Life Sciences 234 (2019) 116738

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

The dipeptidyl peptidase 4 inhibitor sitagliptin improves oxidative stress and ameliorates glomerular lesions in a rat model of type 1 diabetes

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ARTICLE INFO

Keywords:
Diabetic nephropathy
Type 1 diabetes
Sitagliptin
Oxidative stress
Antioxidant defenses
Dipeptidyl peptidase-4

ABSTRACT

Aims: Oxidative stress has been linked to the development and progression of diabetic nephropathy (DN). The present study evaluated whether the dipeptidyl peptidase-4 inhibitor sitagliptin attenuates glomerular lesions and oxidative stress evoked by chronic hyperglycemia, by a mechanism independent of insulin secretion and glycemia normalization.

Main methods: A rat model of DN caused by streptozotocin injection was established and the effects of sitagliptin (5 mg/kg/day) were evaluated after two weeks of treatment.

Key findings: Sitagliptin treatment did not change body weight, glycemic and lipid profiles. However, histopathological observation revealed that sitagliptin attenuates diabetes-induced glomerular lesions on diabetic rats. Sitagliptin also ameliorated the increase in DPP-4 content and promoted the stabilization of GLP-1 in the diabetic kidney. Furthermore, sitagliptin treatment significantly attenuated the increase of free-radical formation and the decrease of antioxidant defenses, attenuating therefore the oxidative stress in the kidneys of diabetic animals.

Significance: The results suggest that sitagliptin treatment alleviates kidney oxidative stress in type 1 diabetic rats, which could play a key role in reducing the progression of DN.

1. Introduction

Diabetic kidney disease (DKD), also named diabetic nephropathy (DN), occurs in 30–40% of patients with diabetes and is a leading cause of end-stage renal disease (ESRD) worldwide [1,2]. DN is one of the most common chronic complications in patients with both type 1 (T1D) and type 2 diabetes (T2D), characterized by microvascular lesions in the renal glomeruli. It typically develops during the first 5–10 years after T1D diagnosis, although it is present in T2D onset [3]. Pathological alterations of the renal glomeruli develop in patients with long-duration diabetes before appearance of microalbuminuria [4] and are considered the most important lesions in DN [5]. Mesangial expansion due to increased matrix production, thickening of the glomerular basement membrane and glomerular sclerosis are the three major histological changes occurring in the glomeruli of patients with DN [5].

Although the pathogenesis of DN is complex, multifactorial, and involves several biochemical pathways, hyperglycemia itself is a major impetus for the progressive and permanent damage of the glomeruli in diabetes. Increasing evidence shows that oxidative stress is the common denominator link of various signaling pathways that are involved in the pathological changes found in DN [6,7]. Diabetes-induced reactive oxygen species (ROS) can be mainly generated by NADPH oxidase (Nox) and at the mitochondrial electron transport chain. An imbalance between the rates of ROS production and their neutralization by antioxidant defensive systems usually results in oxidative stress. An augment of ROS production in the kidneys, as a consequence of high glucose levels, induces damages of various tissue biomolecules such as DNA, proteins, carbohydrates and lipids, inactivation of endogenous antioxidants [8,9]; and podocyte [10], mesangial [11] and tubular [12] cell death, contributing to the pathogenesis of DN [13–15].

Sitagliptin, the first oral dipeptidyl peptidase-4 (DPP-4) inhibitor, has been widely used along with diet and exercise and in conjugation with other medication, to improve glycemic control by lowering blood glucose and HbA1c levels in T2D patients [16–21]. Sitagliptin works by

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https://doi.org/10.1016/j.lfs.2019.116738
Received 4 February 2019; Received in revised form 29 July 2019; Accepted 5 August 2019
Available online 06 August 2019

competitively inhibiting the enzyme DPP-4, preventing the inactivation of incretin hormones, namely glucagon-like peptide-1 (GLP-1) and consequently increasing insulin secretion. We and other authors have demonstrated cytoprotective actions of DPP-4 inhibitors in distinct organs and pathological conditions, such as pancreas, heart, retina and kidney in T2D animal models [22–28]. Although DPP-4 inhibitors have exhibited renal protective effects in T2D, the direct effect of sitagliptin therapy on oxidative stress and on the attenuation of renal lesions development in T1D-evoked DN, through a mechanism independent of insulin secretion and glycemic improvement, remains unexplored.

The present study aimed to investigate the renoprotective effect of sitagliptin on DN in streptozotocin (STZ)-induced diabetic rats, focusing on antioxidant properties.

2. Materials and methods

2.1. Experimental animals

Male Wistar rats (8 weeks old) were housed at approximately 22 °C, and relative humidity of 60%, under a constant 12 h light and dark cycle. Pellet food (standard rat diet; SAFE A04 Augy, France) and water were given *ad libitum*. All procedures involving animals were performed according to the National and European Guidelines for the Use and Care of Laboratory Animals and approved by the Institutional Ethics Committee of the Faculty of Medicine of University of Coimbra for animal care and use (Approval ID: 015-CE-2011). Diabetes was induced with a single intraperitoneal injection of freshly prepared streptozotocin (STZ, Sigma-Aldrich, St. Louis, MO, USA) at the dose of 65 mg/kg body weight (BW) in 10 mM citrate buffer (pH 4.5). On day 2 after STZ injection, blood samples were collected from the tail vein and animals with blood glucose levels above 250 mg/dL were considered diabetic and used in the subsequent studies.

After 2 weeks of diabetes induction, the animals were divided into three groups (number of animals stated in each figure legend): controls, diabetics and diabetics treated with 5 mg/kg/day (*via* oral gavage) sitagliptin (Januvia®, MSD, Portugal) during the following 2 weeks. A set of control animals were also treated with sitagliptin, and the results obtained for the several measured parameters described in this section were similar to those obtained with non-treated control animals.

