



# Glucagon-like peptide-1 counteracts the detrimental effects of Advanced Glycation End-Products in the pancreatic beta cell line HIT-T 15

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## ABSTRACT

Advanced Glycation End-Products (AGEs), a group of compounds resulting from the non-enzymatic reaction of reducing sugars with the free amino group of proteins, are implicated in diabetic complications. We previously demonstrated that exposure of the pancreatic islet cell line HIT-T 15 to high concentrations of AGEs significantly decreases cell proliferation and insulin secretion, and affects transcription factors regulating insulin gene transcription. The glucagon-like peptide-1 (GLP-1) is an incretin hormone that increases proinsulin biosynthesis, stimulates insulin secretion, and improves pancreatic beta-cell viability. The aim of this work was to investigate the effects of GLP-1 on the function and viability of HIT-T 15 cells cultured with AGEs. HIT-T 15 cells were cultured for 5 days in presence of AGEs alone, or supplemented with 10 nmol/l GLP-1. Cell viability, insulin secretion, redox balance, and expression of the AGEs receptor (RAGE) were then determined. The results showed that GLP-1 protected beta cell against AGEs-induced cell death preventing both apoptosis and necrosis. Moreover, addition of GLP-1 to the AGEs culture medium restored the redox balance, improved the responsiveness to glucose, and attenuated AGEs-induced RAGE expression. These findings provide evidence that GLP-1 protects beta cells from the dangerous effects of AGEs.

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## 1. Introduction

Type 2 diabetes is characterised by the resistance of peripheral tissues to insulin and the qualitative and quantitative impairment of insulin secretion that is associated with a progressive loss of pancreatic beta cells. When insulin resistance or beta cell dysfunction exceeds the physiological rate, hyperglycaemia appears and progresses to overt diabetes [1]. Chronic hyperglycaemia leads to an acceleration in the formation of Advanced Glycation End-Products (AGEs), a heterogeneous group of compounds derived from the non-enzymatic reaction of reducing sugars with a free amino group of proteins [2]. AGEs accumulate in extracellular matrix proteins as a physiological process during aging [3,4], and become important pathogenic mediators of almost all diabetes complications [5]. Recently, we demonstrated the direct effect of AGEs on beta-cell viability and dysfunction showing that exposure of the pancreatic islet cell line HIT-T 15 to AGEs decreases cell viability, leads to a significant drop in insulin content and secretion, and affects the expression of transcription factors regulating insulin gene expression [6,7].

Glucagon-like peptide-1 (GLP-1) is an incretin hormone derived from the proglucagon gene and secreted by the intestinal L cell in

response to food ingestion [8]. GLP-1 is of great clinical interest for the management of type 2 diabetes since its anti diabetic actions are mediated at the level of the beta cells, as well as in the peripheral tissues [9]. GLP-1 regulates blood glucose levels both by stimulating glucose-dependent insulin secretion and by inhibiting glucagon secretion, gastric emptying and food intake [10]. Administering either GLP-1 or dipeptidyl peptidase-4 (the enzyme responsible for GLP-1 inactivation) inhibitors, reduces the levels of blood glucose, of HbA<sub>1c</sub> and of the free fatty acids, and improves insulin sensitivity. In the pancreatic beta cells, GLP-1 increases glucose-dependent insulin secretion, stimulates insulin gene transcription, proinsulin mRNA stability, and proinsulin biosynthesis. Furthermore, GLP-1 inhibits apoptosis of pancreatic beta cells by regulating the expression of pro- and anti-apoptotic factors [11–16].

Interestingly, Ishibashi et al. showed that GLP-1 reduced AGEs-induced RAGE (receptor for AGEs) expression in endothelial cells [17].

In this study we investigated the ability of GLP-1 to preserve the viability and function of the pancreatic beta cell line HIT-T 15 when exposed to an AGEs-rich environment.

