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 les cellules β des îlots de Langerhans du pancréas, en termes de viabilité cellulaire, production 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.

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Keywords: Dérivés réactifs de l’oxygène ; Stress oxydant ; Produits terminaux de la glycation avancée ; Cellule INS-1 ; Insulinosécrétion ; Apoptose ; Cellules β des îlots de Langerhans ; 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 degradations 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-piperazineethanesulfonic 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% CO2 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, 10^7/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 MgSO4, 0.15 mmol/L of Na2HPO4, 5 mmol/L of NaHCO3 and 2 mmol/L of CaCl2; 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 490 nm, emission 520 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
in the mitochondria, the MitoSOX Red reagent is oxidized by superoxide and exhibits a red fluorescence (excitation at 510 nm and emission at 580 nm). After drug treatment for specified periods of time, the cells were incubated with 5 μM of MitoSOX Red for 30 min at 37 °C. After incubation, the cells were washed with PBS twice, trypsinized, resuspended and immediately submitted to flow cytometry analysis.

2.6. Measurement of NADPH oxidase activity

NADPH oxidase activity was measured as previously described [14]. In brief, cells were trypsinized, pelleted by centrifugation and resuspended with cold Krebs–HEPES buffer. A cellular suspension (300 μL) was then placed in a 96-well white plate in a luminescence reader, then dark-adapted lucigenin (10 μmol/L, Sigma-Aldrich) added to start the reaction. Chemiluminescence was recorded every 15 s for 10 min. NADPH (final concentration: 100 μmol/L) was added after measuring the background lucigenin chemiluminescence, with further measurements performed for another 10 min. The differences between the values obtained before and after adding NADPH were calculated, and the result represented the activity of NADPH oxidase.

2.7. Quantitative real-time RT-PCR analysis

Total ribonucleic acid (RNA) extraction, cDNA synthesis and real-time polymerase chain reaction (PCR) analyses were performed as previously described [18]. The primers were designed using Primer Express software and synthesized by TaKaRa Biomedicals (Osaka, Japan). Gene-specific primers were designed as follows: Bax (forward: 5′-CTGACGGAATTC-CAAAGG-3′, reverse: CTTCAGATGCTGACCGAGG-3′); Bcl-2 (forward: 5′-GGTGGACACATCGCCCTGTG-3′, reverse: 5′-ATAGCTGTATGAGGTGTCG-3′); and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; forward: 5′-GCAAGTTCACACGCAAG-3′, reverse: 5′-GACCTGCATACTCCACGACAT-3′).

2.8. Apoptosis (ELISA)

Cells were plated on 96-well plates and grown in RPMI 1640, then exposed to a specific concentration of AGEs–BSA (controls), or 200 or 500 mg/L of AGEs for 24 h. In the AGE-treated groups, intracellular fluorescence was clearly increased compared with the controls. Also, the AGE groups showed more marked fluorescence than the BSA control group. Similar results were observed with the DCFH-DA probe, which was detected by flow cytometry (Fig. S2; see supplementary material associated with this article online).

3. Results

3.1. AGEs stimulated intracellular ROS production in INS-1 cells

It was shown in our previous experiment [14] that cellular events mediated by AGEs were associated with enhanced intracellular ROS generation in MIN6 cells. In the present study, ROS generation was tested in AGE-treated INS-1 cells. As shown in Fig. 1 a–c, INS-1 cells were treated with either BSA (control), or 200 or 500 mg/L of AGEs for 24 h. In the AGE-treated groups, intracellular fluorescence was clearly increased compared with the controls. Also, the AGE groups showed more marked fluorescence than the BSA control group. Similar results were observed with the DCFH-DA probe, which was detected by flow cytometry (Fig. S2; see supplementary material associated with this article online).