2.2. Serum biochemical parameters

The rats of all groups were anesthetized with an intraperitoneal injection of a cocktail (2 mg/kg): 2: 1 50 mg/mL ketamine solution in 2.5% chlorpromazine, and blood samples were collected from the jugular vein. Serum glucose and insulin levels were measured using commercial kits (Sigma-Aldrich and Mercodia, Uppsala, Sweden, respectively) and HbA1c levels by using the DCA 2000 + analyzer (Bayer Diagnostics, Barcelona, Spain), according to the instructions of the manufacturer. Blood urea nitrogen (BUN) and serum creatinine concentrations were evaluated as renal function indexes, and serum total cholesterol (Total-c) and triglycerides (TG) were used as lipid profile measures, through automatic validated methods and equipment (Hitachi 717 analyzer, Roche Diagnostics Inc., MA, USA).

2.3. Histopathological examination

Kidneys were harvested and immediately fixed in 10% neutral formalin and then embedded in paraffin for light microscopy. Paraffin sections (3 μm) were stained with periodic acid Schiff (PAS) by standard procedures, as previously described [22]. Kidney lesions were examined by light microscopy using a Microscope Zeiss Mod. Axioplan 2, at x400 magnification. From each kidney, two sections and 10 images were used to analyze the degree of injury. Glomerular damage was assessed by evaluating thickening of glomerular basement membrane and capsule of Bowman, mesangial expansion, nodular sclerosis, global glomerulosclerosis, hypercellularity, atrophy, and hyalinosis of the vascular pole. The tissue sections were randomly selected for analysis using light microscopy. Glomerular lesions were graded using a semi-quantitative scale for each slide ranging from normal (or minimal) to advanced (extensive damage) that was assigned to each component. All imaging and lesion grading were performed in a blinded fashion.

2.4. Western blot analysis

Kidney extracts were prepared as previously described [23]. For the Western blot analysis, 40 μg of protein from the kidney total extracts were loaded per lane on SDS-PAGE. Following electrophoresis and transfer to polyvinylidene difluoride membranes (Boehringer Mannheim, Mannheim, Germany), the membranes were probed with the antibodies rabbit polyclonal anti-DPP-4 (1:1000), mouse monoclonal anti-GLP-1 (1:1000) and rabbit anti-GLP-1 receptor (GLP-1R) (1:500) from Abcam (#ab28340, #ab23468 and #ab39072; Cambridge, UK), and mouse monoclonal anti-β-actin (1:10,000) from Sigma-Aldrich (#A5441). After washing, the membranes were probed with a secondary anti-mouse IgG-HRP-linked antibody (1:10,000; Bio-Rad, Hercules, CA, USA) for 1 h at room temperature. Immunoreactive bands were detected by an enhanced chemiluminescence (ECL) substrate using an imaging system (VersaDoc 4000 MP, Bio-Rad) and quantification was performed using ImageJ software (version 1.47, http://imagej.nih.gov/ij, provided in the public domain by the National Institute of Health, Bethesda, MD, USA).

2.5. Immunofluorescence

Kidney sections (7 μm) were fixed in cold acetone for 10 min. The sections were then washed with phosphate-buffered saline (PBS), permeabilized for 30 min with 0.25% Tx-100 in PBS with 0.02% BSA (PBS/BSA), and blocked for 40 min with 10% normal goat serum or with 5% BSA, prior to incubation overnight at 4 °C with primary antibodies rabbit polyclonal anti-DPP-4 (#ab28340; 1:100), mouse anti-GLP-1 (#ab23468; 1:100) and rabbit anti-GLP-1R from Abcam (#ab39072; 1:100). Sections were rinsed with PBS and then incubated with secondary fluorescent antibodies for 1 h at room temperature. After washing, samples were imaged using a confocal microscope (LSM 710, Carl Zeiss, Gottingen, Germany).

2.6. Antioxidative defense enzyme activities

The antioxidative activity of catalase (CAT) and superoxide dismutase (SOD) was determined in kidney tissue homogenates. Kidneys were homogenized in cold 0.05 M phosphate buffer (pH 7.8). The homogenate was then centrifuged at 10,000 × *g*, for 15 min at 4 °C and the supernatant fraction was used to estimate the CAT and SOD activities, as previously described [29]. CAT activity was determined at 25 °C by monitoring the rate of hydrogen peroxide (0.04% *w*/w) decomposition in 0.05 M phosphate buffer, pH 7.0. One unit of catalase activity was defined by the enzyme quantity that produced an absorbance reduction of 0.43 per minute at 240 nm in this system. SOD activity was calculated considering that one unit of SOD activity represents the inhibition of 50% in the rate of increase in absorbance at 550 nm when compared with control (sample without SOD under the conditions of the assay). CAT and SOD activities were measured in 96-well plates using a microplate reader Synergy HT (BioTek, Winooski, VT, USA). The activity of CAT was expressed as nmol of substrate oxidized per minute per mg of protein (mU/mg of protein). The activity of SOD was defined as U/μg of protein.

2.7. Tissue reduced (GSH) and oxidized (GSSG) glutathione level

Reduced and oxidized glutathione levels as well as reduced: oxidized glutathione (GSH: GSSG) ratio were estimated as previously

described [30]. Briefly, for the GSH assay, 50 µl supernatant and 100 µl phosphate buffer (100 mM Tris-HCl, 1% SDS, 2 mM EDTA, pH 8.2) were mixed. The final assay mixture (100 µl) contained 80 µl of the sample and 20 µl of 3 mM DNTB. After mixing during 15 min at 37 °C, fluorescence was determined at 415 nm using a spectrofluorometer. The assay is based on the reaction of GSH with DNTB that produces the TNB chromophore. The rate of formation of TNB, measured at 415 nm, is proportional to the concentration of GSH in the sample, and this concentration is determined through the linear equation from several standards of GSH. GSSG stock solution 2 mg/mL was prepared by dissolving GSSG in KPE buffer (0.1 M potassium phosphate buffer, 5 mM EDTA, pH 7.5). Serial dilutions were then performed to create standard concentrations ranging from 0 to 32 nmol/mL GSSG. Sulfosalicylic acid and 2-vinylpyridine (2 µl) were added to the tissue extract to derivatize GSH. Triethanolamine (6 µl) was added and samples were assayed using the method describe previously for GSH at 415 nm. Tissue GSH and GSSG levels, expressed as nmol/mg of protein, were measured in 96-well plates using a Biotek Synergy HT spectrophotometer (Biotek).