## 2. Materials and methods

HIT-T 15, a pancreatic beta cell line, was purchased from American Type Culture Collection (Manassas, VA, USA). Insulin ELISA kits

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were provided by Pantec S.r.l. (Torino, Italy). GLP-1 (7–36 amide) was purchased from AnaSpec (Fremont, CA, USA). Antibodies: monoclonal antibody against RAGE was purchased from Chemicon International (Temecula, CA, USA); polyclonal antibody against  $\beta$ -actin was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Cell Titer 96 Aqueous One Solution Cell Proliferation Assay and Cytotox 96 Non-Radioactive Cytotoxicity Assay were from Promega (Milan, Italy). BCA Protein Assay Kit was from Pierce (Rockford, MD, USA). RPMI, PBS and FBS were from Cambrex Bio Science (Walkersville, MD). Nitrocellulose and ECL Plus were from GE Healthcare UK Ltd. (Buckinghamshire, England). Glutathione Assay Kit was from Cayman Chemical Company (Ann Arbor, MI, USA). All other reagents were from Sigma–Aldrich (Milan, Italy).

### 2.1. Preparation of AGEs

Glycated serum (GS) was prepared by adding 50 mmol/l ribose to heat-inactivated (56 °C for one h) FBS, as described previously [6]. Aliquots of FBS were processed the same way but without ribose (non-glycated serum, NGS) and used for standard medium preparation. Pentosidine content was evaluated as a measure of protein glycation, as previously described [18]. The concentration of pentosidine in the experimental media (RPMI containing 10% GS) ranged between 150 and 400 nmol/l, which corresponds to the levels that are observed in the plasma of diabetic patients.

### 2.2. Cell culture and experimental conditions

HIT-T 15 cells were grown in RPMI 1640 supplemented with 10% FBS, 2 mmol/l-glutamine, and antibiotics (100 U/ml penicillin G and 100  $\mu$ g/ml streptomycin sulphate) and maintained at 37 °C in a humidified condition of 95% air and 5% CO<sub>2</sub>. The medium was replaced every 2 days. Cells were grown to confluence, removed with trypsin–EDTA, and then seeded in multiwell plates for all experiments.

Prior to each experiment, confluent cells were washed twice with PBS and fresh medium was added. All the experiments were performed by culturing HIT-T 15 cells for 5 days in media containing either NGS or GS with or without 10 nmol/l GLP-1 (this concentration corresponds to the maximal effective concentration that is broadly used in the literature [19–21]).

### 2.3. Cell viability

In order to evaluate cell proliferation, HIT-T 15 cells were seeded on 96-well plates (3  $\times$  10<sup>4</sup> cells/well) and cultured for 5 days as described. Viable cells were determined using the Cell Titer 96 Aqueous One Solution Cell Proliferation Assay according to the manufacturer's instructions. To evaluate cell apoptosis and cell necrosis, HIT-T 15 cells were plated on 6-well dishes (7  $\times$  10<sup>5</sup> cells/well) for 5 days in media containing NGS or GS with or without 10 nmol/l GLP-1. They were then processed to measure both the activity of caspase-3 (a protease that plays a key role in the apoptosis of mammalian cells (CaspACE Assay System)) and the activity of lactate dehydrogenase (LDH) (a stable cytosolic enzyme that is a marker of cell membrane damage and cell death due to necrosis) using Cytotox 96 Non-Radioactive Cytotoxicity Assay, according to the manufacturer's instructions.

### 2.4. Intracellular Glutathione content

To evaluate the intracellular content of glutathione, HIT-T 15 cells were plated in 6-well dishes (7  $\times$  10<sup>5</sup> cells per well) and cultured for 5 days as described above. Quantification of glutathione was performed using Cayman's GSH Assay Kit according to the manufacturer's instructions. Briefly, the assay utilises an

enzymatic recycling method, using glutathione reductase to quantify glutathione: the sulfhydryl group of glutathione reacts with DTNB (5,5'-dithio-bis-2-(nitrobenzoic acid), Ellman's reagent) and produces a yellow coloured TNB (5-thio-2-nitrobenzoic acid). The mixed disulfide, GSTNB (between glutathione and TNB) that is concomitantly produced, is reduced by glutathione reductase to recycle the glutathione and produce more TNB. Therefore, the rate of TNB production is directly proportional to the concentration of glutathione in the sample. Glutathione is easily oxidized to the disulfide dimer GSSG that is produced during the reduction of hyperoxides by glutathione peroxidase. Since GSSG is reduced to glutathione by glutathione reductase, the assay measures both glutathione and GSSG. We then also quantified the intracellular content of GSSG by first derivatizing glutathione with 2-vinylpyridine and then proceeding with the glutathione assay as described above.