3.2. Mitochondria and NADPH oxidase (NOX) were both sources of ROS induction in INS-1 cells, but the effect of mitochondria was earlier and more important

To determine the source of ROS, MitoSOX Red—the mitochondrial superoxide indicator for mitochondrial ROS generation—was used, whereas the DCFH-DA probe was used to assess cytosolic ROS. As shown in Fig. 2 a, AGEs increased ROS generation in cytosol by 52.8% and 48.8% at 2 h and 24 h, respectively (P < 0.05), whereas co-incubation with an inhibitor of mitochondrial electron transport (thenoyltrifluoroacetone, TTFA) for 2 h decreased ROS generation by 32.7% (P < 0.05). However, pretreatment with rotenone and antimycin A increased ROS generation slightly by 9% and 18%, respectively. The results obtained at 24 h were similar. ROS generation
showed a suppressive effect [16]. TTFA was then used as the ROS inhibitor of the mitochondrial pathway in the subsequent study. As shown in Fig. 2a, apocynin and diphenyleneiodonium (DPI), inhibitors of NADPH oxidase, reduced ROS generation in cytosol by about 22.5% and 17.4%, respectively, at 24 h ($P<0.05$), but with no significant changes observed at 2 h. As shown in Fig. 2b, the MitoSOX Red probe was used to analyze ROS especially via the mitochondrial pathway, and showed that the ROS in mitochondria were reduced by 43.9% and 46.8% at 2 h and 24 h, respectively, in the presence of TTFA in the AGE-treated groups ($P<0.05$). In contrast, rotenone, antimycin A, apocynin and DPI increased ROS generation, but showed only small differences compared with the AGE-treated groups at both 2 and 24 h.

Next, the expression of NOX2, an important member of the NADPH oxidase family, was measured. The result of Western blot tests showed that AGEs upregulated NOX2 expression by 2.32-fold at 24 h ($P<0.05$), while NOX2 expression increased only slightly by 1.2-fold at 2 h (Fig. 2c). The activity of NADPH oxidase was measured by the chemiluminescence method, which found that the activity of NADPH oxidase was significantly raised by approximately 19.2% in the presence of AGEs at 24 h ($P<0.05$), whereas it was only slightly increased by 3% at 2 h (Fig. 2d). TTFA reduced the activity of NADPH oxidase at 2 h and 24 h by 7% and 15%, respectively. However, the activity of NADPH oxidase was completely inhibited by apocynin and DPI at both 2 h (by 44.7% and 48.2%, respectively) and 24 h (by 55.3% and 56.2%, respectively).

3.3. ROS inhibition protected INS-1 cells against apoptosis and partially reversed AGE-induced insulin-secretion dysfunction

INS-1 cells were incubated with either AGEs or BSA for 24 h after pretreatment with TTFA, thus neutralizing anti-RAGE antibody and inhibitors of MAPK. As shown in Fig. 3a, apoptosis was increased 3.21 times by AGEs alone. However, co-culture with the above inhibitors reduced the ratio of apoptosis significantly ($P<0.05$) by 62.1%, 66%, 47.4% and 55.8% with TTFA, anti-RAGE antibody, SP600125 and SB203580, respectively, in the AGE-treated groups. In contrast, ratios in the BSA control group under the same conditions differed only slightly.

Also studied was the effect of AGEs on insulin secretion, using the insulin stimulation index (ISI), and the ratios of insulin content with 25 and 2.8 mmol/L of glucose in Kreb’s buffer, to assess the function of INS-1 cells. Glucose-stimulated insulin release was examined after 2, 12, 24 and 48 h of incubation with and without AGEs. When INS-1 cells were incubated with Kreb’s buffer containing 2.8 mmol/L of glucose, little difference in insulin release was observed (data not shown) whereas, in the presence of 25 mmol/L of glucose, insulin in the AGE-treated groups was 31.6% lower than in the BSA controls at 48 h ($P<0.05$; Fig. 3b). Insulin secretion was around 45%, 42%, 30% and 25% in INS-1 cells pretreated with TTFA, neutralizing anti-RAGE antibody, SP600125 and SB203580, respectively. After 48 h of incubation, the ISI was decreased by 28.8% in the groups treated with AGEs ($P<0.05$, Fig. 3c),

