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Advanced Glycation End-Products affect transcription factors regulating insulin gene expression

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ABSTRACT

Advanced Glycation End-Products (AGEs) are generated by the covalent interaction of reducing sugars with proteins, lipids or nucleic acids. AGEs are implicated in diabetic complications and pancreatic β-cell dysfunction. We previously demonstrated that exposure of the pancreatic islet cell line HIT-T15 to high concentrations of AGEs leads to a significant decrease of insulin secretion and content. Insulin gene transcription is positively regulated by the beta cell specific transcription factor PDX-1 (Pancreatic and Duodenal Homeobox-1). On the contrary, the forkhead transcription factor FoxO1 inhibits PDX-1 gene transcription. Activity of FoxO1 is regulated by post-translational modifications: phosphorylation deactivates FoxO1, and acetylation prevents FoxO1 ubiquitination. In this work we investigated whether AGEs affect expression and subcellular localization of PDX-1 and FoxO1. HIT-T15 cells were cultured for 5 days in presence of AGEs. Cells were then lysed and processed for subcellular fractionation. We determined intracellular insulin content, then we assessed the expression and subcellular localization of PDX-1, FoxO1, phosphoFoxO1 and acetylFoxO1. As expected intracellular insulin content was lower in HIT-T15 cells cultured with AGEs. The results showed that AGEs decreased expression and nuclear localization of PDX-1, reduced phosphorylation of FoxO1, and increased expression and acetylation of FoxO1. These results suggest that AGEs decrease insulin content unbalancing transcription factors regulating insulin gene expression.

1. Introduction

Advanced Glycation End-Products (AGEs) are a group of compounds resulting from the non-enzymatic reaction of reducing sugars with the free amino groups of proteins [1]. Accumulation of AGEs is related to the aging process and is accelerated in diabetes [2–4]. The pathogenic role of AGEs in diabetic complications is widely acknowledged, and we have demonstrated that exposing the pancreatic islet cell line HIT-T15, to high concentrations of AGEs decreases cell viability, and reduces insulin secretion and content [5].

The reduction of insulin gene expression in β-cells that are chronically exposed to high glucose conditions is often accompanied by decreased binding activity of the β-cell specific transcription factor PDX-1 (Pancreatic and Duodenal Homeobox-1) [6]. Transcriptional regulation of the insulin gene involves a great deal of transcription factors [7]. Among them, PDX-1 is essential for conserved regulation of insulin transcription [8]. In turn, transcription of the gene coding for PDX-1 is negatively regulated by the binding of the forkhead transcription factor FoxO1 to the PDX-1 promoter [9]. FoxO1 mainly localizes in the cytoplasm of pancreatic β-cells and shows mutually exclusive nuclear localization with PDX-1. The intracellular distribution of FoxO1 is determined by phosphorylation: FoxO1 is retained in the cytoplasm upon phosphorylation, while conversely, a decrease in FoxO1 phosphorylation allows FoxO1 to enter the nucleus [10]. It has been reported that oxidative stress decreases FoxO1 phosphorylation in β-cells, thus allowing its translocation from the cytoplasm to the nucleus [11]. It has also been reported that oxidative stress induces FoxO1 acetylation, thereby protecting it from ubiquitin-mediated degradation [12]. Besides reducing PDX-1 transcription, nuclear localization of FoxO1 induces nucleocytoplasmic translocation of PDX-1 [13].

Since hyperglycemia increases the formation of AGEs [2], and oxidative stress is a mediator of intracellular AGEs signaling [1], we investigated whether AGEs impair the β-cell insulin content, thereby affecting the expression and localization of the transcription factors involved in regulating insulin gene transcription.

2. Materials and methods

2.1. Preparation of AGEs

Glycated serum (GS) was prepared as previously described [5]. Briefly, heat-inactivated FBS was incubated at 37 °C for 7 days with

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50 mmol/L ribose. The serum was then extensively dialyzed to remove surplus sugar. Aliquots of FBS were processed in the same way but without ribose (non glycated serum, NGS) and used for standard medium preparation. Pentosidine, a well-known marker of glycoxidative stress, was evaluated by HPLC and used as a measurement of protein glycation, as previously described [14]. The concentration of pentosidine in the experimental medium containing GS ranged between 1.5 and 4×10^5 pM, corresponding to the plasma levels found in diabetic patients.