### 2.5. Insulin secretion

Insulin release in response to glucose was evaluated in static conditions. HIT-T 15 cells were plated in 6-well dishes (7  $\times$  10<sup>5</sup> cells per well) and incubated for 5 days with experimental media. Then, after 1 h of pre-incubation in Krebs' Ringer Bicarbonate buffer (118.5 mmol/l NaCl, 2.54 mmol/l CaCl<sub>2</sub>, 1.19 mmol/l KH<sub>2</sub>PO<sub>4</sub>, 4.75 mmol/l KCl, 25 mmol/l NaHCO<sub>3</sub>, 1.19 mmol/l MgSO<sub>4</sub>, 10 mmol/l HEPES, 0.1% BSA, pH 7.4), cells were challenged either with 2.8 or with 16.7 mmol/l glucose for 60 min. Media were collected and stored at –20 °C until the insulin determination was performed. Cells were washed twice with PBS, pH 7.4, lysed in RIPA buffer, and harvested for protein determination using the BCA Protein Assay Kit according to the manufacturer's instructions. Insulin secretion was measured by ELISA and normalised to total protein concentration.

### 2.6. Immunoblotting analysis

HIT-T 15 cells were lysed in RIPA buffer (50 mmol/l Tris–HCl pH 7.5, 150 mmol/l NaCl, 1% NP40, 0.1% SDS, supplemented with protease and phosphatase inhibitor cocktails) and protein concentrations were determined using the BCA Protein Assay Kit. Thirty micrograms of total cell lysate was separated on 10% SDS–PAGE and transferred onto nitrocellulose. Filters were blocked in 5% non-fat dried milk and incubated overnight at 4 °C with primary specific antibodies. Secondary specific horseradish-peroxidase linked antibodies were added for 1 h at room temperature. Bound antibodies were detected using the enhanced chemiluminescence lighting system (ECL Plus), according to the manufacturer's instructions. Each membrane was stripped (1% sodium dodecylsulphate, 0.5% 2-mercaptoethanol) and probed for  $\beta$ -actin to verify equal protein loading. Bands of interest were quantified by densitometry using the NIH program ImageJ.

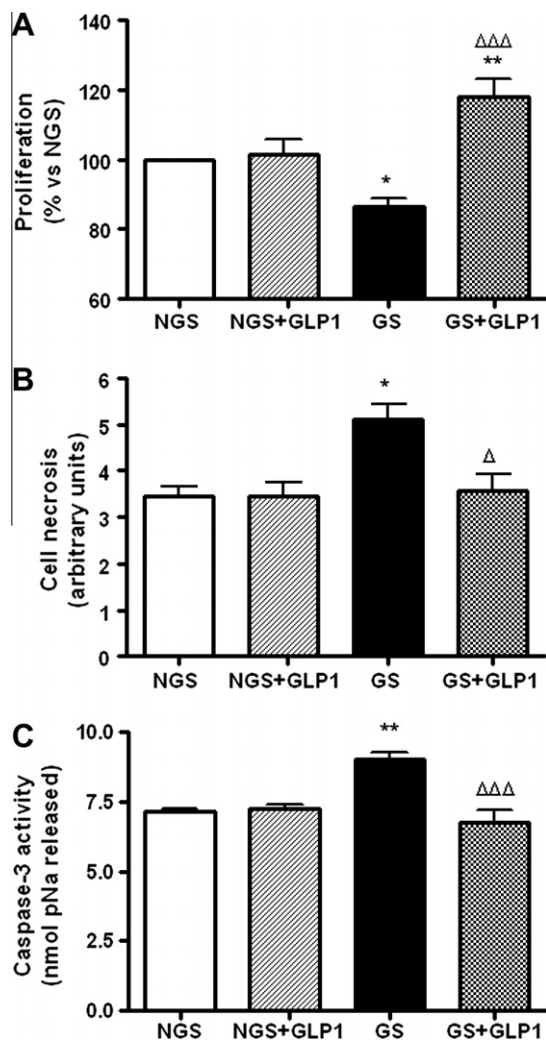
### 2.7. Statistical analyses

The results are representative of at least three experiments. All analyses were carried out with the GraphPad Prism 4.0 software (GraphPad Software, San Diego, CA, USA). Data were expressed as the mean  $\pm$  SEM and then analysed using one-way ANOVA followed by Tukey's Multiple Comparison Test. A *p* value < 0.05 was considered statistically significant.