2.8. Detection of intracellular reactive oxygen species

To detect *in situ* generation of ROS in kidney specimens, fluorescence microscopy with dihydroethidium (DHE) was performed as previously described [31]. Briefly, unfixed frozen sections (7 µm) of kidney were incubated with DHE (200 µM) in PBS in a humidified chamber protected from light at 37 °C for 30 min. Upon reacting with superoxide, DHE is oxidized to ethidium bromide which intercalates within the DNA. After washing steps and fixation in 4% (*w/v*) paraformaldehyde, slides were mounted using Glycergel mounting medium and ROS levels were qualitatively evaluated by confocal fluorescence microscopy (LSM 710, Carl Zeiss, Gottingen, Germany).

ROS in the kidney tissue were also evaluated by a method described by Gupta et al. [32], with some modifications. Briefly, tissue was homogenized in ice-cold 40 mM Tris-HCl buffer (pH 7.4) and 20 µg of protein (in 100 µl) were incubated with either 200 µM of DHE or 2′,7′-dichlorofluorescein diacetate (H_2DCFDA; Invitrogen; Life Technologies, Carlsbad, CA, USA) for 1 h at 37 °C in 96-well plates protected from light. Finally, the fluorescence intensity of the samples was assessed using a fluorescence plate reader. The excitation and emission filters used for the H_2DCFDA probe were 485/20 nm and 528/20 nm, respectively. For the DHE, the excitation and emission filters used were 485/20 nm and 590/35 nm, respectively.

2.9. Statistical analysis

Data are expressed as mean ± standard errors of the mean (SEM). Comparison of values between groups was carried out using analysis of variance (ANOVA) followed by Bonferrroni's *post hoc* tests (GraphPad Prism 5.0 software, La Jolla, CA, USA) and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Sitagliptin treatment has no effect on body weight, glycemic and lipid profiles

Diabetic rats presented less body weight than age-matched control animals at 12 weeks of age (Table 1). Four weeks after STZ injection, the average blood glucose levels of diabetic rats were significantly higher (3.9-fold change of control; $p < 0.001$) than those of control animals. Glycemic deregulation in diabetic animals was confirmed by an increment in HbA1c levels (2.6-fold change of control; $p < 0.001$) and lower levels of insulin (4% of control; $p < 0.001$) when compared to controls. Treatment with sitagliptin for 2 weeks had no effect on body weight, blood glucose, HbA1c or insulin levels (Table 1).

Although no changes were observed in the levels of serum TGs between the three experimental groups, Total-c levels were higher (1.3-fold change of control, $p < 0.05$) in the diabetic rats, an effect that was unchanged with sitagliptin treatment (Table 1). Although serum creatinine levels were unchanged between groups, there was a significantly increased (1.8-fold change of control, $p < 0.01$) BUN concentration in the diabetic rats *vs*. the control ones; sitagliptin treatment was unable to alter BUN levels in the diabetic rats (Table 1).

Table 1
Effects of sitagliptin on body weight, glycemic, insulinic and lipidic profiles, and serum levels of creatine and BUN, in rats with T1D.

Parameters	Control	Diabetic	Diabetic + sitagliptin
Weight (g)	318.3 ± 25.3	226.2 ± 18.2***	229.3 ± 19.3
Glucose (mg/dL)	157.9 ± 20.1	621.0 ± 176.8***	632.4 ± 203.2
HbA1c (%)	3.77 ± 0.06	9.77 ± 0.06***	9.07 ± 0.55
Insulin (mg/dL)	477.1 ± 112.7	19.96 ± 8.393***	23.23 ± 19.87
BUN (mg/dL)	18.56 ± 2.32	33.41 ± 12.70**	46.15 ± 23.10
Serum creatinine (mg/dL)	0.3838 ± 0.046	0.3775 ± 0.068	0.4200 ± 0.066
Serum total–c (mg/dL)	53.00 ± 9.04	70.00 ± 0.63*	63.75 ± 13.19
Serum TG (mg/dL)	94.25 ± 45.78	99.00 ± 65.44	107.8 ± 63.35

Data are expressed as mean ± SEM ($n = 4$–6 per group), *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ as compared to control group. ANOVA followed by Bonferroni's *post hoc* test. BUN, blood urea nitrogen; TG, triglycerides; Total-c, total cholesterol.

3.2. Sitagliptin treatment attenuates glomerular lesions on diabetic rats

PAS staining was performed on renal sections from the three animal groups (Fig. 1). The results clearly show a normal histopathological appearance for the control group without occurrence of glomerular lesions (Table 2).

Contrary to the control group, thickening of Bowman's capsule, hyalinosis of the vascular pole, glomerular atrophy, hypercellularity, dilatation of the Bowman's space and advanced glomerular lesions were found in renal tissues of diabetic animals. The renoprotective effects of sitagliptin were evident on diabetic rats with amelioration of almost all the moderate and advanced lesions (Table 2).

