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Inhibition of the receptor for advanced glycation endproducts (RAGE) protects pancreatic β-cells

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ABSTRACT

Advanced glycation endproducts (AGEs) and the receptor for AGEs (RAGE) have been linked to the pathogenesis of diabetic complications, such as retinopathy, neuropathy, and nephropathy. AGEs may induce β-cell dysfunction and apoptosis, another complication of diabetes. However, the role of AGE-RAGE interaction in AGE-induced pancreatic β-cell failure has not been fully elucidated. In this study, we investigated whether AGE–RAGE interaction could mediate β-cell failure. We explored the potential mechanisms in insulin secreting (INS-1) cells from a pancreatic β-cell line, as well as primary rat islets. We found that glycated serum (GS) induced apoptosis in pancreatic β-cells in a dose- and time-dependent manner. Treatment with GS increased RAGE protein production in cultured INS-1 cells. GS treatment also decreased bcl-2 gene expression, followed by mitochondrial swelling, increased cytochrome *c* release, and caspase activation. RAGE antibody and knockdown of RAGE reversed the β-cell apoptosis and bcl-2 expression. Inhibition of RAGE prevented AGE-induced pancreatic β-cell apoptosis, but could not restore the function of glucose stimulated insulin secretion (GSIS) in rat islets. In summary, the results of the present study demonstrate that AGEs are integrally involved in RAGE-mediated apoptosis and impaired GSIS dysfunction in pancreatic β-cells. Inhibition of RAGE can effectively protect β-cells against AGE-induced apoptosis, but cannot reverse islet dysfunction in GSIS.

1. Introduction

Diabetes mellitus is a disorder characterized by hyperglycemia due to an absolute or relative deficiency of insulin. Patients with diabetes are prone to complications such as nephropathy, atherosclerosis, neuropathy, retinopathy, and cataracts. Though hyperglycemia plays a role in the pathogenesis of diabetic complications, the mechanisms by which it does so have yet to be clarified. One potential mechanism that has been suggested is non-enzymatic protein glycation [1]. Proteins glycated early in hyperglycemic states will be further modified by the formation of advanced glycation end products (AGE).

As a result of chronically elevated blood glucose in diabetes, AGEs are generated and accumulated. Both AGEs and their receptors have been shown to play a key role in the pathogenesis of diabetic complications [2–7]. The receptor for AGE (RAGE) is a member of the immunoglobulin superfamily of cell surface molecules [8]. AGE–RAGE interaction triggers the activation of critical cell signaling pathways, such as p21ras, mitogen-activated protein kinases (MAPKs), and nuclear factor-κB (NF-κB), leading to the activation of proinflammatory responses and the cellular damage that underlies the complications of diabetes [9–12]. Beta-cell failure is one of the many complications of diabetes [13]. However, it is not known to what extent AGE directly affects pancreatic β-cell viability and function.

In this study, we utilize isolated islets and a rat insulin-secreting β-cell line (INS-1) to test the hypothesis that AGEs contribute to β-cell apoptosis via the interaction of AGE–RAGE, which reduces bcl-2 family gene expression and activates the caspase signaling cascade.

2. Materials and methods

2.1. AGE-fetal bovine serum preparation

GS was prepared and the concentration of AGEs within the GS was measured as described in the electronic supplementary material (ESM).

2.2. Cell culture

INS-1 cells were cultured to near confluence in RPMI-1640 medium (Invitrogen, NY) with 11.1 mmol/l D-glucose supplemented

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with 10% fetal bovine serum (FBS) and 50 μmol/l β-mercaptoethanol (Sigma–Aldrich, MO). All tissue culturing was performed in a thermo tissue-culture incubator that provided an environment of 95% O_2/5% CO_2 gas. GS or NG was added in the appropriate experiments and cells were incubated for an additional 24, 48, 72, or 96 h. The cells were harvested for flow cytometry and DNA laddering experiments by using 1 ml 0.25% trypsin-0.02% EDTA solution.

2.3. Pancreatic islet isolation

All animal studies were performed according to guidelines established by the Research Animal Care Committee of Nanjing Medical University. Male Sprague–Dawley rats (200–250 g) were purchased from Shanghai Laboratory Animal Centre (Chinese Academy of Sciences, China). Islet isolation and culturing techniques have been described previously [14]. Freshly isolated islets were transferred to sterile 6 cm dishes and cultured in RPMI 1640 containing 11.1 mmol/l glucose supplemented with 10% FBS, 10 mmol/l HEPES, 100 U/ml penicillin and 100 μg/ml streptomycin. The islets were allowed to equilibrate for 3 h, at which point they were counted and moved into a 48-well plate (10 islets/well). They were cultured overnight at 37 °C. The next morning, the islets were treated with NG or 10% GS in the depletion medium. GSIS studies were performed 48 h later.