## 3. Results

### 3.1. Cell viability

Adding GLP-1 to GS during the culture restored cell proliferation to the same rate as the control cells (Fig. 1A). The ability of GLP-1 to prevent AGEs-impaired viability was investigated by

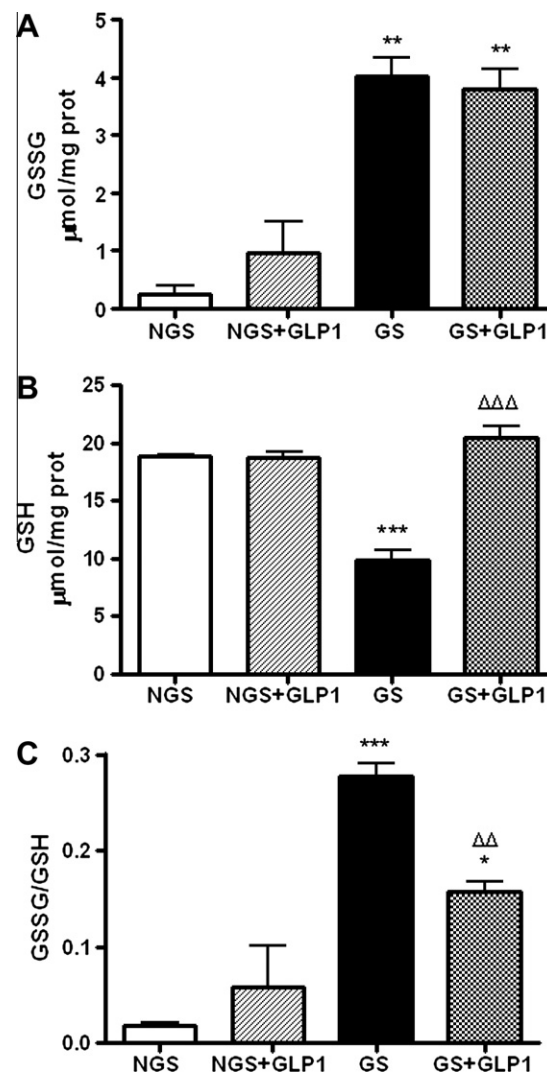


**Fig. 1.** Cell viability. HIT-T 15 cells cultured for 5 days in RPMI supplemented either with NGS or GS and 10 nmol/l GLP-1. Then (A) cell proliferation rate was determined by a colorimetric method based on the formazan product of the tetrazolium compound MTS. Values shown indicate the percentage of absorbance compared to NGS culture ( $n = 8$ , \* $p < 0.05$ , \*\* $p < 0.01$  vs NGS;  $\Delta\Delta\Delta p < 0.001$  compared to the same treatment without GLP-1). (B) Cell necrosis was measured by quantification of lactate dehydrogenase (LDH) released in culture supernatants by the cells; values represent the ratio between LDH release/cells in every condition ( $n = 3$ , \* $p < 0.05$  vs NGS;  $\Delta p < 0.05$  compared to the same treatment without GLP-1). (C) Cell extracts were tested for caspase 3 activity using its colorimetric substrate N-acetyl-Asp-Glu-Val-Asp p-nitroanilide (pNa); bars represent nmol pNa released in the medium by  $2 \times 10^6$  cells ( $n = 3$ , \*\* $p < 0.01$  vs NGS;  $\Delta\Delta\Delta p < 0.001$  compared to the same treatment without GLP-1). Values shown indicate the mean SEM of at least three independent experiments.

measuring LDH release, a marker of necrosis, and caspase-3 activation, a marker of apoptosis. Quantification of LDH showed that GS treatment significantly increased the number of necrotic cells. This effect was completely abrogated by adding GLP-1 to the GS culture medium (Fig. 1B). Addition of GLP-1 to the GS medium completely prevented any GS-induced increment in caspase-3 activation, thereby restoring caspase-3 activity to the same levels as the control cells (Fig. 1C).

