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Glycated fetal calf serum affects the viability of an insulin-secreting cell line in vitro

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Abstract

The purpose of the present study was to evaluate the direct effects of advanced glycation end products (AGEs) on β-cells by their exposure to a glycated serum to estimate the cellular viability and the related insulin secretion. Glycation of fetal calf serum was obtained by incubation with 50 mol/L ribose at 37°C for 7 days; at the end of this incubation period, the pentosidine content ranged between 15 and 16 × 10^5 pmol/L. HIT-T15 cells, a pancreatic islet cell line, were grown and cultured for 5 days in Roswell Park Memorial Institute (RPMI) medium containing either not glycated (NGS) or glycated (GS) fetal calf serum. Cellular oxidative stress (ie, thiobarbituric acid-reactive substances) was assessed by high-performance liquid chromatography. Cellular viability was evaluated by detection of proliferation, cell necrosis, and cell apoptosis rate. The insulin secretion and the related intracellular content were evaluated by enzyme-linked immunosorbent assay. The present study reported, after 5 days of exposure to the glycation environment, a moderately reduced cellular proliferation (−20.44% ± 2.92%) with a corresponding increase of cell necrosis (+67.7% ± 1.56%) and cell apoptosis (+39.83% ± 2.92%) rate in comparison with the untreated cells. Oxidative intracellular stress was higher in GS conditions compared with the NGS ones (+293.3% ± 87.53%). Insulin release from GS-treated HIT-T15 cells was lower than that of NGS-treated cells both when cells were stimulated with low glucose concentration (2.8 mmol/L, −30.3% ± 4.91%) or when they were challenged with high glucose concentration (16.7 mmol/L, −29.2% ± 5.82%). Incubation of HIT-T15 cells with glycated serum also caused a significant decrease of insulin intracellular content (−44.47% ± 9.98%). Thus, AGEs were shown to exert toxic effects on insulin-secreting cells. Chronically high intracellular oxidative stress, due to accumulation of AGEs, affects the insulin secretion machinery. The present data suggest a pivotal role of the non-enzymatic glycation process in the onset and progression of diabetes during aging and a direct adverse effect of a glycated environment on the pancreatic islet cells.

1. Introduction

Type 2 diabetes mellitus is one of the most diffused diseases in Western countries, representing more than 90% of all diagnosed forms of diabetes [1]. It is characterized by 2 interrelated factors: the resistance of peripheral tissues to insulin action and both the qualitative and quantitative impairments of insulin secretion [2]. The balance between insulin sensitivity and insulin secretion maintains blood glucose levels within the reference range; a decrease of insulin sensitivity is counterbalanced by an increase of insulin secretion. However, when insulin resistance or β-cell dysfunction exceeds the physiological rate, hyperglycemia appears and progresses to overt diabetes [3].

Insulin resistance is mediated by several factors, including obesity with a modulation of hormones, cytokines, and metabolic energy; abnormalities of insulin receptor substrate proteins and their downstream signaling cascades; activation of inflammatory signaling pathways; and mitochondrial dysfunction, among others [4-8]. Moreover, amyloid accumulation, in addition to lipo- and glucotoxicity, may also affect insulin secretion [9-12]. Glucotoxicity, the concept according to which high glucose concentrations lead to the deterioration of β-cells, involves the production of reactive oxygen species (ROS) both in extra- and intracellular spaces stimulating cellular responses and/or apoptosis [13,14]. These events were also observed in human pancreatic islets and insulin-secreting cells that became unable to function

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properly when cultured at high concentrations of glucose [15]. Recently, a similar cellular defect after a short-term exposure to potent reducing sugars such as ribose [15] was observed, whereas the prevention of cellular impairment through the administration of antioxidants such *N*-acetyl-L-cysteine was recorded both in cell lines and in rat models of diabetes [16]. Therefore, oxidative stress plays a primary role in glucotoxicity. Despite this, the underlying mechanism that causes this phenomenon is not yet fully elucidated.

The development of irreversible damage to β-cells resulting from an exposure to high concentration of glucose or other reducing sugars may also stem, in our opinion, from the non-enzymatic glycoxidation reaction [17,18]. This process leads to the formation of molecules called *advanced glycation end products* (AGEs), which are irreversibly modified molecules [17-19]. Glycation seems to be interrelated to the molecular defects that are recognized at the basis of the impaired insulin gene expression resulting from long-term exposure to high glucose concentrations [15]. The process of glycation produces ROS, favoring both intra- and extracellular oxidative environments. Moreover, the binding of AGEs to their specific receptors induces a further intracellular release of ROS, disrupting the cellular redox balance [20,21].

