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Original article

Advanced glycation end-products induce injury to pancreatic beta cells through oxidative stress

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Abstract

Aim. – This study evaluated the direct effects of advanced glycation end-products (AGEs) on pancreatic β cells, including cellular viability, generation of reactive oxygen species (ROS) and insulin secretion, and also looked for the main source of ROS in INS-1 cells and the possible molecular mechanism(s) of cell injury by AGEs.

Methods. – INS-1 cells were cultured with 100, 200 and 500 mg/L of AGEs for specific periods of time. Cell apoptosis was determined by ELISA and real-time PCR assays. ROS were detected by DCFH-DA and MitoSOX Red probes with a flow cytometer, NADPH oxidase activity was measured by lucigenin chemiluminescence and MAPK phosphorylation was measured by Western blot tests.

Results. – Both cell apoptosis and ROS generation increased in AGE-treated cells in a dose-dependent way, and both the mitochondrial electron transport chain and NADPH oxidase pathway participated in ROS generation, although the role of the mitochondrial pathway was earlier and more important. AGEs exerted a toxic effect on insulin secretion that could be largely reversed by inhibiting ROS.

Conclusion. – AGEs injured INS-1 cells by oxidative stress mainly through the mitochondrial pathway, although the JNK and p38 MAPK signaling pathways were also key modulators in ROS-mediated β-cell death.

Keywords: Reactive oxygen species; Oxidative stress; Advanced glycation end-products; INS-1 cells; Insulin secretion; Apoptosis; β-cell

Résumé

Les produits terminaux de la glycation avancée lèsent les cellules β des îlots de Langerhans du pancréas par le stress oxydant.

Objectif. – Le but de notre étude était d'évaluer l'effet direct des produits terminaux de la glycation avancée (AGEs, en anglais) sur des cellules β des îlots de Langerhans du pancréas, en termes de viabilité cellulaire, génération des dérivés réactifs de l'oxygène (ROS, en anglais) et insulinosécrétion, de détecter la source principale de ROS dans les cellules INS-1, et de déterminer le mécanisme moléculaire des lésions causées par les AGEs.

Méthodes. – Les cellules INS-1 ont été cultivées en présence d'AGEs aux doses de 100, 200 et 500 mg/L. L'apoptose cellulaire a été déterminée par la méthode immuno-enzymatique ELISA et la PCR en temps réel. Les ROS ont été détectés par les sondes DCFH-DA et MitoSOX Red en cytométrie en flux. L'activité de la NADPH oxydase a été mesurée par la chémiluminescence. Le niveau de la MAPK phosphorylation a été déterminé par le Western blot.

Résultat. – La culture des cellules β en présence d'AGEs a provoqué une augmentation des ROS et l'apoptose cellulaire de manière dépendante de la concentration. La chaîne de transport d'électrons dans la mitochondrie et la voie de la NADPH oxydase intervenaient dans la génération des ROS, et le rôle de la voie mitochondriale était plus précoce et plus important. Les AGEs ont exercé un effet toxique sur la sécrétion de l'insuline, cet effet pouvant être en grande partie annulé par l'inhibition de la production des ROS.

Conclusion. – Les AGEs induisent des dommages aux cellules INS-1 par l'intermédiaire du stress oxydant provoqué principalement par la voie mitochondriale. La voie JNK et la voie P38 MAPKs jouent un rôle modulateur dans l'apoptose des cellules β des îlots de Langerhans du pancréas causée par les ROS.

Mots clés : Dérivés réactifs de l'oxygène ; Stress oxydant ; Produits terminaux de la glycation avancée ; Cellule INS-1 ; Insulinosécrétion ; Apoptose ; Cellules β des îlots de Langherans ; Pancréas

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1. Introduction

Advanced glycation end-products (AGEs) are modifications of proteins or lipids that become non-enzymatically glycated and oxidized after coming into contact with aldose sugars, and then initiate a complex series of rearrangements and dehydrations to produce a class of irreversibly cross-linked and fluorescent moieties [1]. Recently, several researchers have pointed out the detrimental effects of glycation and AGEs on diabetes-associated cataract formation, nephropathy, retinopathy, neuropathy, periodontal disease, and impaired dermal and osseous wound-healing. However, there have been few studies of the direct effects of AGEs on β-cell function and whether or not AGEs can impair pancreatic islets or other insulin-secreting cells [2].

It is generally believed that oxidative stress is an important mechanism of β-cell degeneration [3,4]. It is capable of reacting with and damaging various molecular targets, including deoxyribonucleic acid (DNA), proteins and lipids [5]. More important, oxidative stress is a known apoptosis trigger and modulator activating the pro-apoptotic mitogen-activated protein kinase (MAPK) and other signaling pathways [6]. As β cells have very low intrinsic levels of antioxidant proteins, they are extremely vulnerable to reactive oxygen species (ROS) [7,8].