### 3.2. Intracellular content of glutathione

As shown in Fig. 2, exposure of HIT-T 15 cells to GS induced a significant increment in the oxidized form of glutathione and a significant decrease in the reduced form (Fig. 2A and B). Adding GLP-1 to the GS culture medium did not affect GSSG content, but fully restored the amounts of GSH to the level of the control cells (Fig. 2A



**Fig. 2.** Evaluation of the oxidized (GSSG) (A) and the reduced (GSH) (B) forms of glutathione and of their ratio (C) in HIT-T 15 cells that were cultured for 5 days in RPMI supplemented either with NGS or GS and 10 nmol/l GLP-1 ( $n = 3$ , \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs NGS;  $\Delta\Delta\Delta p < 0.001$ ,  $\Delta\Delta p < 0.01$  compared to the same treatment without GLP-1).

and B). Consequently, adding GLP-1 attenuated the GSSG/GSH ratio in cells cultured with GS (Fig. 2C).

### 3.3. Insulin secretion

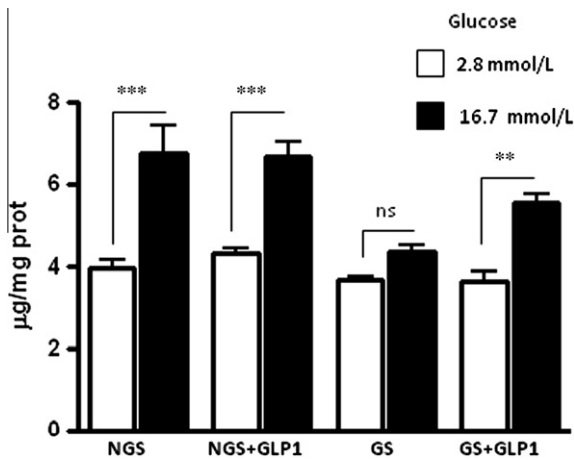
As previously demonstrated [6], GS negatively affects insulin secretion in HIT-T 15 cells. The presence of GLP-1 during the 5 days of culture with GS increased insulin secretion in response to the glucose stimulus as compared to the response from cells cultured with GS alone (Fig. 3).

### 3.4. RAGE expression

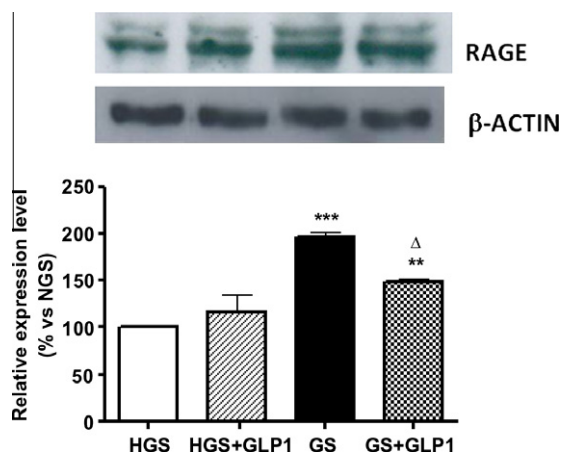
The intracellular effects of AGEs are mediated by RAGE [22]. RAGE expression increased significantly in HIT-T 15 cells that were cultured for 5 days with GS; adding GLP-1 to the GS culture medium counteracted the GS-induced RAGE expression (Fig. 4).

## 4. Discussion

In this study we show that GLP-1 was able to counteract AGEs-induced pancreatic beta cell death and dysfunction.