3.3. Sitagliptin attenuates diabetes-mediated increase of DPP-4 content and modulates the incretin axis in the kidney of diabetic rats

DPP-4, GLP-1 and GLP-1R were analyzed by Western Blotting and immunofluorescence (Fig. 2). We have previously reported, in the same type 1 diabetes animal model, that an identical treatment protocol (two weeks with 5 mg/kg of sitagliptin daily) leads to an inhibition of plasma DPP-4 activity of about 70% and that it also prevents the increase in circulating DPP-4 levels induced by diabetes [33]. Accordingly, we found a statistically significant increase in DPP-4 protein levels in diabetic kidney extracts (from 100.0 ± 7.32% to 144.4 ± 22.45%; $p < 0.01$) (Fig. 2A). Confocal microscopy images also revealed that diabetes led to increased glomerular DPP-4 levels, as revealed by higher fluorescence intensity (Fig. 2D). Treatment with sitagliptin decreased DPP-4 protein levels (85.68 ± 8.75%; $p < 0.01$) and its immunofluorescence staining in diabetic rats (Fig. 2A and D).

Although there were no significant changes in the levels of GLP-1 in total protein extracts among control and diabetic animals (from 100.0 ± 10.9 to 61.5 ± 11.5%) (Fig. 2B), sitagliptin treatment markedly increased its levels (122.0 ± 11.4%; $p < 0.01$) in the kidneys of diabetic animals (Fig. 2B). Immunofluorescence results were in agreement with Western blot analysis showing that treatment with sitagliptin increased the staining for GLP-1 in diabetic kidney (Fig. 2E). Although no significant changes were found for GLP-1R levels in kidney total protein extracts (Fig. 2C and E), we found that sitagliptin was able

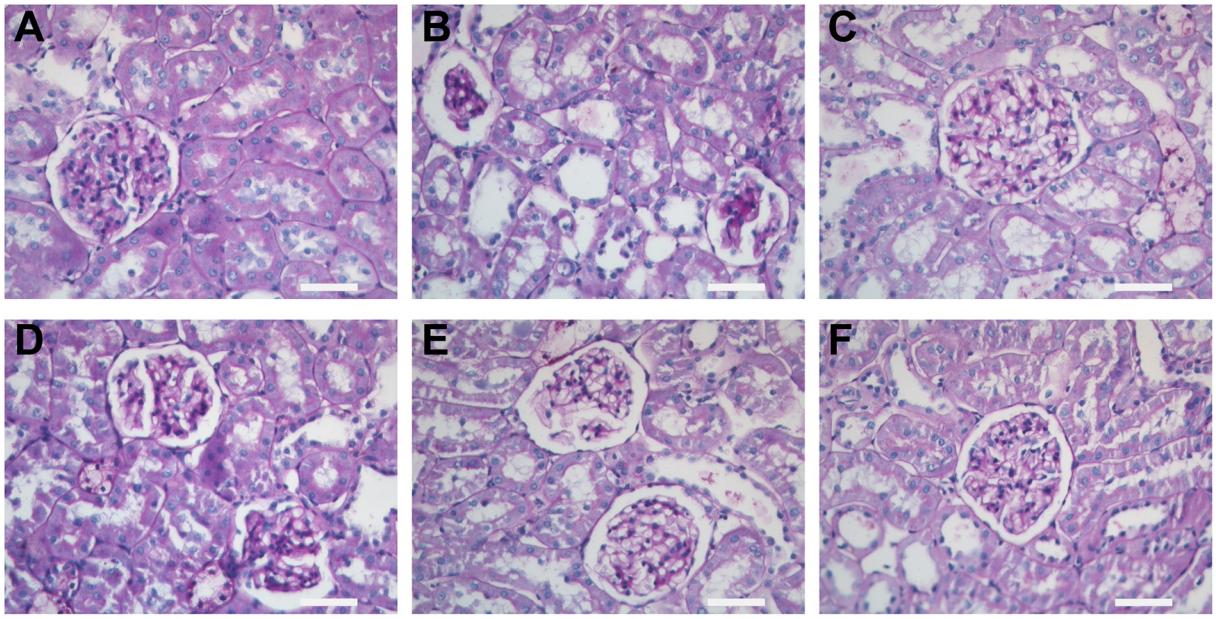

Fig. 1. – Effects of sitagliptin treatment on glomerular lesions induced by diabetes. Representative photomicrographs of kidney histology: A - Control group, with a normal glomerulus; B, C, D and E - STZ diabetic group, with glomerular atrophy and sclerosis (B), hypercellularity (C), hyalinosis of vascular pole (D) and thickening of glomerular basement membrane (E); F - STZ diabetic group treated with sitagliptin, without significant glomerular lesions. Sita, sitagliptin. PAS staining (Bars = 100 μm).

Table 2
Scoring of glomerular lesions in control and diabetic rat kidneys (2 weeks of vehicle or sitagliptin treatment).

Morphology changes	Control	Diabetic	Diabetic + sitagliptin
Thickening of Bowman's capsule	–	+	–
Hyalinosis of vascular pole	–	+	–
Glomerular atrophy	–	+ +	+ +
Hypercellularity	–	+ +	+
Dilatation of Bowman's space	–	+ +	+
Advanced lesions	–	+ + +	+

–: Normal, +: Mild, + +: Moderate; + + +: Severe levels, revealing no < 25, 50, 75% histopathological lesions of glomerular tissues, respectively.

to upregulate GLP-1 immunoreactivity and the colocalization of GLP-1 and GLP-1R in the glomeruli of diabetic animals (Fig. 2E).

3.4. Sitagliptin ameliorates the oxidative stress in the kidneys of diabetic animals

Oxidative stress has a key role in the pathogenesis of DN and therefore, the degree of renal oxidative stress was assessed using the oxidation-sensitive fluorescent dyes DHE and H_2DCFDA (Fig. 3). The levels of superoxide and ROS in total renal extracts were significantly higher in diabetic kidneys (118.3 ± 0.7% and 136.4 ± 4.8%, respectively) when compared to controls ($p < 0.001$) (Fig. 3A, B), an effect that was attenuated with sitagliptin treatment (95.1 ± 1.9%, $p < 0.001$ and 108.6 ± 6.6%, $p < 0.01$, respectively). As shown in Fig. 3C, the glomeruli of diabetic animals showed higher DHE fluorescence than that observed in control animals, indicating increased glomerular superoxide production. Sitagliptin treatment attenuated superoxide anion production in the diabetic glomeruli.