2.4. Flow cytometry analysis

INS-1 cells (2×10^6 cells per well) were cultured in 6-well plates and treated with NG or GS for 24 h, 48 h, 72 h, or 96 h (time gradient) or with 10%NG, 1%, 2%, 5% or 10% GS for 72 h (dose gradient). The cells were then harvested and fixed with 1 ml 75% ice-cold ethanol at −20 °C overnight. After fixation, the cells were washed in PBS and stained with 500 μl propidium iodide solution (50 μg/ml, Sigma) containing 25 μg/ml RNase. The cells were incubated at room temperature for 0.5 h in the dark, and analysed using a FACS Calibur flow cytometer and Cellquest Pro software (Becton Dickinson Immunocytometry Systems, CA). For determination of RAGE expression on the cell surface, INS-1 cells were treated with GS or NG control for 24 h, then suspended in PBS (1×10^6) and incubated with 1 μg RAGE antibody (Santa Cruz, CA) for 1 h at room temperature. After incubation with FITC conjugated goat anti-mouse secondary antibody (Chemicon, CA) for 45 min at room temperature, cells were analyzed with flow cytometer.

2.5. DNA laddering assay

INS-1 cells were treated with NG or GS, as described above. Cellular DNA isolation and ladder detection were performed according to the manufacturer's instructions (Roche Molecular Biochemicals, Indianapolis, IN). DNA laddering was run on 2% agarose gels along with a molecular weight marker.

2.6. Adenovirus-mediated RNAi

The sequences of DNA nucleotides used to create rat small interfering RNA (siRNA) are shown in Supplement data (Table 1). The sequences were synthesized, annealed and subcloned into pShuttle-H1 according to the method of Shen et al. [15]. To allow infection efficiency to be conveniently monitored, the H1 siRNA fragments were cut from pShuttle-H1 and ligated into the pAdTrack plasmid upstream of the CMV-green fluorescent protein (GFP) cassette. The AdTrack-H1 siRNA plasmid was recombined with back-bone pAdEasy-1 in BJ5183 bacteria. Adenovirus generation, amplification, and titration were performed as described previously [16]. INS-1 cells were infected with adenovirus at a multiplicity of infection of 50 at 37 °C. Two hours after infection, the cells were cultured in fresh medium for another 24 h before being treated with GS for 48 h.

2.7. Real-time RT-PCR

Total RNA was extracted using Trizol reagent (Invitrogen). First-strand cDNA synthesis was performed using 1 μg of total RNA and an avian myeloblastosis virus reverse transcription system. Real-time quantitative PCR was performed using the SYBR Green PCR Master Mix and ABI Prism 7000 Sequence Detection System. All data were analyzed using the expression of β-actin as a reference. The sequences of the primers used are available in Supplement data (Table 2).

2.8. GSIS assay

Isolated rat islets were moved to 48-well plates (10 islets/well) and treated with NG or GS for 48 h. The islets were pre-incubated for 1 h in HEPES-balanced Krebs–Ringer bicarbonate buffer (KRBH) containing 3.3 mmol/l glucose and 1 g/l bovine serum albumin (BSA). The islets were incubated for 1 h in KRBH containing basal (3.3 mmol/l) or stimulatory (16.7 mmol/l) concentrations of glucose. After the static incubation, the supernatants were obtained and frozen at −70 °C for subsequent determination of insulin concentration. The insulin levels were measured using RIA as described previously [17].

2.9. Caspase-9 assay

Caspase-9 activity in the cytosolic fraction was determined as described in the ESM.

2.10. Mitochondrial fraction preparation

INS-1 cells were cultured in 10 cm dishes at 60% confluence for 24 h and treated with NG or GS for 48 h. The cells were harvested and then mitochondrial and cytosolic fractions were isolated using a mitochondrial fractionation kit (Active Motif, Carlsbad, CA) according to the manufacturer's protocol [18]. Protein concentration was measured with a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA).

2.11. Transmission electron microscopy

We used transmission electron microscopy to monitor the mitochondrial morphology as described in the ESM.

2.12. Western blot analysis

INS-1 cells were cultured and treated as described above, and lysed with ice-cold lysis buffer. After protein content determination, western blotting was performed as described [17]. Individual immunoblots were probed with antibodies to mouse anti-RAGE monoclonal antibody, mouse anti-cytochrome *c* monoclonal antibody, rabbit anti-PARP-1 polyclonal antibody, or mouse anti-Bcl-2 monoclonal antibody. Target protein levels were quantified relative to levels of control protein, mouse anti-β-actin monoclonal antibody.

2.13. Statistical analysis

Comparisons were performed using Student's *t* test between pairs of groups, or ANOVA for multiple group comparison. Results are presented as means ± SEM. A p value of less than 0.05 was considered to be statistically significant.