**Fig. 3.** Glucose-induced insulin secretion in HIT-T 15 cells cultured for 5 days in RPMI supplemented either with NGS or GS and 10 nmol/l GLP-1. HIT-T 15 cells were incubated for 1 h in the presence of 2.8 mM (□) or 16.7 mM (■) glucose concentrations after 1 h of pre-incubation in glucose-free buffer ( $n = 4$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ). The amount of insulin in the collected samples was determined by ELISA. Data are expressed as  $\mu\text{g}$  insulin released/mg protein and represent the mean SEM of at least three independent experiments.



**Fig. 4.** Western immunoblotting of RAGE in HIT-T 15 cells following 5 days of culture with NGS or GS and 10 nmol/l GLP-1. Cells were lysed and lysates were processed for immunoblot. Bands were visualised by an ECL detection system, intensity was quantified by densitometric analysis using NIH Image software and expressed as fold induction relative to  $\beta$ -actin. Data shown are representative of at least three experiments (mean  $\pm$  SEM) ( $n = 3$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  vs NGS;  $\Delta p < 0.05$  compared to the same treatment without GLP-1).

The decreased viability we observed in AGEs-exposed HIT-T 15 cells derives both from the increase in caspase-3 activity and from the increase in the rate of necrosis. GLP-1 is known to improve pancreatic beta cell survival by preventing apoptosis, and the ability of GLP-1 to prevent caspase-3 activation has been described in several previous studies [11,13,16]. Consistent with these data, AGEs-induced caspase-3 activation was prevented by culturing cells with GLP-1. Furthermore, in agreement with a previous observation by Li et al. [23] who found that exendin-4, a long acting GLP-1 receptor agonist, plays a protective role against cytokine-induced necrosis in beta cells, our results show that GLP-1 is able to counteract AGEs-induced pancreatic beta cell necrosis.

Generation of oxidative stress has a key role in AGEs-induced cellular dysfunction. It is well known that expression levels of antioxidant enzymes are low in pancreatic beta cells [24], thereby making these cells susceptible to oxidative stress. It is also known that over-expression of the antioxidant enzymes confers a

beneficial effect to islets exposed to reactive oxygen species [25]. Glutathione is an important factor in maintaining redox balance, and an increased ratio between the oxidized (GSSG) and the reduced (GSH) form is considered indicative of oxidative stress [26]. As expected our data showed that exposure to a glycated environment increases oxidative stress, as revealed by the levels of GSSG. Furthermore, we found that AGEs exert a direct effect on reducing the availability of GSH in HIT-T 15 cells, consequently leading to an increase in the GSSG-to-GSH ratio. Taken together, our results suggest that AGEs can impair intracellular redox imbalance both by increasing oxidative stress and by decreasing the expression levels of antioxidant defences. Since GLP-1 does not reduce the AGEs-induced GSSG increment, the possible mechanism involved in the protective effect of GLP-1 on the survival of HIT-T 15 cells exposed to AGEs is likely related to the increased GSH availability rather than to the decreased GSSG formation. This would suggest that GLP-1 is involved in regulating the GSH levels in beta cells. These results are comparable to those of Kimura et al. [27] in rat pheochromocytoma cells, suggesting that there may be a common mechanism of action of GLP-1 in maintaining redox balance. The interaction between AGEs and RAGE induces the intracellular release of reactive oxygen species, thus disrupting the cellular redox balance [22]. We found that GLP-1 decreased GS-induced RAGE expression, consequently this may attenuate the detrimental effects of AGEs in HIT-T 15 cells.

Besides improving beta cell survival, GLP-1 is also known to improve pancreatic beta cell function [13]. Here we provided evidence that GLP-1 was able to ameliorate insulin secretion also when pancreatic beta cells are exposed to AGEs. The observed improved glucose-induced insulin secretion when GLP-1 is added to the GS medium may be related to increased sensitivity to glucose. This interpretation is in agreement with evidence that one of the mechanisms through which GLP-1 increases glucose stimulated insulin secretion involves up-regulation of glucose-sensing elements [19].

In summary, we showed for the first time that GLP-1 counteracts the detrimental effects of AGEs on pancreatic beta-cell viability and function decreasing oxidative stress and attenuating RAGE expression. These results suggest that GLP-1 may be able to defend pancreatic beta cells from the dangerous memory of glucose (AGEs).

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