Fig. 1. AGE-stimulated ROS generation in INS-1 cells cultured with different concentrations of AGEs for 24 h as detected by an HE probe on fluorescence microscopy ($\times$ 200): (a) INS-1 cells with BSA at 200 mg/L (control); (b) INS-1 cells with AGEs at 200 mg/L; and (c) INS-1 cells with AGEs at 500 mg/L.
Fig. 2. Mitochondria played a major role on ROS generation by AGEs in the early stages. INS-1 cells were incubated with BSA or AGEs (500 mg/L) for 2 and 24 h with/without 30-min pretreatment with various inhibitors: rotenone (Rot, 0.1 μM); TTFA (0.5 μM); antimycin A (Anti A, 10 nM); apocynin (Apo, 0.2 mM); and DPI (2 μM). ROS levels were measured by flow cytometry. DCFH-DA (a) and MitoSOX Red (b) probes were used separately; (c) NOX2 expression was detected by Western blot tests; and (d) the activity of NADPH oxidase was measured by the chemiluminescence method. The results of three separate experiments were pooled and the means ± SD calculated. *P < 0.05 vs control; #P < 0.05 vs AGE-treated groups.

whereas this was reversed by pretreatment with TTFA and neutralizing anti-RAGE antibody, with increases of 30.9% and 32.4%, respectively (P < 0.05). The inhibitor of JNK and p38 MAPK also showed some improvement (17.9% and 20.2%, respectively).

4. Discussion

Direct immunochemical evidence shows that there are six distinct AGE structures (AGE-1 to -6) within the AGE-modified proteins and peptides that circulate in the serum of diabetic patients [19], including glyceraldehyde-derived AGEs (AGE-2) and glycolaldehyde-derived AGEs (AGE-3), also called ‘toxic AGEs’ (TAGEs). TAGE-mediated apoptosis has been described by others in endothelium-derived cells, leukocytes, neurons and lung cells [20–23]. AGE-induced apoptosis is mainly mediated by RAGE and activation of caspase-3 [24]. In our present experiments, the ratio of the apoptosis-related genes Bax and Bcl-2, another apoptotic gene family besides caspases, was used to evaluate injury caused by AGEs.
Our present results have demonstrated that the interaction of AGEs with their primary receptors increased intracellular ROS in INS-1 cells, while the presence of the apoptosis-related genes Bax/Bcl-2 was clearly upregulated in AGE-treated cells. These effects were mediated by RAGE, which was suggested by the suppression of AGE-mediated ROS production in the presence of anti-RAGE IgG (Fig. S3; see supplementary material associated with this article online). This was also confirmed in other studies of human umbilical-vein endothelial cells (HUVEC) [16].

ROS are also an essential element for certain biological responses at physiological concentrations [25,26]. Indeed, there is strong evidence to support a role for ROS in the regulation of pivotal cellular signaling events such as homoeostasis, cell proliferation and differentiation, and inflammatory and immune responses. However, because of their toxicity, there is only a narrow concentration range within which they can function effectively as secondary messengers. In the literature, RAGE protein was markedly blocked by antibody, while the level of ROS generation was only reduced to normal levels, which suggests that the generation of physiological ROS is independent of RAGE.