2.2. Cell culture

The pancreatic islet cell line HIT-T15 (American Type Culture Collection, Manassas, VA) grown in RPMI 1640 medium supplemented with 10% FBS and 4 mM L-glutamine, 100 IU penicillin-G, and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. Culture media was changed every 48 h. Before each experiment, HIT-T15 cells were plated in 6-well dishes (7×10^5 cells per well) and maintained for 5 days in RPMI containing either NGS or GS.

2.3. Insulin content

After 5 days of culture, a set of cells were washed twice with PBS, pH 7.4, at 0 °C, extracted with acid/ethanol (0.15 M HCl in 75% ethanol in H_2O) for 16 h at 0 °C, then centrifuged at 15,000*g* at 4 °C. Supernatants were collected and stored at −20 °C until insulin determination was carried out by ELISA. The results were normalized to total protein concentration.

2.4. Cell lysis and subcellular fractionation

At the end of the experiments, a set of HIT-T15 cells were lysed in RIPA buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% NP40, 0.1% SDS, supplemented with protease and phosphatase inhibitors), and protein concentrations were determined using the BCA Protein Assay Kit. Another set of HIT-T15 cells was processed for subcellular fractionation using the Qproteome Cell Compartment Kit (QIAGEN, Milan, Italy) according to the manufacturer's instructions. Briefly, various cellular compartments were isolated by sequential addition of different extraction buffers to the cell pellet. Each subcellular fraction was collected after centrifugation and stored at −80 °C. Nuclear and cytosolic fractions obtained from each experimental condition were used for immunoblot analysis.

2.5. Immunoblot analysis

Thirty-micrograms of total cell lysate and subcellular fractions were separated on an SDS–PAGE and transferred onto nitrocellulose. Filters were blocked in 5% BSA and incubated overnight at 4 °C with primary specific antibodies (PDX-1, FoxO1, Ser256 phosphorylated FoxO1, acetylated FoxO1, β-Actin). Secondary specific horseradish-peroxidase linked antibodies were added for 1 h at room temperature. Bound antibodies were detected using the enhanced chemiluminescence lighting system LiteAblot Plus (EuroClone, Milan, Italy), according to the manufacturer's instructions. Bands of interest were quantified by densitometry using the NIH program, ImageJ.

2.6. Statistical analysis

All analyses were carried out using the GraphPad Prism 4.0 software package (GraphPad Software, San Diego, CA, USA). Data were expressed as means ± SD and then analyzed by the unpaired *t*-test. The results are representative of at least 3 experiments.

3. Results

Insulin content was significantly lower in HIT-T15 cells cultured in the presence of AGEs as compared to cells cultured in standard conditions (Fig. 1).

The reduction of insulin gene expression is often accompanied by decreased availability of the transcription factor PDX-1 [6]. To verify whether decreased insulin content was associated with a change in PDX-1 expression, we quantified the amount of PDX-1 by immunoblot analysis. As expected, PDX-1 expression was lower in protein lysates of cells cultured with GS for 5 days as compared to those of cells cultured in standard medium (Fig. 2).

Next, to investigate the effects of AGEs on the relative intracellular distribution of PDX-1, FoxO1 and phosphoFoxO1, we performed Western blot analysis using nuclear and cytoplasmic protein fractions isolated from HIT-T15 cells that had been cultured for 5 days in media containing either NGS or GS. Analysis of subcellular fractions revealed that PDX-1 expression decreased

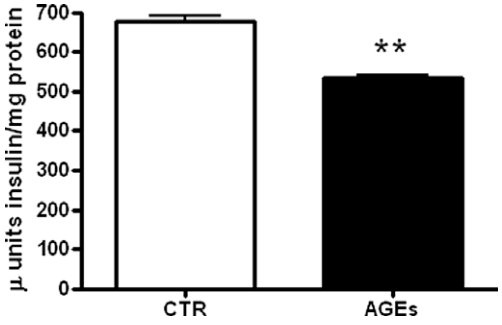

Fig. 1. Intracellular insulin content in HIT-T15 cells cultured for 5 days in RPMI supplemented either with NGS (CTR) or GS (AGEs). Intracellular insulin was measured after acidified ethanol extraction; $^{**}p < 0.01$. Data represent the means ± SD of at least 3 independent experiments.

