

Sitagliptin prevents the development of metabolic and hormonal disturbances, increased β -cell apoptosis and liver steatosis induced by a fructose-rich diet in normal rats

Bárbara MAIZTEGUI¹, María I. BORELLI¹, Viviana G. MADRID¹, Héctor DEL ZOTTO¹, María A. RASCHIA, Flavio FRANCINI, María L. MASSA, Luis E. FLORES, Oscar R. REBOLLEDO and Juan J. GAGLIARDINO
CENEXA – Centro de Endocrinología Experimental y Aplicada (UNLP-CONICET LA PLATA, Centro Colaborador OPS/OMS), Facultad de Ciencias Médicas UNLP, 60 y 120 (1900) La Plata, Argentina

A B S T R A C T

The aim of the present study was to test the effect of sitagliptin and exendin-4 upon metabolic alterations, β -cell mass decrease and hepatic steatosis induced by F (fructose) in rats. Normal adult male Wistar rats received a standard commercial diet without (C) or with 10% (w/v) F in the drinking water (F) for 3 weeks; animals from each group were randomly divided into three subgroups: untreated (C and F) and simultaneously receiving either sitagliptin (CS and FS; 115.2 mg/day per rat) or exendin-4 (CE and FE; 0.35 nmol/kg of body weight, intraperitoneally). Water and food intake, oral glucose tolerance, plasma glucose, triacylglycerol (triglyceride), insulin and fructosamine concentration, HOMA-IR [HOMA (homoeostasis model assessment) for insulin resistance], HOMA- β (HOMA for β -cell function) and liver triacylglycerol content were measured. Pancreas immunomorphometric analyses were also performed. IGT (impaired glucose tolerance), plasma triacylglycerol, fructosamine and insulin levels, HOMA-IR and HOMA- β indexes, and liver triacylglycerol content were significantly higher in F rats. Islet β -cell mass was significantly lower in these rats, due to an increase in the percentage of apoptosis. The administration of exendin-4 and sitagliptin to F animals prevented the development of all the metabolic disturbances and the changes in β -cell mass and fatty liver. Thus these compounds, useful in treating Type 2 diabetes, would also prevent/delay the progression of early metabolic and tissue markers of this disease.

INTRODUCTION

T2DM (Type 2 diabetes mellitus) represents a serious health problem worldwide due to its high and growing incidence and to the negative impact of its associated complications upon quality of life and healthcare costs [1]. To cope with this problem, two different approaches

have been implemented: the improvement of care quality and the development of new therapeutic tools, and the primary prevention of the disease in people at risk [2].

The development of incretin mimetic and incretin enhancer drugs represents a promising therapeutic tool to achieve treatment goals [3–5], considering that people with T2DM have a significant decrease of incretin

Key words: β -cell mass, exendin-4, hypertriglyceridaemia, insulin resistance, pre-diabetes, sitagliptin.

Abbreviations: AUC, area under the curve; DPP-4, dipeptidyl peptidase-4; F, fructose; GLP-1, glucagon-like peptide-1; HOMA, homoeostasis model assessment; HOMA- β , HOMA for β -cell function; HOMA-IR, HOMA for insulin resistance; IGT, impaired glucose tolerance; IR, insulin resistance; OGTT, oral glucose tolerance test; PCNA, proliferating cell nuclear antigen; T2DM, Type 2 diabetes mellitus; Vvi, volume density.

¹ These authors contributed equally to the study.

Correspondence: Dr Juan J. Gagliardino (email cenexa@speedy.com.ar).

effect [6]. GLP-1 (glucagon-like peptide-1), a prominent active compounds of the incretin family, modulates many processes in pancreatic islet: it potentiates insulin synthesis and secretion [5], inhibits glucagon secretion [7], increases islet cell proliferation, and decreases cell apoptosis [8,9]; further, exendin-4 (a GLP-1 receptor agonist), reverses the loss of islet mass grafts after transplantation [10]. The mechanisms whereby GLP-1 may exert these effects have been reviewed by Drucker and Nauck [3].

The pleiotropic effects of GLP-1 suggest that it could potentially slow down or prevent the continuous deterioration of β -cell mass and function characteristic of T2DM and it could potentially prevent its development in people at risk. While many reports support the usefulness of incretin mimetic and incretin enhancer drugs for the effective treatment of T2DM, there is not sufficient evidence of their effectiveness to prevent/delay its development.

To provide such evidence, it would be useful to have an animal model with clinical/metabolic characteristics similar to those of people at high risk for T2DM. In this regard, it has been proved that F (fructose) administration to normal rats for different time periods sequentially induces IGT (impaired glucose tolerance) and T2DM [11–16]. These rats also have fatty liver and progressively decreased β -cell mass and function due to an increase of β -cell apoptosis, thus resembling the changes described in people with T2DM [17,18].

In an attempt to elucidate this issue, we currently studied the effect of sitagliptin, a DPP-4 (dipeptidyl peptidase-4) inhibitor, and exendin-4 upon the metabolic, β -cell mass and hepatic changes induced by F administration to normal rats for 21 days. Results demonstrate that the simultaneous administration of sitagliptin or exendin-4 with F prevented the development of all the recorded metabolic disturbances induced by this diet and their negative impact upon β -cell mass.

MATERIALS AND METHODS

Chemicals and drugs

Collagenase was obtained from Serva Feinbiochemica; BSA fraction V and other reagents were from Sigma. F was obtained from Corn Products International. Exendin-4 was obtained from Bachem, and sitagliptin was provided by Process Research, Merck Research Laboratories.