It is not yet known whether AGEs can impair insulin secretion from insulin-secreting cells. To study the direct effects of AGEs on β-cells, we exposed the pancreatic islet cell line HIT-T15 cells to high concentrations of AGEs to evaluate their effects on cellular viability and insulin production.

2. Materials and methods

2.1. Materials

Cell Titer 96 Aqueous One Solution Cell Proliferation Assay, Cytotox 96 Non-Radioactive Cytotoxicity Assay, and CaspACE Assay System were from Promega Italia (Milan, Italy). Bio-Rad Protein Assay was from Bio-Rad Laboratories (Milan, Italy). Mercodia Mouse Insulin Enzyme-Linked Immunosorbent Assay (ELISA) was from PANTEC (Turin, Italy). Fetal calf serum (FCS) was from Seromed (Berlin, Germany). All other reagents were from Sigma-Aldrich (Milan, Italy).

2.2. Glycation of FCS

Fifty millimoles per liter ribose was added to heat-inactivated (56°C for 1 hour) FCS. Penicillin and streptomycin were supplemented to inhibit bacterial growth. After a 1-week incubation at 37°C, FCS was then extensively dialyzed against 0.1 mol/L sodium phosphate-buffered saline (PBS, pH 7.4) to remove the free ribose. Aliquots of FCS were processed in the same way but without the ribose solution.

Pentosidine content, a well-known AGE, was evaluated as a measure of proteins' glycation, as previously described [22]. In the experimental RPMI medium containing 10% glycated serum (GS), the concentration of pentosidine ranged between 1.5 and 1.6 × 10^5 pmol/L.

Endotoxin content of GS and not-glycated serum (NGS) was determined by the Limulus Amebocyte Lysate assay and was found to be lower than 15 EU/L, defining it as a negative test.

2.3. Cells

HIT-T15 cells, a pancreatic islet cell line, were obtained from the American Type Culture Collection (Manassas, VA); cultured in RPMI 1640 medium supplemented with 10% FCS, 100 mU/L penicillin, and 100 mg/L streptomycin; and maintained at 37°C in 5% CO_2 humid atmosphere. The medium was changed every 48 hours. Before each experiment, cells were maintained for 5 days in RPMI medium containing either NGS or GS. HIT-T15 cells cultured in medium supplemented with NGS were used as a control culture.

2.4. Cell viability

To evaluate cell proliferation, HIT-T15 cells were plated in a 96-well plate (3 × 10^4 cells per well) and cultured for 5 days in a medium containing either NGS or GS. Viable cells were determined using the Cell Titer 96 Aqueous One Solution Cell Proliferation Assay according to the manufacturer's instructions. Briefly, it is a colorimetric method determining the number of viable cells via MTS tetrazolium reduction measured through formazan production. The MTS tetrazolium compound is bioreduced by cells into a colored formazan product directly proportional to the number of living cells in culture.

To evaluate cell apoptosis and cell necrosis, HIT-T15 cells were plated on 6-well dishes (7 × 10^6 cells per well) for 5 days in a medium containing NGS or GS and then processed for measuring the activity of caspase-3, a protease that plays a key role in the apoptosis of mammalian cells (CaspACE Assay System), and of lactate dehydrogenase, a stable cytosolic enzyme and marker of cell membrane damage and cell death due to necrosis, using Cytotox 96 Non-Radioactive Cytotoxicity Assay according to the manufacturer's instructions.

2.5. Cellular oxidative stress

After a 5-day incubation in RPMI medium containing either NGS or GS, 1 × 10^6 cells were treated with a lysis buffer (50 mmol/L Tris-HCl [pH 7.4], 150 mmol/L NaCl, 5 mol/L dithiothreitol, and 1% Triton X-100) to evaluate the intracellular content of thiobarbituric acid-reactive substances (TBARS), as markers of lipoxidative stress, according to the Wong method [22]. Samples were mixed with 0.440 mol/L phosphoric acid solution at a 1:15 ratio. Afterward, thiobarbituric acid and distilled water were added to each sample, which was then heated at 100°C for 60 minutes and then put in an ice-water bath (0°C) until the high-performance liquid chromatography (HPLC) analysis was performed. Ten minutes before the HPLC run, all the samples were neutralized with a

methanol-NaOH solution. The mobile phase contained HPLC-grade methanol in 50 mmol/L potassium phosphate buffer (pH 6.8). The detector was set, respectively, at the 532-nm excitation and 553-nm emission wavelength. The TBARS content, of which malondialdehyde is the prevalent component, was calculated using a standard synthetic malondialdehyde calibration curve.