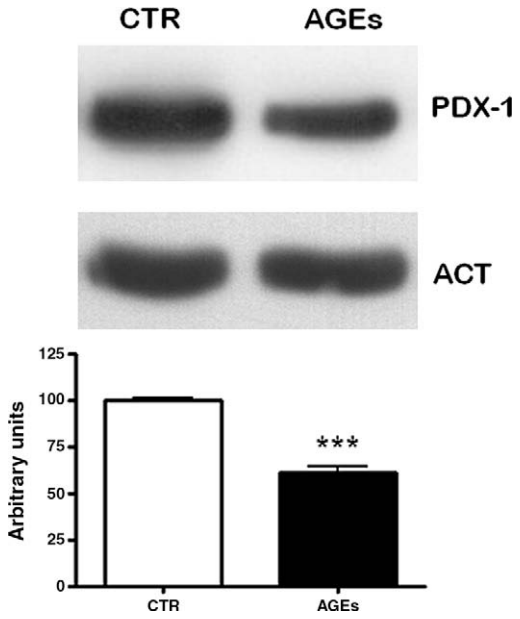

Fig. 2. Effects of AGEs on PDX-1 expression. Western blot analysis of PDX-1 in protein extracts from HIT-T15 cells cultured for 5 days in standard medium (CTR) or in medium containing GS (AGEs). Nitrocellulose membrane was probed with a specific antibody that recognizes PDX-1, then stripped and reprobed with an antibody against β-actin to check for equal loading. The relative ratio between PDX-1 and β-actin was calculated by densitometry. The bar graph depicts the averages of data obtained from 3 individual experiments (means ± SD; $^{***}p < 0.001$).

both in the cytoplasm and in the nucleus when cells were cultured with AGEs (Fig. 3, upper panel). Unlike PDX-1, when HIT-T15 is cultured in the presence of AGEs, FoxO1 accumulates both in the cytosolic and in the nuclear fractions (Fig. 3, middle panel). At the same time, we observed a decrease in the amount of phosphoFoxO1 in the cytosolic fractions of HIT-T15 cells that were cultured in the presence of AGEs (Fig. 3, lower panel).

Since acetylation protects FoxO1 from ubiquitin-mediated degradation [12], we investigated the acetylFoxO1 expression in protein extracts from HIT-T15 cells cultured in NGS or GS. Immunoblot analysis revealed that acetylFoxO1 increased considerably when cultured with AGEs (Fig. 4).

4. Discussion

In this study we demonstrated that exposure to AGEs impairs the expression and distribution of transcription factors involved in regulating insulin gene transcription.

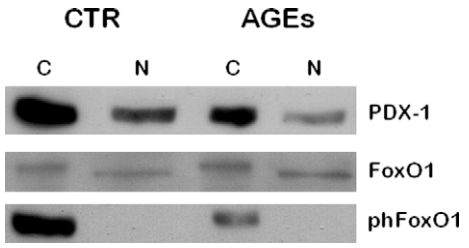

Fig. 3. Effects of AGEs on intracellular distribution of PDX-1, FoxO1 and phospho-FoxO1. Nuclear (N) and cytoplasmic (C) expression of PDX-1, FoxO1 and phospho-FoxO1 were evaluated by Western blot analysis using specific primary antibodies. The same membrane was excised at the level of the molecular weight representing 60 kDa and divided into two parts. The fragments were immunoblotted, respectively, for PDX-1 (40 kDa) and phFoxO1 (80 kDa). The fragment containing the higher molecular weights was then stripped and reprobed for FoxO1. The panel is representative of 3 individual experiments.