Experimental groups

Normal male Wistar rats (180–200 g) were divided into two groups: a control group that was fed with a standard commercial diet (C), and another group that received the same diet plus 10% (w/v) F in the drinking water for 3 weeks. Animals from C and F were randomly divided

into three subgroups ($n = 20$ each group): untreated (C and F), treated with sitagliptin (CS and FS), or treated with exendin-4 (CE and FE). All the animals were housed in a room with controlled temperature (25°C) and lighting (12 h light/dark cycle). CE and FE rats were injected with exendin-4 (0.35 nmol/kg of body weight, intraperitoneally) twice a day, while sitagliptin was administered orally (115.2 mg/day per rat) premixed with the milled pellet at 0.6% (w/w). Water and food intake were measured daily, while individual body weight was recorded once a week.

Animal experiments and handling were performed according to the “Ethical principles and guidelines for experimental animals” of the Swiss Academy of Medical Sciences (3rd Edition 2005).

OGTT (oral glucose tolerance test)

OGTT was performed in 12-h fasted rats from each experimental group 24 h prior to sacrifice. Glucose (1 g/kg of body weight in saline solution) was given through a gavage tube placed into the stomach and blood samples were obtained from the retro-orbital plexus under light pentobarbital anaesthesia (48 mg/kg of body weight) at 0, 30, 60 and 120 min following the glucose load. In these samples, glucose concentration was measured with test strips (One touch Ultra; Lifescan) and plasma insulin by RIA using a specific antibody against rat insulin, rat insulin standard (Linco Research) and highly purified porcine insulin labelled with ^{125}I [19]. Results for glucose were expressed as the AUC (area under the curve) in $\text{mmol} \cdot \text{l}^{-1} \cdot \text{min}^{-1}$. HOMA-IR [HOMA (homoeostasis model assessment) for insulin resistance] was calculated according to the formula: $\text{insulin } (\mu\text{-units/ml}) \times \text{glucose (mmol/l)} / 22.5$; the formula for HOMA- β (HOMA for β -cell function) was: $\text{insulin } (\mu\text{-units/ml}) \times 20 / \text{glucose (mmol/l)} - 3.5$ [20]. Values correspond to fasting serum glucose and insulin levels. Fasting plasma triacylglycerol (triglyceride) and fructosamine levels were measured using commercial kits (BioSystems) implemented in an automated clinical analyser.

Liver triacylglycerol content

Liver triacylglycerol extraction was performed following the Schwartz and Wolins protocol [21]; concentration was measured with a commercial enzymatic assay kit (TG Color GPO/PAP AA, Wiener Laboratory, Rosario, Argentina).

Immunohistochemical studies

The whole pancreas from three animals of each experimental group was carefully dissected and removed and its wet weight was recorded; thereafter, a piece of the tail of each pancreas was obtained, fixed in 10% formaldehyde and embedded in paraffin. Serial sections of each one of the fixed pancreases (5 μm)

were obtained from three different depths of the blocks with a rotatory microtome and mounted on silanized slides (3-amino-propyltriethoxy-silane; Sigma). Sections were deparaffinized, incubated for 30 min in 3% (v/v) hydrogen peroxide in methanol to block the endogenous peroxidase activity and rehydrated in a descending ethanol series, followed by incubation in 2.5% porcine serum to reduce non-specific binding. The slides were then incubated for 24 h at 4°C in a humidified chamber with our own anti-guinea pig insulin antibody (1:20 000 dilution) [16]. The final staining was performed by incubating the slides for 30 min with appropriately diluted streptavidin–biotin complex (1:40 and 1:20 dilution respectively; Sigma); thereafter, the sections were stained with haematoxylin.

Cell replication

Sequential double-staining of sections was performed using these combinations: (i) PCNA (proliferating cell nuclear antigen) antibody (1:4000 dilution; Sigma) [22], and (ii) β -cells (our own guinea pig-insulin antibody; 1:20 000 dilution). First, we performed PCNA staining as described above using carbazole as chromogen; the same section was then immunostained for β -cells, except that alkaline phosphatase and Fast Blue (Sigma) were used as chromogens. Alkaline phosphatase conjugate was applied to each section for 30 min at room temperature (25°C); the sections were then washed and alkaline phosphatase was applied for a further 30 min. Within a given cell type, the replication rate was quantified and expressed as the percentage of PCNA-labelled cells among the total β -cells counted (no less than 3000 cells of each type). The immunohistochemical staining has been previously validated [23]. For every immunostaining performed, simple controls were done by omitting the primary antibody.

β -Cell apoptosis

The propidium iodide technique was used to identify apoptotic bodies [24]. For this purpose, deparaffinized–rehydrated pancreas sections were incubated for 30 min in a dark humidified chamber with a propidium iodide (4 μ g/ml; Sigma) and RNase A (100 μ g/ml; Sigma) solution. Then, the sections pretreated with guinea pig non-immune sera diluted in Tris-buffered saline (pH 7.4) were incubated for 1 h with the insulin antibody. To measure the fluorescence labelling of the primary antibody, the slides were incubated at room temperature for 45 min in the dark chamber with the IgG-specific, fluorescein-conjugated, affinity-purified guinea pig antibody (against heavy and light IgG chains; Santa Cruz Biotechnology). The sections were then washed with PBS and mounted in Tris-glycerol (pH 8.4) for analysis with an immunofluorescent Zeiss Axiolab epifluorescence microscope equipped with an HBO50 mercury lamp and two different

filters. For the quantitative assessment of apoptosis, positively immunofluorescence-labelled β -cells were counted under a $\times 40$ objective lens in sections obtained from different levels of the paraffin blocks. The number of apoptotic β -cells was expressed as the percentage of the total number of β -cells counted.

