai.

Table 1
Sequences of primers for PCR

Gene	Forward primer	Reverse primer	Accession number
Actin	AGCCATGTACGTAGCCATCC	CTCTCAGCTGTGGTGGTGAA	NM_007393
Catalase	ACATGGTCTGGGACTTCTGG	CAAGTTTTTGATGCCCTGGT	NM_009804
HO-1	CACGCATATACCCGCTACCT	CCAGAGTGTTCATTCGAGCA	NM_010442
GPx	GTCCACCGTGTATGCCTTCT	TCTGCAGATCGTTCATCTCG	NM_008160
SOD	CCAGTGCAGGACCTCATTTT	CACCTTTGCCCAAGTCATCT	NM_011434.1
NQO1	TTCTCTGGCCGATTCAGAGT	GGCTGCTTGGAGCAAAATAG	NM_008706
iNOS	CACCTTGGAGTTCACCCAGT	ACCACTCGTACTTGGGATGC	NM_010927
BAX	TGCAGAGGATGATTGCTGAC	GATCAGCTCGGGCACTTTAG	NM_007527
Bcl-2	CTGGCATCTTCTCCTTCCAG	GACGGTAGCGACGAGAGAAG	AY416381.1