2.6. Insulin secretion and content

Insulin release was evaluated in static condition. HIT-T15 cells were plated in 6-well dishes (7×10^5 cells per well) and incubated for 5 days in medium containing either NGS or GS. After 1-hour preincubation in Krebs-Ringer bicarbonate buffer (118.5 mmol/L NaCl, 2.54 mmol/L $CaCl_2$, 1.19 mmol/L KH_2PO_4, 4.75 mmol/L KCl, 25 mmol/L $NaHCO_3$, 1.19 mmol/L $MgSO_4$, 10 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 0.1% bovine serum albumin [pH 7.4]), cells were stimulated either with 2.8 or with 16.7 mmol/L glucose for 60 minutes.

Media were collected and stored at −20°C until the insulin determination was performed. Cells were then washed twice with PBS (pH. 7.4), treated with 1 mol/L NaOH, and harvested for protein determination using the Bio-Rad Protein Assay Kit according to the Bradford method. Insulin secretion was measured by ELISA and normalized to total protein concentration. To evaluate insulin content, another set of cells, grown in the same conditions, were washed twice with PBS (pH 7.4) at 0°C; extracted with acid/ethanol (0.15 mol/L HCl in 75% ethanol in H_2O) for 16 hours at 0°C; and then centrifuged at 15 000*g* at 4°C. Supernatants were collected and stored at −20°C until the insulin determination was performed by ELISA. The results were normalized to total protein concentration.

2.7. Statistical analyses

Data were expressed as the mean ± SEM for the 3 different experiments unless otherwise indicated. To verify statistical differences between samples, the Mann-Whitney *U* test for non-parametric unpaired data was used. The statistical significance of the differences among the percentages of vital, apoptotic, and necrotic cells was checked with χ^2 test; a $P < .05$ was considered statistically significant.

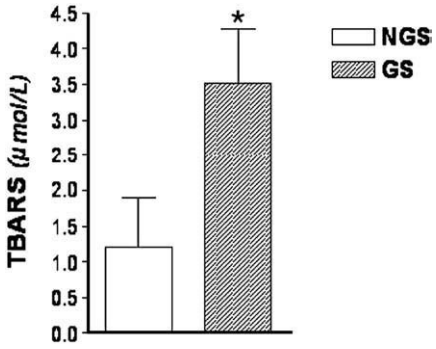

Fig. 1. Oxidative stress. HIT-T15 cells were lysed after 5 days of incubation with RPMI medium supplemented either with 10% NGS or GS. Intracellular levels of TBARS (in micromoles per liter), as markers of lipoxidative stress, were determined according to the Wong method. Data are expressed as the mean ± SEM of at least 3 independent experiments. *$P < .05$ vs NGS.