A statistically significant decrease in CAT and SOD activities was detected in the kidneys of the diabetic group (catalase: 16.23 ± 1.26 mU/mg protein, SOD: 505.2 ± 0.03 U/μg protein) compared to the control group (catalase: 27.08 ± 1.82 mU/mg protein, SOD: 708.6 ± 38.8 U/ug protein) ($p < 0.01$) (Fig. 4A, B). After two weeks of sitagliptin treatment, CAT and SOD activities were significantly higher (27.08 ± 1.82 mU/mg protein, $p < 0.05$ and 706.5 ± 53.80 U/μg protein, $p < 0.001$) than in the diabetic group (Fig. 4A, B). Given that GSH is one of the most important ROS scavengers, its ratio with GSSG was assessed as a key factor to determine oxidative stress (Fig. 4C). The GSH level in the diabetic group (9.47 ± 0.05 nmol/mg protein) was significantly decreased compared to the control group (18.44 ± 0.90 nmol/mg protein) ($p < 0.001$) (Fig. 4C). GSSG levels were found significantly higher (58.00 ± 13.13 nmol/mg protein) in diabetic rats compared to the control animals (21.06 ± 2.25 nmol/mg protein) ($p < 0.001$). In agreement with these results, an increased ratio of GSSG to GSH was found in the diabetic group ($p < 0.001$) (Fig. 4C). Sitagliptin significantly increased GSH level (17.22 ± 1.33 nmol/mg protein, $p < 0.001$) and decreased GSSG level (16.24 ± 1.87, $p < 0.001$), and thus GSSG/GSH decreased in the kidneys of diabetic rats (Fig. 4C).

4. Discussion

In the present study, we demonstrate that sitagliptin, a DPP-4 inhibitor, has beneficial effects on the kidney of diabetic animals by a mechanism independent of glucose normalization and enhanced insulin secretion. We show that daily oral administration of sitagliptin for two weeks attenuated glomeruli changes in an animal model of T1D, concomitantly with attenuation of hyperglycemia-mediated oxidative stress.

There are several reports from our group and other authors showing cytoprotective effects of DPP-4 inhibitor sitagliptin, on various organs and tissues affected by diabetes, including pancreas, retina and kidney, in experimental T2D [22,26,33–42]. Our research group has previously found that T2D animal models treated with sitagliptin preserved renal tissue with decreased inflammatory state and cell death [23]. Although incretin-based therapies such as sitagliptin may offer renoprotective effects in T2D, there was no evidence in the literature of whether these effects may be, at least in part, attributed to antioxidant actions in an independent-manner of glycemic control. Therefore, the therapeutic potential of sitagliptin in the diabetic kidney was investigated in the STZ-induced rat model of T1D.