Morphometric analysis

Morphometric analysis was performed by videomicroscopy using a Jenamed 2 Carl Zeiss light microscope and a RGB CCD Sony camera, together with the OPTIMAS software (Bioscan). The following parameters were measured: total pancreatic area (excluding connective tissue), insulin-positive ductal cell and islet β -cell area [Vvi (volume density)]. To estimate islet and ductal cell mass, we multiplied the respective Vvi by the weight of the total pancreas [25].

The tissue sections were examined by two blinded independent observers who were oblivious to which study group the section came from. In case the inter-observer measurement of any variable in any sample was higher than 10%, they independently re-evaluated the section and rescored the variable.

Statistical analysis

The experimental data were analysed using variance analysis (one-way ANOVA) and post-hoc Bonferroni and Tamhane tests for multiple comparisons. Since insulin levels and the HOMA-IR and HOMA- β indexes did not have a normal distribution, the natural logarithm of the values was used to perform the analysis. Differences were considered significant when $P < 0.05$.

RESULTS

Body weight, food consumption and water intake

Comparable body weight and body increase were recorded in C and F animals after the 3-week treatment period (Table 1). Exendin-4 but not sitagliptin administration reduced significantly body weight gain in both C and F rats.

F animals drank a larger volume of water than C ones, while the ingested amount of solid food was significantly larger in the latter group (Table 1). This fact resulted in a different percentage of daily intake of nutrients by F compared with C rats (carbohydrates/proteins/lipids, 59:32:9 compared with 45:43:12 respectively), with a comparable caloric intake (54.1 ± 0.70 compared with 61.4 ± 3.22 kcal/day respectively). Neither exendin-4 nor sitagliptin affected the calorie intake in C and F animals (CE: 56.8 ± 7.2 ; CS: 52.5 ± 2.5 ; FE: 48.9 ± 0.8 ; FS: 52.9 ± 1.8 kcal/day).

Table 1 Changes in body weight, food consumption and water intake in the experimental groupsValues are means $\pm$ S.E.M. ($n = 20$). * $P < 0.01$ compared with F; † $P < 0.01$ compared with C.

Group	Body weight (g)			Food consumption (g)	Water intake (ml)
	Initial	Final	Δ g		
C	206 $\pm$ 6.7	278 $\pm$ 7.9	72 $\pm$ 4.0	21.3 $\pm$ 1.1	25.9 $\pm$ 1.2
CE	207 $\pm$ 2.9	255 $\pm$ 4.1	47 $\pm$ 6.0†	19.6 $\pm$ 2.5	28 $\pm$ 3.0
CS	217 $\pm$ 5.2	300 $\pm$ 5.6	83 $\pm$ 4.9	21.6 $\pm$ 0.8	28 $\pm$ 1.8
F	208 $\pm$ 8.6	278 $\pm$ 10.7	70 $\pm$ 4.2	15.1 $\pm$ 0.1†	49.8 $\pm$ 5.9†
FE	202 $\pm$ 4.3	251 $\pm$ 3.3	49 $\pm$ 2.1*	14.3 $\pm$ 0.3	38.6 $\pm$ 3.4
FS	211 $\pm$ 2.3	281 $\pm$ 2.5	70 $\pm$ 1.4	15.1 $\pm$ 0.6	45.6 $\pm$ 8.8

Table 2 Blood glucose, triacylglycerol, insulin and fructosamine levels, and AUC during the OGTT in the experimental groupsValues are means $\pm$ S.E.M. ($n = 20$). * $P < 0.03$, ** $P < 0.01$, *** $P < 0.003$, **** $P < 0.001$ and ***** $P < 0.0001$; † $P < 0.02$, †† $P < 0.01$ and ††† $P < 0.0001$ compared with C.

Group	Glucose (mmol/l)	Triacylglycerol (mmol/l)	Insulin (ng/ml)	Fructosamine (μ mol/l)	AUC (mmol $\cdot$ l ⁻¹ $\cdot$ min ⁻¹)
C	4.48 $\pm$ 0.12	0.47 $\pm$ 0.01	0.30 $\pm$ 0.02	175.1 $\pm$ 8.5	1.51 $\pm$ 0.08
CE	4.17 $\pm$ 0.09	0.48 $\pm$ 0.06	0.25 $\pm$ 0.03	150.6 $\pm$ 6.3	1.49 $\pm$ 0.14
CS	4.45 $\pm$ 0.16	0.63 $\pm$ 0.09	0.27 $\pm$ 0.03	190.7 $\pm$ 10	1.35 $\pm$ 0.17
F	4.94 $\pm$ 0.22	1.14 $\pm$ 0.12†††	1.09 $\pm$ 0.28†	218.6 $\pm$ 8.2††	3.05 $\pm$ 0.23†††
FE	4.19 $\pm$ 0.18	0.72 $\pm$ 0.09***	0.30 $\pm$ 0.05*	154.6 $\pm$ 8.1*****	1.37 $\pm$ 0.15*****
FS	4.68 $\pm$ 0.27	0.67 $\pm$ 0.05*****	0.31 $\pm$ 0.05*	172.5 $\pm$ 6.8**	1.54 $\pm$ 0.07*****

OGTT

The AUC during the OGTT was significantly higher in F as compared with C animals (Table 2). These values decreased significantly to almost control levels in F rats treated with exendin-4 or sitagliptin.

Blood glucose, triacylglycerol, insulin and fructosamine levels

While blood glucose levels were comparable in all the groups, triacylglycerol, fructosamine and insulin levels were significantly higher in F compared with C rats (Table 2).