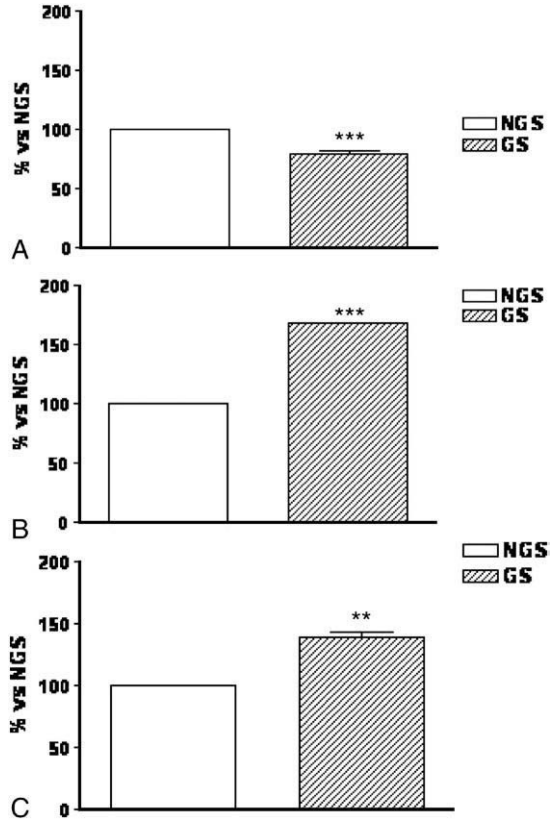

Fig. 2. Cell viability. HIT-T15 cells cultured for 5 days in RPMI medium supplemented either with 10% NGS or GS. A, Afterward, cell proliferation rate was determined by a colorimetric method based on the formazan product of the tetrazolium compound MTS; ***$P < .0005$. B, Cell necrosis was measured through quantification of lactate dehydrogenase released in culture supernatants by the cells; ***$P < .001$. C, Cell extracts were tested for caspase 3 activity using the colorimetric substrate N-Acetyl-Asp-Glu-Val-Asp p-nitroanilide; **$P < .01$. Values shown indicate the percentage of absorbance compared with NGS culture and represent the mean ± SEM of at least 3 independent experiments.

3. Results

3.1. Validation of the model

HIT-T15 cells cultured with NGS did not reveal differences in morphology, proliferation, or level of insulin secretion compared with the cells cultured with FCS (standard culture, data not shown).

3.2. Glycation of FCS and HIT-T15 cell culture

Pentosidine concentration formed from glycated FCS was assessed initially. Glycated serum contained $15.81 \pm 0.63 \times 10^5$ vs $0.614 \pm 0.023 \times 10^5$ pmol pentosidine per liter of NG (ie, a 1-week incubation at 37°C was sufficient nevertheless to significantly increase the pentosidine concentration

in serum without ribose) (n = 3, $P < .01$). The final pentosidine concentration in GS-treated medium ranged between 1.5 and 1.6×10^5 pmol/L. The GS-treated HIT-T15 cells did not affect cellular morphology.

3.3. Oxidative stress

After 5 days of culture, cells were lysed to determine the intracellular amount of TBARS as marker of lipoxidative stress. The TBARS concentration in GS-treated cells was significantly higher than that in NGS-treated cells (3.52 ± 0.76 vs 1.20 ± 0.70 μmol/L, $P < .05$, n = 7) (Fig. 1).

3.4. Cell viability

After 5 days of culture, the GS-treated HIT-T15 cells proliferation rate significantly decreased (−20.44% ± 2.92%, n = 5, $P < .0005$) paralleled by an increase of necrosis (+67.7% ± 1.56%, $P < .001$) and apoptosis (+39.83% ± 2.92%, $P < .01$) rates compared with NGS-treated cells (Fig. 2).

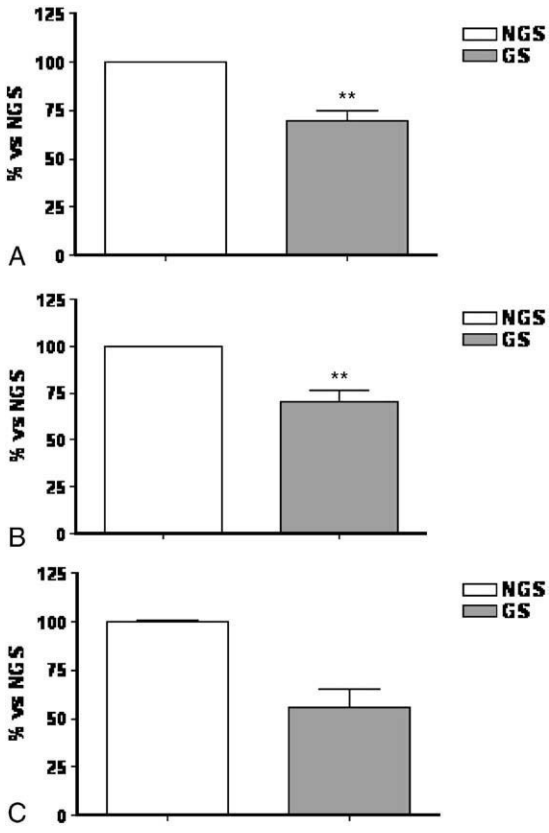

Fig. 3. Glucose-induced insulin secretion (A, B) and intracellular insulin (C) in HIT-T15 cells cultured for 5 days in RPMI medium supplemented either with 10% NGS or GS. HIT-T15 cells were incubated for 1 hour in the presence of 2.8 mmol/L (A) or 16.7 mmol/L (B) glucose concentrations after 1 hour of preincubation in glucose-free buffer; $^{**}P < .005$. C, Intracellular insulin was measured after acidified ethanol extraction; $^{*}P < .05$. Insulin amount of collected samples was determined by ELISA. Data are expressed as the percentage of NGS value and represent the mean ± SEM of at least 3 independent experiments.