Both mitochondrial and NOX enzymes are important sources of ROS in INS-1 cells. In the present research, the use of inhibitors of mitochondrial electron transport, including rotenone (a specific inhibitor of complex I), TTFA (a specific inhibitor of complex II) and antimycin A (a specific inhibitor of complex III), to block the mitochondrial electron transport chain
demonstrated that rotenone and antimycin A markedly promoted endogenous ROS rather than inhibited ROS generation. Other studies have also found that rotenone and antimycin A increased ROS generation by exogenous compounds [16]. By inhibiting electron transport in the respiratory chain and causing more electron leakage, they combined with oxygen to generate more ROS. However, TTFA displayed a negative effect on ROS generation. Our present study showed that, in the early phase of ROS generation, the mitochondrial pathway was the main source, while the NOX family of NADPH oxidase showed little influence. However, after exposure of cells to AGEs for more than 12 h, both pathways took part in ROS generation. Recently, some investigators have indicated that ROS from the mitochondrial pathway increased NOX1 expression and enhanced its activity [27]. Lee et al. [28] found that ROS generated from mitochondria during the early phase appeared to trigger the late phase of ROS induction by stimulating the PI3 K/Rac1/NOX1 pathway in human 293 T cells. We believe that mitochondria exert an earlier and more prominent effect on ROS generation in INS-1 cells.

To determine whether there were any common pathways in AGE-induced INS-1 cell apoptosis, the MAPK pathway, one of several reported upstream signaling pathways by AGEs, was also investigated [29]. The functional role of MAPK in mediating AGE-induced ROS activation has been demonstrated using inhibitors of JNK and p38 MAPK, which was consistent with the observation that AGEs activated JNK and p38 MAPK (Fig. S4; see supplementary material associated with this article online). Inhibition of JNK and p38 MAPK signals reduced AGE-stimulated apoptosis. These results suggest that JNK and p38 MAPK signals play a vital part in AGE-induced INS-1 cell apoptosis. AGEs increased the formation of intracellular ROS as well as MAPK which, through intermediate molecules, activates different targets, including transcription factors such as nuclear factor kappa-B (NF-KB) and activator protein (AP)-1 [30–32]. Activation of these transcription factors was found to subsequently increase the expression of different molecules such as inducible nitric oxide synthase, and inflammatory cytokines such as tumour necrosis factor (TNF) and interleukin (IL)-1, and consequently led to cell death [30,32].

Chronic oxidative stress (accumulation of ROS) due to glucose toxicity and lipotoxicity has been increasingly implicated in the impaired state of β cells in diabetes [3,33,34]. Coughlan et al. [35] found that AGEs directly caused insulin secretory defects in MIN6 cells and mouse islets in vivo most likely by impairing mitochondrial function. Zhao et al. [36] also reported that AGE treatment in mice and in isolated islets impaired GSIS by inducing nitric oxide, which eventually led to inhibition of cytochrome oxidase and adenosine triphosphate (ATP) synthesis. In the present experiments, our results showed that high concentrations of AGEs impaired insulin secretion in INS-1 cells, and that this effect could be partially reversed by inhibiting ROS generation. These results suggest that oxidative stress within mitochondria may be the main cause of β-cell impairment. Methylglyoxal (MGO), the most reactive precursor of AGEs, also impaired both insulin action and secretion by enhancing the activity of glycogen synthase kinase-3β (GSK-3β) in INS-1 cells [37]. However, this effect was independent of ROS production, which suggests that other factors may also be involved in AGE-induced dysfunction of pancreatic β cells. Our present data support the idea that AGEs impair the secretion of pancreatic β cells at least in part through ROS.

In summary, AGEs injured INS-1 cells through oxidative stress. Although both the mitochondrial respiratory chain and NADPH oxidase systems were implicated in AGE–RAGE signaling of rat β cells, the effect of the mitochondrial pathway may be earlier and more important. We suggest that the large accumulation of AGEs works with high levels of sugar, lipids and other factors to affect the insulin-secretion mechanisms by disrupting redox status and promoting cell apoptosis, which aggravates peripheral insulin resistance [38] and, ultimately, leads to the progression of type 2 diabetes.

Disclosure of interest

The authors declare that they have no conflicts of interest concerning this article.

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Appendix A. Supplementary data

Supplementary material (Figs. S1–S4) associated with this article can be found at http://www.sciencedirect.com, at doi:10.1016/j.diabet.2012.01.003.

References