In this study, induction of diabetes with STZ was associated with

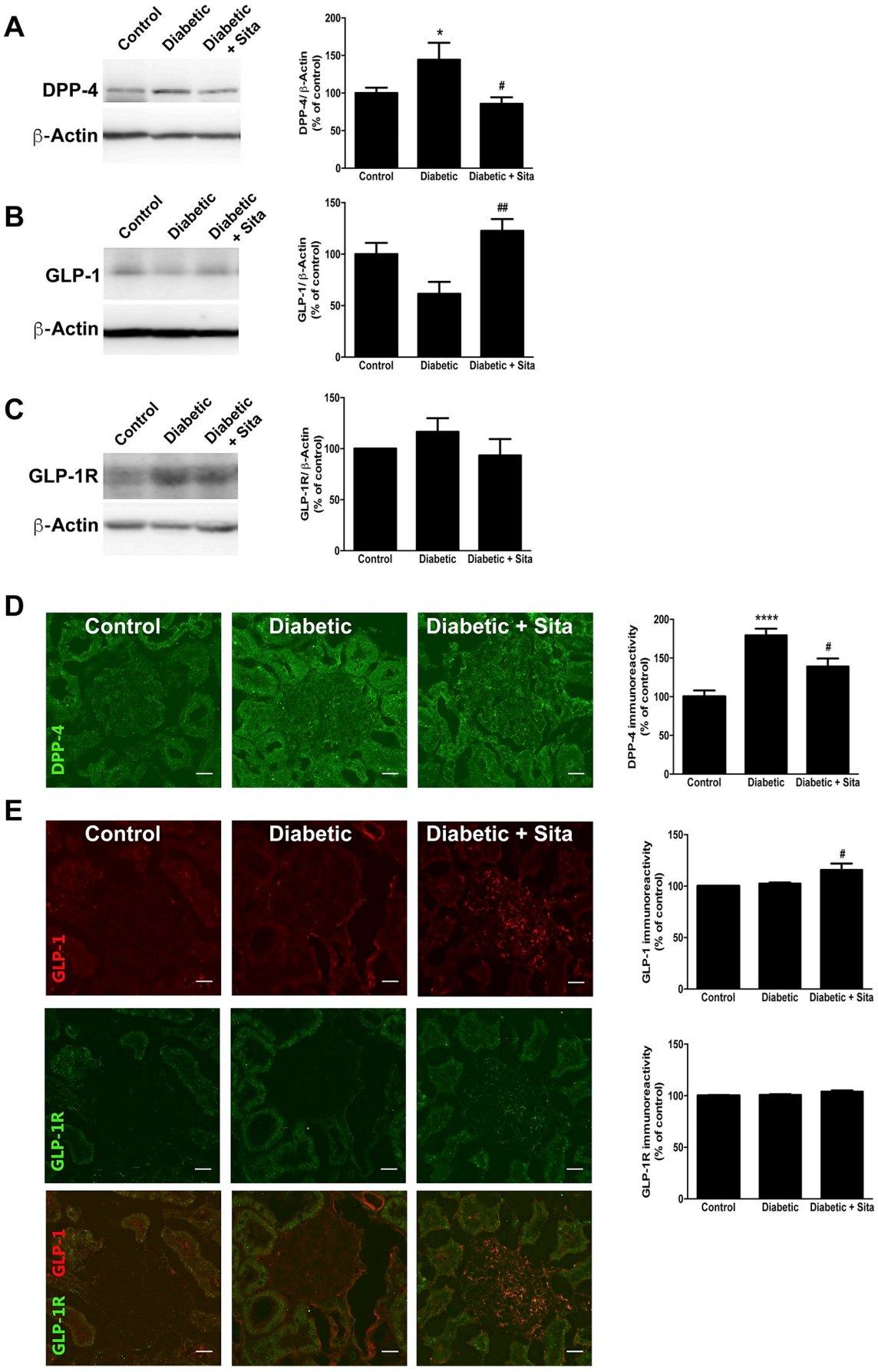

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Fig. 2. - Sitagliptin attenuates the upregulation of DPP-4 content and modulates the incretin axis in the kidney of diabetic rats. The protein levels of (A) DPP-4, (B) GLP-1 and (C) GLP-1R were assessed in total kidney cell lysates by Western Blotting in Control, STZ nontreated or treated with sitagliptin. The Western blots presented are representative of each group of animals. Data are expressed as percentage of control and represent the mean ± SEM ($n = 4$–7 per group); $^{*}p < 0.05$ significantly different from control; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ significantly different from diabetic. (D) Representative confocal images for each group of animals showing DPP-4 (green) immunoreactivity in kidney sections, as well as immunoreactivity quantification for DPP-4. (E) Representative confocal images for each group of animals showing GLP-1 (red) and GLP-1R (green) immunoreactivity in kidney sections, as well as immunoreactivity quantification for GLP-1 and GLP-1R. STZ diabetic rats treated with sitagliptin showing colocalization areas of GLP-1 and GLP-1R (yellow) in the glomeruli. Sita, sitagliptin. Bars = 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

significant reduction in the body weight, together with hyperglycemia, hypoinsulinemia, and changes in lipid profile.

Our results showing glomerular structural abnormalities in STZ-induced diabetic rats are consistent with persistent albuminuria and progressive glomerulosclerosis [43,44] observed in human DN and experimental diabetes [43–48]. However, serum creatine concentrations were unchanged between groups, as previously reported by others in preclinical models [49]. Although plasma BUN and creatinine are often measured together, it has been suggested that the former is a more sensitive marker for kidney damage, and that, creatinine levels require long time to increase, being thus reported only at the end stage of renal disease [49,50].

In our diabetic model, classical signs of renal injury were present. Advanced glomerular lesions, glomerular atrophy, hypercellularity and dilatation of Bowman's space were observed in the kidneys of diabetic rats. Thickening of Bowman's capsule and hyalinosis of vascular pole were observed in less extent in the kidneys of diabetic rats. The renal lesions were essentially confined to the glomeruli, without severe tubular cell damage or tubulointerstitial fibrosis (data not shown). This is consistent with previous reports showing that STZ-induced diabetic rats exhibit little or no nodular lesions, interstitial inflammation and severe tubular cell damage, thus suggesting that this diabetic animal model may be useful to study the early stages of DN [48,51]. Although we found no reversion or improvement in biochemical parameters by using sitagliptin in our rat model of T1D, the DPP-4 inhibitor was successful in ameliorating glomerular lesions.

Previous work from our group in an experimental model of T2D demonstrated that a low-dose chronic treatment with sitagliptin