3.5. Insulin secretion and content

Glucose-stimulated insulin release was examined after 5 days of culture in the presence of either NGS or GS. When cells were stimulated with Krebs-Ringer bicarbonate buffer containing 2.8 mmol/L of glucose, insulin release from GS-treated HIT-T15 cells was lower than that in NGS-treated cells (−30.3% ± 4.91%, n = 5, $P < .005$). When HIT-T15 cells were challenged with a high concentration of glucose (16.7 mmol/L), insulin secretion was again lower in GS-treated cells compared with NGS-treated cells (−29.2% ± 5.82%, n = 5, $P < .005$). In addition, the intracellular insulin content was significantly decreased in GS-treated HIT-T15 cells (−44.47% ± 9.98%, n = 5, $P < .05$) (Fig. 3).

4. Discussion

Aging is a risk factor for impaired glucose tolerance and onset of diabetes. It is known that fasting blood glucose levels increase slowly but consistently with aging in nearly all subjects; this feature is independent of other factors, such as obesity, reduced physical activity, and eating disorders [24-26], commonly found in elderly people. Moreover, a positive family history confers an increased risk for type 2 diabetes mellitus, meaning that gene expression defects play a relevant role in likely subjects [27].

There is general agreement that all the aforementioned factors contribute to the onset of glucose intolerance during aging, inducing a continuous pattern of metabolic pathologies.

Robertson and other authors [12,16,21,28] stated that high glucose concentration is responsible for glucotoxicity and that the continuous exposure to high glucose conditions and its related duration, rather than a "threshold function of glucose concentration," adversely affect β-cells function [9]. However, it is also well known that glycoxidation end products increase both in blood [29] and nearly all tissues [30] of aging people.

We suppose that, during aging, a dangerous vicious circle occurs. The AGEs may play a remarkable role in the age-related dysfunction of β-cells, favoring the onset or the progression of diabetes: the continuous exposure to high glucose, which stems from a β-cell deterioration for genetic or environmental causes, might also be the first occurrence and the starting point of an enhanced production of AGEs, which in turn worsens the β-cell functions. Therefore, wherever the metabolic derangement starts, it is mutually amplified by the other factor.

Therefore, to verify our hypothesis and to look at the direct effects of glycoxidation end products on β-cell activity, we exposed HIT-T15 cells to pentosidine concentration (roughly 1.5×10^5 pmol/L), similar to those reported for plasma levels of uremic subjects [22,29]; moreover, we avoided further increasing glucose concentrations compared with the standard medium to prevent any possible direct toxic effect of reducing sugars (glucotoxicity) on the β-cells.

We first observed that, after a 5-day incubation period, the glycated environment did not cause any change in cellular morphology. Nevertheless, 2 major consequences were observed. First, the incubation of HIT-T15 cells with AGEs compared with the incubation with NGS showed a reduction of proliferation rate (−20%) with a higher apoptosis (+40%) and necrosis (+68%) occurrence, indicating an interference with cell survival. The boost in the direction of cell death is probably due to the higher intracellular release of ROS; accordingly, we observed a significant increase of intracellular TBARS after the incubation with glycated serum. This result is interesting taking into account the enhanced susceptibility of β-cell line to oxidative stress because of a very low amount of antioxidant defenses [31].

Binding of AGEs with their specific receptors is followed by free radical generation; the high intracellular oxidative stress activates nuclear factor–κB release and a cascade of signaling proteins, triggering a functional modification and a change of cells condition [32]. Therefore, the present study supports the toxic effect of AGEs on an endocrine cell line as described for other cell types. Our previous study, using NT2 neurons, is consistent with the present results: the incubation with a glycated serum induces an increase of apoptotic death mediated by ROS release and protein kinase C δ activation [33].

Advanced glycation end product–mediated apoptosis was also described by other authors in endothelium-derived cells [34,35], leukocytes [36], neurons [37], and lung cells [38].