The HOMA-IR and HOMA- β indexes were also significantly higher in F animals, indicating the presence of an IR (insulin resistance) state and a β -cell attempt to cope with the increased insulin demand in these rats (Figures 1A and 1B). All these high levels decreased to almost control values in F rats treated with either exendin-4 or sitagliptin. Neither exendin-4 nor sitagliptin affected those parameters in C animals (Table 2 and Figures 1A and 1B).

Liver triacylglycerol content

F rats had higher liver triacylglycerol content than C animals (476.4 $\pm$ 29.9 compared with 186.8 $\pm$ 11.8 μ g/100 mg of tissue respectively; $P < 0.001$). While neither exendin-4 nor sitagliptin affected hepatic triacylglycerol content in C animals, both drugs decreased it significantly

in F rats (FE: 374.7 $\pm$ 29.9 μ g/100 mg of tissue, $P < 0.05$; FS: 299.8 $\pm$ 16.7 μ g/100 mg of tissue; $P < 0.001$).

Morphometric analysis and β -cell apoptosis

Pancreas sections from C, F, FE and FS rats are shown in Figures 2(A)–2(D) respectively. It can be seen that β -cell mass markedly decreased in F rats, but this mass recovered a normal profile after exendin-4 and sitagliptin administration.

The morphometric analysis demonstrates that, in fact, islet β -cell mass was significantly lower (37%) in F rats (Table 3). Exendin-4 and sitagliptin did not markedly affect this parameter in C animals; however, both compounds increased significantly islet β -cell mass in F rats (201% and 68% respectively), attaining values close to those measured in C rats.

Insulin-positive duct-cell mass also increased in F rats, suggesting a possible neogenesis reaction, but it was not affected by exendin-4 or sitagliptin administration.

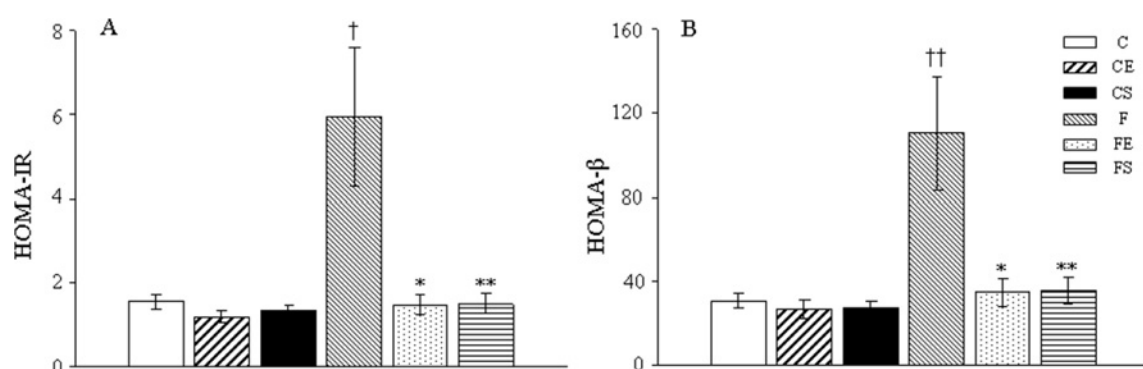
Although no significant differences were recorded in PCNA labelling among groups, higher values were recorded in F rats.

The β -cell apoptotic percentage was significantly higher (167%) in F rats (Figures 2E and 2F, and Table 3). Such increased percentage of apoptosis fell to almost control values after exendin-4 or sitagliptin

Table 3 Morphometric analysis and β -cell apoptosis in the experimental groups

Values are means $\pm$ S.E.M. from three different levels of the block corresponding to three animals from each group. * $P < 0.01$ and ** $P < 0.001$ compared with F; † $P < 0.01$ and †† $P < 0.001$ compared with C. ND, not detectable.

Group	Islet β -cell mass (mg)	Insulin-positive duct cell mass (mg)	β -Cell replication (% PCNA)	Apoptotic β -cell (%)
C	5.00 $\pm$ 0.45	ND	1.81 $\pm$ 1.31	0.18 $\pm$ 0.02
CE	6.59 $\pm$ 1.32	ND	2.29 $\pm$ 0.7	0.19 $\pm$ 0.04
CS	4.54 $\pm$ 0.5	ND	2.79 $\pm$ 0.76	0.14 $\pm$ 0.02
F	3.17 $\pm$ 0.37†	0.02 $\pm$ 0.007	2.43 $\pm$ 0.95	0.48 $\pm$ 0.02††
FE	9.54 $\pm$ 1.8*	0.02 $\pm$ 0.005	1.65 $\pm$ 0.77	0.14 $\pm$ 0.03**
FS	5.32 $\pm$ 0.7*	0.02 $\pm$ 0.008	1.16 $\pm$ 0.27	0.18 $\pm$ 0.02**

**Figure 1 HOMA-IR (A) and HOMA- β (B) indexes in the experimental groups**

Values are means $\pm$ S.E.M. ($n = 20$ in each case). † $P < 0.05$ and †† $P < 0.01$ compared with C; * $P < 0.05$ and ** $P < 0.02$ compared with F.

administration (71 % and 63 % decrease respectively; Figures 2G and 2H, and Table 3).