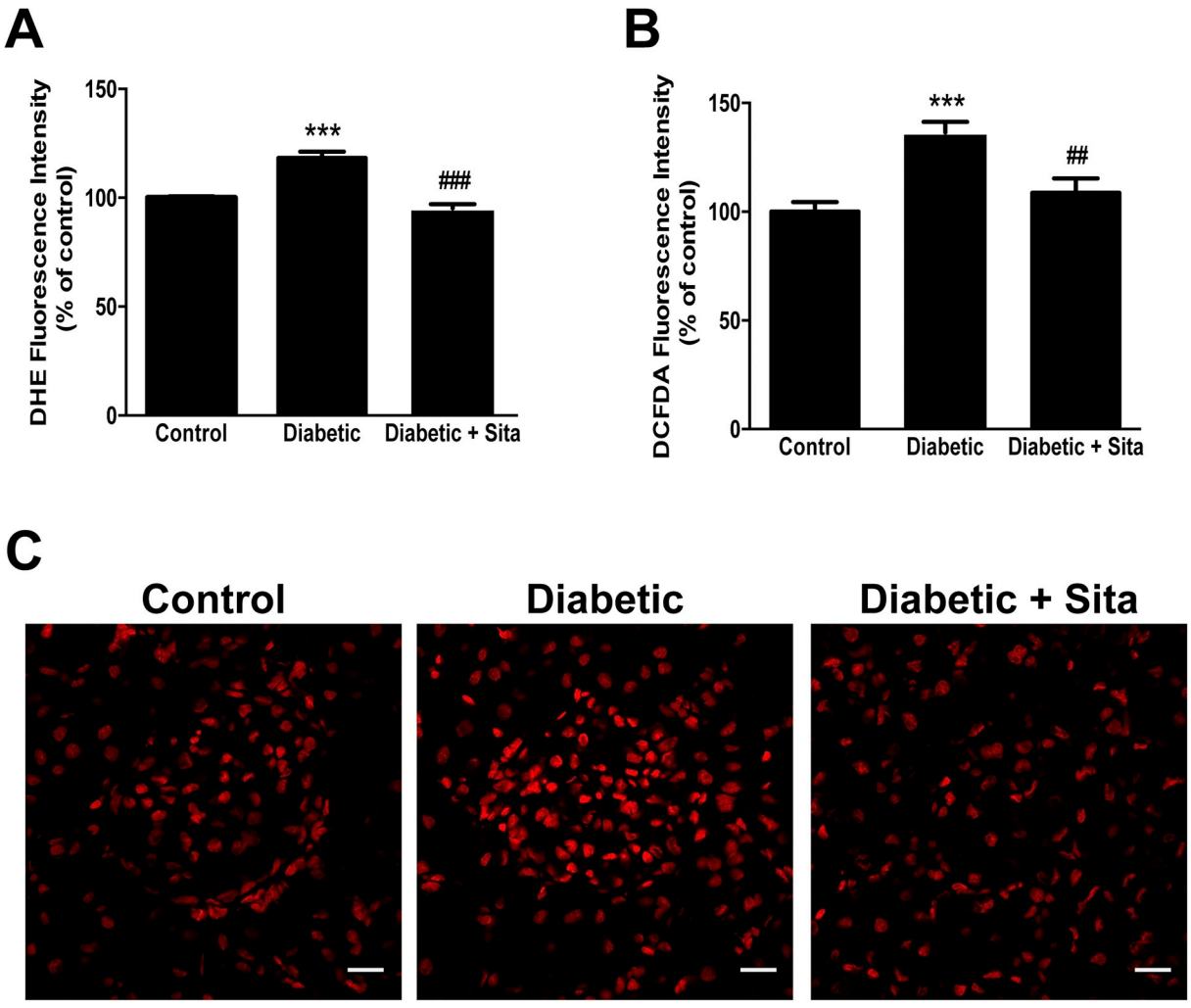

Fig. 3. – Sitagliptin attenuates the production of reactive oxygen species (ROS) in the kidney tissues of diabetic rats. ROS were measured using the fluorescent probes DHE and H_2DCFDA. Quantification of DHE (A) and DCF (B) fluorescence increase (as a measure of ROS production) in the total extracts of kidney of control, STZ nontreated and treated with sitagliptin. Data are the mean ± SEM ($n \geq 6$ per group); $^{***}p < 0.001$ significantly different from control. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ significantly different from diabetic. (C) Representative images of DHE fluorescence in glomeruli of control, STZ nontreated and treated with sitagliptin. Scale bars 20 μm. Sita, sitagliptin.

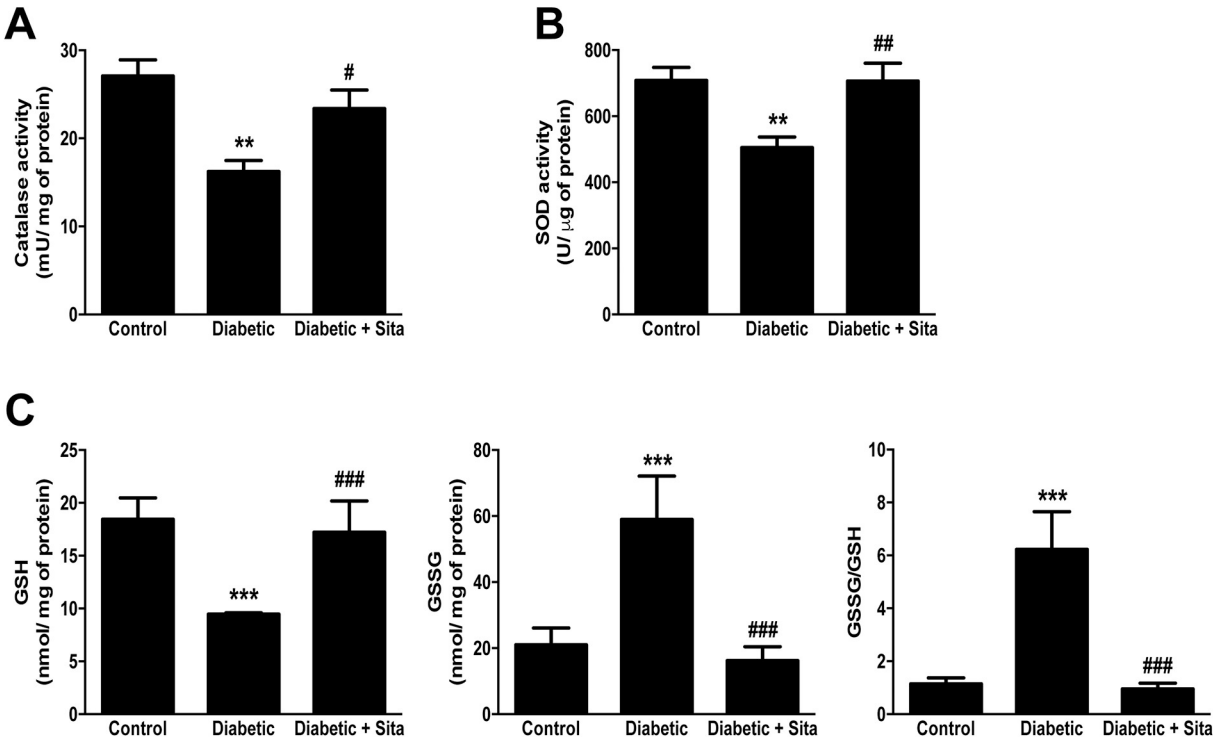

Fig. 4. – Sitagliptin attenuates the decrease on the antioxidant enzymes activity in the kidney tissue induced by diabetes. Effects of sitagliptin on catalase (CAT) (A) and superoxide dismutase (SOD) (B) activity and oxidative markers GSH, GSSG and GSSG/GSH (C) in kidney total extracts of rats with T1D. Data are presented as the mean ± SEM; $^{**}p < 0.01$, $^{***}p < 0.001$ significantly different from control cohort. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ significantly different from diabetic cohort. Sita, sitagliptin.