The molecular mechanism responsible for the cytotoxic effect may be related to the intracellular release of oxygen free radical [39] already mentioned; however, another possible explanation lies on the activation of an inflammatory process in the islet through a direct up-regulation of the expression of prostaglandin-endoperoxide synthase 2 leading to β-cell dysfunction and eventually to death [40].

In our study, hyperglycemia cannot play a significant role in the cell toxicity because of its steady concentration for the entire observation period.

The second relevant result is related to the significant decrease of insulin synthesis and secretion from HIT-T15 cells after the incubation with GS.

Exogenous AGEs impaired insulin content (−44%) and secretion both in basal condition (−30%) and after a glucose challenge (−29%), revealing an unequivocal functional impairment.

It is therefore conceivable to hypothesize a pathogenic role of AGEs in β-cell impairment of insulin secretion even if it is not possible to exclude additional contributions of early glycation products on this process. The present results, obtained after a short-term exposure of β-cells to AGEs, reinforce the hypothesis of a direct toxicity of the glycoxidation cascade as suggested by previous reports [15,19,41].

Matsuoka et al [15] and Kajimoto [18] reported that nonphysiological levels of ribose (40-60 mmol/L) in the medium induced, in the HIT-T15 cells, the suppression of insulin gene promoter activity or the inhibition of glucokinase gene expression. The authors suggested that the glycation in itself was responsible for the observed cells damages. Kaneto et al [41] reported an increased apoptosis rate, higher oxidative stress, and enhanced glycation of proteins after the incubation with high levels of fructose (50-100 mmol/L) or ribose (50 mmol/L).

However, these damages are likely to be caused by the glycation of certain substrates (DNA, enzyme, etc) instead of being ascribed to the level of AGEs in the medium. In the same way, Kooptiwut et al [42] also reported that a long-term exposure to hyperglycemia (40 mmol/L glucose for 10 days) decreased glucose-stimulated insulin secretion in mouse islets, an indirect, in this experiment, evidence of a pathogenetic role of AGEs. On the contrary, in our experiment, we maintained a steady level of glucose; and according to it, the present results are imputable to the increase of AGEs, supporting their direct toxicity on HIT-T15 cells.

Together with reducing sugars, lipid can also play a detrimental role on β-cell function: a sustained increase of free fatty acids [43] and hyperlipidemia [44] are considered an inducing pathway able to worsen the β-cell dysfunction. The mechanism is poorly understood, and there is no overall agreement if the contemporaneous occurrence of hyperglycemia is pathogenetic [43-45]. Moreover, lipids are precursors of AGEs [46]; thus, in our opinion, their pathogenetic contribution to the progressive β-cell deterioration is to be considered critical.

We therefore suggest that the accumulation of AGEs works synergically with high levels of sugar affecting the insulin secretion machinery through a disruption of the redox status and the programmed cellular death, promoting peripheral insulin resistance [46] that leads to the onset or progression of type 2 diabetes mellitus. Because diabetes itself boosts the formation of AGEs, a vicious circle is produced, capable of self-maintenance (Fig. 4).

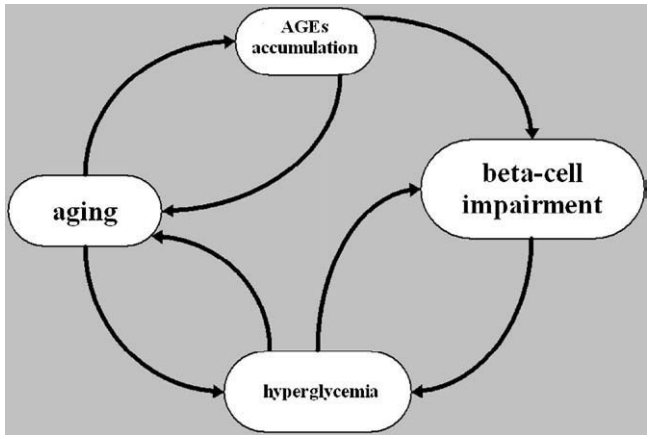

Fig. 4. Hypothesis of the vicious circle that favors the onset or progression of diabetes.

Our study should be expanded to acquire further scientific evidence: genetic backgrounds are needed to assess the existence of the metabolic syndrome, and the molecular mechanism of injury must be unveiled.

Acknowledgment

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