DISCUSSION

As previously shown, F administration to normal rats for 21 days induces hypertriglyceridaemia, IR (hyperinsulinaemia, high insulin/glucose molar ratio and increased HOMA-IR), IGT, increased protein glycosylation (high serum fructosamine levels) and a significant 37 % decrease in β -cell mass; the latter could be mainly ascribed to an increased percentage of apoptosis (167 %) [16] that was not compensated for by a non-significant increase in β -cell replication percentage and an apparent neogenesis reaction (increased percentage of insulin-positive duct cells). Two other organs were also markedly affected in these rats: (i) the mass of abdominal adipose tissue increased significantly, showing a higher proportion of saturated fatty acid composition together with a higher release of these fatty acids, leptin and adiponectin, and significant changes in glycoxidative stress markers [15,26], and (ii) the liver showed an abnormal glucose metabolism shifted towards lipid synthesis and deposit [27]. Altogether, these changes resemble those described in human pre-diabetes, T2DM and the metabolic syndrome [28]. We have postulated

that the underlying mechanism of all these changes was the development of a glycoxidative stress that probably starts in abdominal adipose tissue and in the liver [15,26].

In the current study, exendin-4 injection to F rats maintained the triacylglycerol, fructosamine and insulin plasma levels within the range of values recorded in C rats. The HOMA-IR and HOMA- β indexes also remained within normal range, indicating that IR was not developed in these animals and thus preventing the consequent β -cell function overload. β -Cell apoptosis percentage and mass values also lay within the range of those measured in C rats. These changes were associated with the normalization of IGT and liver triacylglycerol content recorded in F rats.

Similar and even more pronounced changes were observed when F rats were treated with sitagliptin. Conversely, administration of either exendin-4 or sitagliptin to C rats did not modify significantly any of the above mentioned parameters.

The beneficial effects of GLP-1 receptor agonists and DPP-4 inhibitors upon hyperglycaemia and β -cell mass present in different models of T2DM have been largely and consistently documented [8,29–36]. Conversely, the available evidence of their effect upon IGT is scarce and not conclusive, and under such metabolic condition the incretin effect upon β -cell mass depends apparently on the experimental

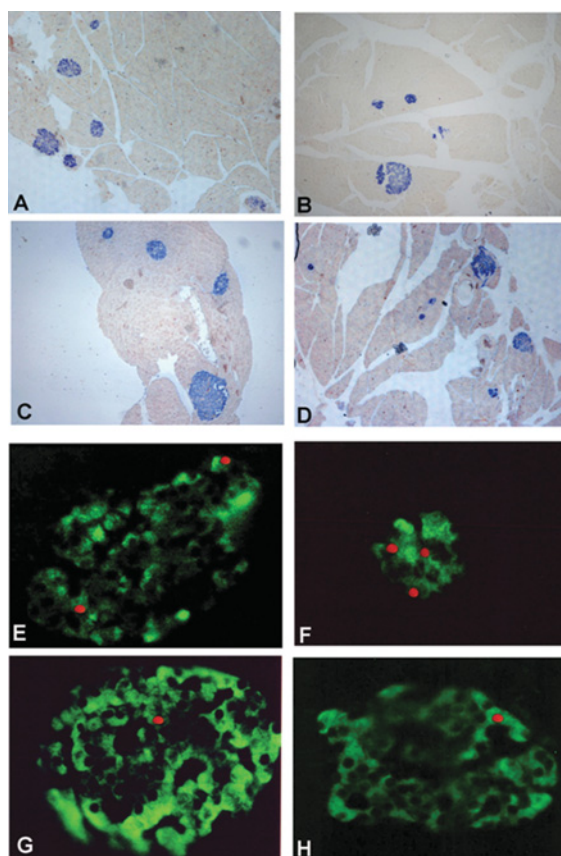


Figure 2 Representative sections of the pancreas from the experimental groups showing β -cell mass (A–D) and β -cell apoptosis (E–H)

(A–D) Pancreas sections from C (A), F (B), FE (C) and FS (D) rats. The β -cell mass appears markedly decreased in F as compared with C, while exendin-4 and sitagliptin gave β -cell mass a profile comparable with that observed in C rats. Magnification, $\times 10$. (E–H) β -cell apoptosis in C (E), F (F), FE (G) and FS (H). It greatly increased in F compared with C. After exendin-4 and sitagliptin administration, the profile of this process was comparable with that observed in C rats. Magnification, $\times 40$.

model used. Winzell and Ahrén, using mice with IGT induced by administration of a high fat diet, improved such impairment by increasing the serum insulin levels [37]; they did not mention however, any effect upon β -cell mass. Alogliptin (another DPP-4 inhibitor) administered alone to prediabetic *db/db* mice produced neither robust hyperglycaemia prevention nor sufficient β -cell preservation, but, associated with voglibose (an α -glucosidase inhibitor), it improved diabetic indexes and prevented diabetes development [38]. Although these authors did not perform quantitative analysis, they mentioned that combined treatment preserved pancreatic β -cells and islet structure [38]. In this study, the combined treatment did not affect significantly plasma triacylglycerol or total cholesterol levels. On the other hand, administration of P32/98 (a DPP-4 inhibitor) for 3 weeks to fatty Zucker rats

with manifest IGT improved their day–night blood glucose profile, reduced significantly their triacylglycerol and non-esterified fatty acids levels, and increased the glucose-induced insulin release, without affecting β -cell Vvi [39]. Xu et al. also showed that exendin-4 administration improved IGT developed in rats with 90–95% pancreatectomy and increased β -cell mass due to an increase in their percentage of replication/neogenesis; however, they did not measure β -cell apoptosis [29]. Conversely, other authors showed no significant changes in β -cell area or islet morphology [40–42].

In our study, performed in an animal model that portrays characteristics similar to those recorded in human pre-diabetes, exendin-4 and sitagliptin administration induced similar and significant beneficial effects upon the multiple F-induced metabolic disturbances and decreased β -cell mass. The latter effect was due to a significant decrease in β -cell apoptosis. These two agents also decreased liver steatosis.