(10 mg/kg bw/day during 6 weeks) has beneficial effects on metabolic and lipid profiles together with reduction in systemic chronic inflammation and oxidative stress markers. Alterations in the metabolism and lipids have a key role in the pathophysiology of diabetes and may precede and further potentiate renal injury [52]. Moreover, sitagliptin treatment promoted an amelioration in renal lesions in T2D rats, including glomerular, tubulointerstitial, and vascular lesions, which were accompanied by reduced lipid peroxidation [22]. Although in that model of T2D, sitagliptin showed capacity to improve the lipid measures, in this study using a T1D model the changes on lipid profile are very modest and, so, it is not surprising that 2 weeks of sitagliptin treatment did not significantly alter this slight change on Total-c found in the diabetic rats.

In this report, we clearly show that sitagliptin is also able to significantly ameliorate glomerular lesions in the STZ-induced model of T1D. Although our study did not evaluate the renal function, clinical reports suggest that sitagliptin is able to reduce albumin excretion [53,54]. Previous works have described increased serum DPP-4 activity in both T2D and T1D patients [55,56]. We and other authors have previously observed increased levels and serum DPP-4 activity in the same diabetic animal model that was used in the present study [33,57]. We have also previously reported that in diabetic rats given the same dose for two weeks, sitagliptin significantly reduced DPP-4 activity (around 70%) compared to untreated STZ-induced diabetic animals, which may be correlated with decreased levels of the protein present in the serum. In the kidney, we found that diabetes induced an increase in DPP-4 protein levels and immunofluorescence staining, when compared to nondiabetic animals. Previous work using the same T1D model as the one reported in the current study demonstrated a positive correlation between enhanced DPP-4 expression levels in the kidney tissue and the amount of the soluble form of the protein present in blood plasma, although DPP-4 activity was reduced [58]. Accordingly, other studies have demonstrated that renal lesions induced by chronic hyperglycemia, augment biosynthesis and secretion of DPP-4 by endothelial cells [59,60]. Moreover, *in vitro* studies performed with glomerular endothelial cells exposed to high glucose have shown increased DPP-4 mRNA expression and activity [59]. Our results show that sitagliptin is able to restore DPP-4 protein levels and promote an accumulation of GLP-1 in the kidney of diabetic rats, suggesting that kidney DPP-4 protein content and/or activity may be responsible for the changes seen in GLP-1 levels in kidneys of diabetic rats. Although no significant changes were observed in GLP-1R protein levels, we found a restored expression of GLP-1 and a co-localization of GLP-1 with GLP-1R in the sitagliptin-treated diabetic rats, implying a role for GLP-1/GLP-1R signaling activation in the amelioration of nephropathy. These pleiotropic (renoprotective) effects may derive, at least in part, from GLP-1/GLP-1R activation, which are in agreement with previous studies from our group and other reports [23,61–63]. Whether the kidney GLP-1 levels are increased as a result of gut production, which may arise in the kidney *via* the circulation or, alternatively, renal cells produce GLP-1 locally, needs to be better understood. However, our results clearly show that accumulation of GLP-1 and colocalization with GLP1-1R in the glomeruli of a T1D animal model, without decreased blood glucose levels, may have an important role in the observed renoprotective effects, thus highlighting the existence of a direct effect of sitagliptin on glomerular cells.

Chronic hyperglycemia induces activation of multiple metabolic pathways able to disrupt redox homeostasis [64]. Activation of these metabolic pathways can lead to inflammation and renal fibrosis [65,66]. Overproduction of ROS, mainly superoxide anion by mitochondria, links the activation of these metabolic pathways with altered glomerular hemodynamics [67,68]. Chronic hyperglycemia and consequent increased intracellular ROS production decrease the antioxidative defense cell systems, thus making the cell more prone to oxidative damage. Preclinical studies have demonstrated that the inability of some cells to maintain intracellular glucose homeostasis lead to the transport of large amounts of glucose to the inside of renal cells, including glomerular cells, which leads to the enhancement of

glycolysis and excessive ROS production [7,69]. Hyperglycemia-induced ROS production can oxidize lipids, proteins and nucleic acids, inducing profound alterations in their structure and function, which contributes to cell death by apoptosis or necrosis [7,70]. Herein, intracellular ROS production was significantly increased in the kidney of diabetic rats. Sitagliptin treatment was able to reduce ROS generation in rats with STZ-induced diabetes. Therefore, we hypothesized that sitagliptin may exert a direct protective effect, possibly *via* modulation of antioxidant response in the diabetic kidney. By inhibiting DPP-4 activity, sitagliptin increases renal GSH levels and the enzymatic activity of CAT and SOD, thus suggesting an antioxidant role for sitagliptin.

5. Conclusion

This study shows that sitagliptin exhibits renoprotective effects in STZ-induced diabetic rats. Sitagliptin is able to prevent the increase in DPP-4 levels and promotes the stabilization of GLP-1 and possibly activation of GLP-1R in the diabetic kidney. These effects seem to occur *via* attenuation of hyperglycemia-induced oxidative stress. However, further study is needed to investigate the exact underlying cellular and molecular mechanisms.

Authors contributions

CM, AG and PMRP contributed equally to this work. RF designed the research study. CM, AG, PMRP, DA and BM performed the experiments. RF and FR analyzed the data. RF and CFR wrote the manuscript. All authors reviewed the final version of the manuscript.

Acknowledgments

The work was supported by the Foundation for Science and Technology (FCT, Portugal) and Strategic Project UID/NEU/04539/2013 and UID/NEU/04539/2019 (CNC.IBILI Consortium), COMPETE-FEDER (POCI-01-0145-FEDER-007440) and Centro 2020 Regional Operational Programme: HEALTHYAGING 2020 (CENTRO-01-0145-FEDER-000012); BRAINHEALTH 2020 (CENTRO-01-0145-FEDER-000008); PPBI-POCI-01-0145-FEDER-022122. PMRP and AG were supported by Ph.D fellowships from FCT.

Declaration of competing interest

The authors state no conflicts of interest.

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