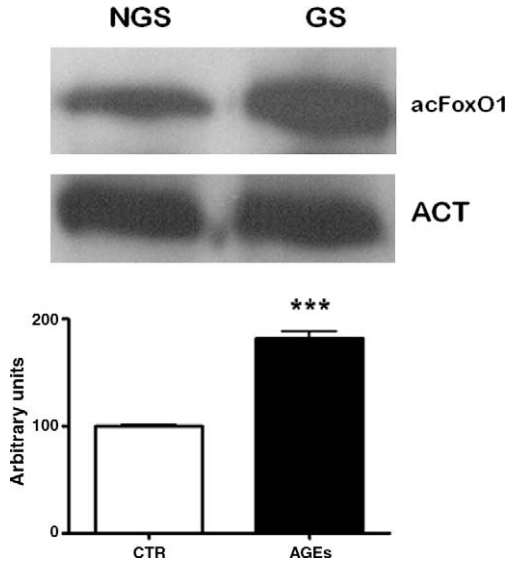

Fig. 4. Effects of AGEs on acetylFoxO1 expression. Western blot analysis of acetylFoxO1 in protein extracts from HIT-T15 cells cultured for 5 days in standard medium (CTR) or in medium containing GS (AGEs). Nitrocellulose membrane was probed with a specific antibody that recognizes acetylFoxO1, then stripped and reprobed with an antibody against β-actin to check for equal loading. The relative ratio between acetylFoxO1 and β-actin was calculated by densitometry. The bar graph depicts the averages of data obtained from 3 individual experiments (means ± SD; $^{***}p < 0.001$).

Transcriptional regulation of the insulin gene results not only from the binding of activators or repressors to the insulin promoter, but also from their relative nuclear concentrations [7]. In this context, the relative expression of PDX-1 and FoxO1 is especially important because PDX-1 is one of the most important positive regulators of insulin gene transcription [8], and because FoxO1 not only down-regulates PDX-1 expression [9], thereby decreasing its availability, but also because FoxO1 shows mutually exclusive nuclear localization with PDX-1 [13].

We previously reported that exposure to AGEs reduces the intracellular insulin content in the pancreatic β-cell line HIT-T15 [5]. Herein, we demonstrated that the reduced insulin content is associated with decreased PDX-1 expression and reduced availability of PDX-1 in the nuclear fraction.

Our results show that the reduction in PDX-1 expression that occurs in HIT-T15 cells cultured in the presence of AGEs is coupled with the increment of FoxO1. Moreover, we found that treatment with AGEs up-regulates acetylation of FoxO1. Since acetylation protects FoxO1 from ubiquitination, the AGEs-increased amount of FoxO1 may be due to greater protection against proteasomal degradation. Furthermore, since phosphorylation is important in determining the cytoplasmic localization of FoxO1 [10], the AGEs-induced nuclear translocation of FoxO1 is caused by the decreased FoxO1 phosphorylation. As expected, increased FoxO1 in the nucleus of HIT-T15 cells cultured in the presence of AGEs is coupled with reduced nuclear localization of PDX-1. Taken together, these findings suggest that AGEs reduce HIT-T15 insulin content decreasing phosphorylation of FoxO1 and thus allowing its nuclear translocation. At the same time, the increased AGEs-induced acetylation of FoxO1 leads to an increment of its nuclear content, thus resulting in a decrease in both the PDX-1 expression and in its nuclear localization. In summary, AGEs decrease the availability of transcription factors that positively regulate insulin gene transcription, thus favoring nuclear localization of the factors that have a negative effect on insulin gene transcription. The result of the imbalance of these transcription factors is a decrease in insulin gene transcription, and, consequently, in the insulin content of the β-cells.

Our hypothesis is that AGEs-induced pancreatic β-cell dysfunction shares common mechanisms of action with hyperglycemia. PDX-1 expression is reduced in animal models of diabetes [15]. Recently, Del Guerra reported that decreased insulin expression in type 2 diabetic islet cells is associated with increased FoxO1 expression [16]. Furthermore, chronic hyperglycemia results in oxidative stress that induces FoxO1 translocation to the nucleus in mouse β-cells [13]. Some speculations may be made concerning these analogies. In fact, in type 2 diabetes, the aim is to "cure" hyperglycemia, but often without taking AGEs into consideration. However, not all AGEs are removed when the euglycemic condition is restored, therefore, they might be considered the memory of hyperglycemia. In conclusion, we suggest that the use of AGEs formation inhibitors and/or of AGEs scavengers may be taken into consideration in order to fully remove sources of β-cell dysfunction.

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