We did not currently study the molecular mechanism by which sitagliptin decreased F-induced β -cell apoptosis. In this regard, Maida et al. [43] have recently reported that sitagliptin administration for 60 days to streptozotocin diabetic mice significantly reduced plasma DPP-4 activity and increased the percentage of β -cell area. The latter increase was associated with significantly lower levels of cleaved caspase-3 immunopositivity within the islets. These authors also reported that sitagliptin increased the pancreatic mRNA levels of IGF-1 and Akt-1, two genes involved in the control of β -cell survival [44]. We could thus assume that in our F model, sitagliptin exerted its beneficial effects upon β -cell apoptosis and mass using similar intracellular pathways.

We have previously shown that F animals portray increased markers of glycoxidative stress in abdominal adipose tissue and in the liver [15,26]. Based on the unifying hypothesis proposed by Brownlee [45], we could assume that such stress is the underlying common cause of IR and of the increased rate of non-enzymatic protein glycosylation observed in our F rats. Complementarily, the F load would shift liver carbohydrate metabolism towards fat deposition and triacylglycerol release, thus promoting fatty liver, hypertriglyceridaemia and IR. The combination of glycoxidative stress, lipotoxicity (high plasma triacylglycerol levels) and increased demand of insulin (IR) would stimulate β -cell apoptosis [46], with the consequent decrease in its functional mass; IGT would be the consequence of such β -cell dysfunction. The beneficial effect of both exendin-4 and sitagliptin upon the overall metabolic disturbances and β -cell dysfunction strongly suggests that both compounds can successfully counteract the mentioned pathogenic events. We cannot at the moment define, however, the possible specific metabolic target/s on which these compounds work.

In brief, our data show for the first time that administration of exendin-4 and sitagliptin prevents, to a

similar extent, F-induced hypertriglyceridaemia, IR and IGT; these two drugs also prevent the decrease in β -cell mass through a main decrease in their apoptosis. Since these results were obtained in an animal model with metabolic as well as islet and liver characteristics that resemble those observed in human pre-diabetes, exendin-4 and sitagliptin could be useful therapeutic tools to prevent the progression of this metabolic state towards T2DM.

AUTHOR CONTRIBUTION

Bárbara Maiztegui, María Borelli, Héctor Del Zotto and Juan Gagliardino conceived and designed the study, performed the statistical analyses and drafted the manuscript. Bárbara Maiztegui, María Borelli and Héctor Del Zotto carried out the oral glucose tolerance experiments and metabolic determinations. Viviana Madrid and Héctor Del Zotto performed the immunohistochemical studies and morphometric analyses. María Massa and Flavio Francini carried out the liver triacylglycerol content determinations. Moría Massa, Luis Flores and Oscar Rebolledo handled the experimental animals and carried out the biochemical determinations. All authors read and approved the final manuscript.

ACKNOWLEDGEMENTS

We thank Adrián Díaz for animal handling and insulin assays, César Bianchi for technical assistance, Jorge Elgart for his contribution in statistical analysis, and Adriana Di Maggio for careful manuscript editing prior to submission.

FUNDING

This study was partially supported by an unrestricted grant provided by Merck, Sharp & Dohme, Argentina and the National Research Council [grant number R1550/07].