Mitochondria and the NOX family of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase are known to be two major sources of ROS induced by external stimuli [9,10]. It has been largely established that the mitochondrial respiratory chain is an important site of ROS production within most cells [11]. However, superoxide-producing enzymes, including NADPH oxidase, have been implicated as the most important source of ROS production in phagocytes [12]. Recently, several studies have demonstrated the presence of NADPH oxidase in pancreatic β cells and the role of phagocyte-type NADPH oxidase components in oxidative stress [13]. However, the relationship between mitochondria and the NOX family of NADPH oxidase remains unclear. Our previous study showed that AGEs increased ROS in the mouse pancreatic β-cell line MIN6 partially through the NADPH oxidase pathway [14]. To further study the effects of AGEs on β cells, rat pancreatic β-cell insulin-secreting (INS-1) cell lines were exposed to AGEs to determine the possible mechanism(s) behind cell injury and degeneration.

2. Research design and methods

2.1. Cell culture

INS-1 cells were cultured in Roswell Park Memorial Institute (RPMI) medium 1640, supplemented with 10% fetal bovine serum (FBS), 10 mmol/L of glucose, 10 mmol/L of HEPES (4-2-hydroxyethyl-1-piperazineethanesulphonic acid), 2 mmol/L of L-glutamine, 1 mmol/L of sodium pyruvate, 50 μmol/L of β-mercaptoethanol, 100 U/mL of penicillin and 100 μg/mL of streptomycin, at 37 °C in a humidified 5% CO_2 atmosphere. After removing the medium, the cells were incubated in RPMI medium supplemented with 1% FBS and either bovine serum albumin (BSA) alone (as control) or various concentrations of AGEs (100, 200 or 500 mg/L), as indicated elsewhere [15,16].

2.2. Preparation of AGEs (from D-glyceraldehyde)

AGEs were prepared as described elsewhere [17]. Briefly, 50 mg/mL of BSA (Sigma-Aldrich, St. Louis, MO, USA) were incubated under sterile conditions with 0.1 M of D-glyceraldehyde (Sigma-Aldrich) in 0.2 M of phosphate buffer (pH 7.4) for seven days. The unincorporated sugar was removed by dialysis against 0.2 M of PBS (pH 7.4). Non-glycated BSA was incubated under the same conditions except for the absence of D-glyceraldehyde as a negative control. Preparations were tested for endotoxin using a limulus amebocyte lysate (LAL) reagent (Associates of Cape Cod, Inc., East Falmouth, MA, USA); if the endotoxin was less than 15 EU/L, it was considered a negative test.

2.3. Measurement of insulin secretion and glucose-stimulated insulin secretion (GSIS) of INS-1 cells

Cells were cultured overnight in RPMI 1640 medium with 0.5% BSA and 11.1 mmol/L of glucose, then incubated with AGEs for different periods of time. For each culture, 10^5/mL of cells per well were seeded onto 24-well plates, and preincubated for 30 min at 37 °C in Kreb's buffer (140 mmol/L of NaCl, 30 mmol/L of HEPES, 4.6 mmol/L of KCl, 1 mmol/L of $MgSO_4$, 0.15 mmol/L of Na_2HPO_4, 5 mmol/L of $NaHCO_3$ and 2 mmol/L of $CaCl_2$; pH 7.4) with 0.2% BSA and 3.3 mmol/L of glucose, which was then stimulated with either 2.8 or 25 mmol/L of glucose for 60 min. Insulin was measured using a radioimmunoassay (RIA) kit (Linco Research, St. Charles, MO, USA) with rat insulin as the standard.

2.4. Analysis of cytosolic ROS levels

Cytosolic ROS levels were analyzed using the following two methods. For fluorescent microscopy, cells were treated with 10 μmol/L of dihydroethidium (DHE), incubated for 1 h at 37 °C and then washed three times with PBS. The fluorescence of INS-1 cells was observed by fluorescent microscopy (excitation 520 nm, emission 610 nm). For flow cytometry, cytosolic ROS levels were measured by 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma-Aldrich). The INS-1 cells were washed and incubated with 10 μM of DCFH-DA for 40 min. Following this, the INS-1 cells were trypsinized, harvested, washed twice with PBS and directly collected before the immediate detection of the mean fluorescence intensity (MFI) of DCF for 1×10^5 cells per sample to measure cellular ROS levels (excitation 490 nm, emission 520 nm).

2.5. Measurement of mitochondrial superoxide by MitoSOX Red

Mitochondrial ROS were measured using a MitoSOX Red probe (Invitrogen Corp., Carlsbad, CA, USA), a live-cell permeant that rapidly and selectively targets mitochondria. Once