REFERENCES

- 1 Diabetes Atlas, 4th edn (2009) International Diabetes Federation. www.eatlas.idf.org
- 2 Gillies, C. L., Abrams, K. R., Lambert, P. C., Cooper, N. J., Sutton, A. J., Hsu, R. T. and Khunti, K. (2007) Pharmacological and lifestyle interventions to prevent or delay type 2 diabetes in people with impaired glucose tolerance: systematic review and meta-analysis. *BMJ* **334**, 299
- 3 Drucker, D. J. and Nauck, M. A. (2006) The incretin system: glucagon-like peptide-1 receptor agonists and dipeptidyl peptidase-4 inhibitors in type 2 diabetes. *Lancet* **368**, 1696–1705
- 4 Ahrén, B. (2007) Dipeptidyl peptidase-4 inhibitors: clinical data and clinical implications. *Diabetes Care* **30**, 1344–1350
- 5 Holst, J. J., Vilsbøll, T. and Deacon, C. F. (2009) The incretin system and its role in type 2 diabetes mellitus. *Mol. Cell. Endocrinol.* **297**, 127–136
- 6 Knøp, F. K., Vilsbøll, T., Højberg, P. V., Larsen, S., Madsbad, S., Vølund, A., Holst, J. J. and Krarup, T. (2007) Reduced incretin effect in type 2 diabetes: cause or consequence of the diabetic state? *Diabetes* **56**, 1951–1959
- 7 Toft-Nielsen, M. B., Damholt, M. B., Madsbad, S., Hilsted, L. M., Hughes, T. E., Michelsen, B. K. and Holst, J. J. (2001) Determinants of the impaired secretion of glucagon-like peptide-1 in type 2 diabetic patients. *J. Clin. Endocrinol. Metab.* **86**, 3717–3723
- 8 Farilla, L., Hui, H., Bertolotto, C., Kang, E., Bulotta, A., Di Mario, U. and Perfetti, R. (2002) Glucagon-like peptide-1 promotes islet cell growth and inhibits apoptosis in Zucker diabetic rats. *Endocrinology* **143**, 4397–4408
- 9 Li, Y., Hansotia, T., Yusta, B., Ris, F., Halban, P. A. and Drucker, D. J. (2003) Glucagon-like peptide-1 receptor signaling modulates β cell cell apoptosis. *J. Biol. Chem.* **278**, 471–478
- 10 Toyoda, K., Okitsu, T., Yamane, S., Uonaga, T., Liu, X., Harada, N., Uemoto, S., Seino, Y. and Inagaki, N. (2008) GLP-1 receptor signaling protects pancreatic β cells in intraportal islet transplant by inhibiting apoptosis. *Biochem. Biophys. Res. Commun.* **367**, 793–798
- 11 Thorburn, A. W., Storlien, L. H., Jenkins, A. B., Khouri, S. and Kraegen, E. W. (1989) Fructose-induced *in vivo* insulin resistance and elevated plasma triglyceride levels in rats. *Am. J. Clin. Nutr.* **49**, 1155–1163
- 12 Gutman, R. A., Basilico, M. Z., Bernal, C. A., Chicco, A. and Lombardo, Y. B. (1987) Long-term hypertriglyceridemia and glucose intolerance in rats fed chronically an isocaloric sucrose-rich diet. *Metab. Clin. Exp.* **36**, 1013–1020
- 13 Catena, C., Giacchetti, G., Novello, M., Colussi, G., Cavarape, A. and Sechi, L. A. (2003) Cellular mechanisms of insulin resistance in rats with fructose-induced hypertension. *Am. J. Hypertens.* **16**, 973–978
- 14 Delbosc, S., Paizanis, E., Magous, R., Araiz, C., Dimo, T., Cristol, J. P., Cros, G. and Azay, J. (2005) Involvement of oxidative stress and NADPH oxidase activation in the development of cardiovascular complications in a model of insulin resistance, the fructose-fed rat. *Atherosclerosis* **179**, 43–49
- 15 Rebolledo, O. R., Marra, C. A., Raschia, A., Rodriguez, S. and Gagliardino, J. J. (2008) Abdominal adipose tissue: early metabolic dysfunction associated to insulin resistance and oxidative stress induced by an unbalanced diet. *Horm. Metab. Res.* **40**, 794–800
- 16 Maiztegui, B., Borelli, M. I., Raschia, M. A., Del Zotto, H. and Gagliardino, J. J. (2009) Islet adaptive changes to fructose-induced insulin resistance: β -cell mass, glucokinase, glucose metabolism, and insulin secretion. *J. Endocrinol.* **200**, 139–149
- 17 Butler, A. E., Janson, J., Bonner-Weir, S., Ritzel, R., Rizza, R. A. and Butler, P. C. (2003) β -cell deficit and increased β -cell apoptosis in humans with type 2 diabetes. *Diabetes* **52**, 102–110
- 18 Kahn, S. E., Zraika, S., Utzschneider, K. M. and Hull, R. L. (2009) The β cell lesion in type 2 diabetes: there has to be a primary functional abnormality. *Diabetologia* **52**, 1003–1012
- 19 Linde, S., Hansen, B. and Lernmark, A. (1980) Stable iodinated polypeptide hormones prepared by polyacrylamide gel electrophoresis. *Anal. Biochem.* **107**, 165–176
- 20 Matthews, D. R., Hosker, J. P., Rudenski, A. S., Naylor, B. A., Treacher, D. F. and Turner, R. C. (1985) Homeostasis model assessment: insulin resistance and β -cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* **28**, 412–419
- 21 Schwartz, D. M. and Wolins, N. E. (2007) A simple and rapid method to assay triacylglycerol in cells and tissues. *J. Lipid Res.* **48**, 2514–2520

- 22 Connolly, K. M. and Bogdanffy, M. S. (1993) Evaluation of proliferating cell nuclear antigen (PCNA) as an endogenous marker of cell proliferation in rat liver: a dual-stain comparison with 5-bromo-2'-deoxyuridine. *J. Histochem. Cytochem.* **41**, 1–6
- 23 Del Zotto, H., Massa, L., Rafaeloff, R., Pittenger, G. L., Vinik, A., Gold, G., Reifel-Miller, A. and Gagliardino, J. J. (2000) Possible relationship between changes in islet neogenesis and islet neogenesis-associated protein-positive cell mass induced by sucrose administration to normal hamsters. *J. Endocrinol.* **165**, 725–733
- 24 Scaglia, L., Cahill, C. J., Finegood, D. T. and Bonner-Weir, S. (1997) Apoptosis participates in the remodeling of the endocrine pancreas in the neonatal rat. *Endocrinology* **138**, 1736–1741
- 25 Bonner-Weir, S. and Smith, F. E. (1994) Islet cell growth and the growth factors involved. *Trends Endocrinol. Metab.* **5**, 60–64
- 26 Alzamendi, A., Giovambattista, A., Raschia, A., Madrid, V., Gaillard, R. C., Rebollo, O., Gagliardino, J. J. and Spinedi, E. (2009) Fructose-rich diet-induced abdominal adipose tissue endocrine dysfunction in normal male rats. *Endocrine* **35**, 227–232
- 27 Francini, F., Castro, M. C., Gagliardino, J. J. and Massa, M. L. (2009) Regulation of liver glucokinase activity in rats with fructose-induced insulin resistance and impaired glucose and lipid metabolism. *Can. J. Physiol. Pharmacol.* **87**, 702–710
- 28 Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (2001) Executive Summary of The Third Report of The National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, And Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III). *JAMA, J. Am. Med. Assoc.* **285**, 2486–2497
- 29 Xu, G., Stoffers, D. A., Habener, J. F. and Bonner-Weir, S. (1999) Exendin-4 stimulates both β -cell replication and neogenesis, resulting in increased β -cell mass and improved glucose tolerance in diabetic rats. *Diabetes* **48**, 2270–2276
- 30 Young, A. A., Gedulin, B. R., Bhavsar, S., Bodkin, N., Jodka, C., Hansen, B. and Denaro, M. (1999) Glucose-lowering and insulin-sensitizing actions of exendin-4: studies in obese diabetic (ob/ob, db/db) mice, diabetic fatty Zucker rats, and diabetic rhesus monkeys (*Macaca mulatta*). *Diabetes* **48**, 1026–1034
- 31 Pospisilik, J. A., Martin, J., Doty, T., Ehses, J. A., Pami, N., Lynn, F. C., Piteau, S., Demuth, H. U., McIntosh, C. H. and Pederson, R. A. (2003) Dipeptidyl peptidase IV inhibitor treatment stimulates β -cell survival and islet neogenesis in streptozotocin-induced diabetic rats. *Diabetes* **52**, 741–750
- 32 Mu, J., Woods, J., Zhou, Y. P., Roy, R. S., Li, Z., Zychband, E., Feng, Y., Zhu, L., Li, C., Howard, A. D. et al. (2006) Chronic inhibition of dipeptidyl peptidase-4 with a sitagliptin analog preserves pancreatic β -cell mass and function in a rodent model of type 2 diabetes. *Diabetes* **55**, 1695–1704
- 33 Mu, J., Petrov, A., Eiermann, G. J., Woods, J., Zhou, Y. P., Li, Z., Zychband, E., Feng, Y., Zhu, L., Roy, R. S. et al. (2009) Inhibition of DPP-4 with sitagliptin improves glycemic control and restores islet cell mass and function in a rodent model of type 2 diabetes. *Eur. J. Pharmacol.* **623**, 148–154
- 34 Moritoh, Y., Takeuchi, K., Asakawa, T., Kataoka, O. and Odaka, H. (2009) The dipeptidyl peptidase-4 inhibitor alogliptin in combination with pioglitazone improves glycemic control, lipid profiles, and increases pancreatic insulin content in ob/ob mice. *Eur. J. Pharmacol.* **602**, 448–454
- 35 Tahara, A., Matsuyama-Yokono, A., Nakano, R., Someya, Y., Hayakawa, M. and Shibasaki, M. (2009) Antihyperglycemic effects of ASP8497 in streptozotocin-nicotinamide induced diabetic rats: comparison with other dipeptidyl peptidase-IV inhibitors. *Pharmacol. Rep.* **61**, 899–908
- 36 Ferreira, L., Teixeira-de-Lemos, E., Pinto, F., Parada, B., Mega, C., Vala, H., Pinto, R., Garrido, P., Sereno, J., Fernandes, R. et al. (2010) Effects of sitagliptin treatment on dysmetabolism, inflammation, and oxidative stress in an animal model of type 2 diabetes (ZDF rat). *Mediators Inflamm.* **2010**, 592780
- 37 Winzell, M. S. and Ahren, B. (2004) The high-fat diet-fed mouse: a model for studying mechanisms and treatment of impaired glucose tolerance and type 2 diabetes. *Diabetes* **53**, S215–S219
- 38 Moritoh, Y., Takeuchi, K. and Hazama, M. (2010) Combination treatment with alogliptin and voglibose increases active GLP-1 circulation, prevents the development of diabetes and preserves pancreatic β -cells in prediabetic db/db mice. *Diabetes Obes. Metab.* **12**, 224–233
- 39 Augstein, P., Berg, S., Heinke, P., Altmann, S., Salzsieder, E., Demuth, H. U. and Freyse, E. J. (2007) Efficacy of the dipeptidyl peptidase IV inhibitor isoleucine thiazolidide (P32/98) in fatty Zucker rats with incipient and manifest impaired glucose tolerance. *Diabetes Obes. Metab.* **10**, 850–861
- 40 Pospisilik, J. A., Stafford, S. G., Demuth, H. U., Brownsey, R., Parkhouse, W., Finegood, D. T., McIntosh, C. H. and Pederson, R. A. (2002) Long-term treatment with the dipeptidyl peptidase IV inhibitor P32/98 causes sustained improvements in glucose tolerance, insulin sensitivity, hyperinsulinemia, and β -cell glucose responsiveness in VDF (fa/fa) Zucker rats. *Diabetes* **51**, 943–950
- 41 Pospisilik, J. A., Stafford, S. G., Demuth, H. U., McIntosh, C. H. and Pederson, R. A. (2002) Long-term treatment with the dipeptidyl peptidase IV inhibitor improves hepatic and peripheral insulin sensitivity in the VDF Zucker rat: a euglycemic-hyperinsulinemic clamp study. *Diabetes* **51**, 2677–2683
- 42 Pederson, R. A., White, H. A., Schlenzig, D., Pauly, R. P., McIntosh, C. H. and Demuth, H. U. (1998) Improved glucose tolerance in Zucker fatty rats by oral administration of the dipeptidyl peptidase IV inhibitor isoleucine thiazolidide. *Diabetes* **47**, 1253–1258
- 43 Maida, A., Hansotia, T., Longuet, C., Seino, T. and Drucker, D. J. (2009) Differential importance of glucose-dependent insulinotropic polypeptide vs glucagon-like peptide 1 receptor signaling for β cell survival in mice. *Gastroenterology* **137**, 2146–2157
- 44 Rhodes, C. J. (2005) Type 2 diabetes – a matter of β -cell life and death? *Science* **307**, 380–384
- 45 Brownlee, M. (2005) The pathobiology of diabetic complications: a unifying mechanism. *Diabetes* **54**, 1615–1625
- 46 Poitout, V. and Robertson, R. P. (2008) Glucolipotoxicity: fuel excess and β -cell dysfunction. *Endocr. Rev.* **29**, 351–366

Received 13 July 2010/19 August 2010; accepted 26 August 2010

Published as Immediate Publication 26 August 2010, doi:10.1042/CS20100372