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Dipeptidyl peptidase-IV inhibition prevents blood–retinal barrier breakdown, inflammation and neuronal cell death in the retina of type 1 diabetic rats

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ABSTRACT

Diabetic retinopathy, a leading cause of vision loss in working-age population, is often associated with inflammation and apoptosis. We have previously reported that sitagliptin, a DPP-IV inhibitor, exerts beneficial effects in the retina of type 2 diabetic animals. The present study aimed to evaluate whether sitagliptin can exert protective effects in the retina of type 1 diabetic animals by a mechanism independent of insulin secretion and glycemia normalization.
Streptozotocin-induced diabetic rats were treated orally with sitagliptin (5 mg/kg/day) for the last two weeks of 4 weeks of diabetes. Sitagliptin treatment did not change the weight and glucose, HbA_{1c} or insulin levels. However, it prevented the diabetes-induced increase in DPP-IV/CD26 activity and levels in serum and retina. Sitagliptin also prevented the increase in blood–retinal barrier (BRB) permeability and inhibited the changes in immunoreactivity and endothelial subcellular distribution of occludin, claudin-5 and ZO-1 proteins induced by diabetes. Furthermore, sitagliptin decreased the retinal inflammatory state and neuronal apoptosis.
Sitagliptin inhibited the BRB breakdown in a type 1 diabetic animal model, by a mechanism independent of normalization of glycemia, by preventing changes in TJ organization. Sitagliptin also exerted protective effects against inflammation and pro-apoptotic state in the retina of diabetic rats. Altogether, these results suggest that sitagliptin might be envisaged to be used to prevent or delay some of the alterations associated with the development of diabetic retinopathy.

1. Introduction

Diabetes is associated with the development of microvascular complications, being the most common diabetic retinopathy. Chronic hyperglycemia leads to retinal endothelial cell dysfunction resulting in, among other effects, pericyte loss, formation of acellular capillaries, increased vessel permeability and leukocyte adhesion [1]. In streptozotocin-induced diabetic mice and rats, as well as in diabetic humans, it has been demonstrated an increase in blood–retinal barrier (BRB) permeability, which is the hallmark of the early stages of diabetic retinopathy progression [2–4]. Diabetes-induced vascular permeability seems to be correlated with the disruption of tight junctions (TJs), which form a complex network structure between the endothelial cells, comprising the inner BRB [5,6]. It has been described that chronic hyperglycemia induces changes in the levels and distribution of TJ proteins within the retinal vascular endothelium, which seem to directly contribute to increased vascular permeability [3,4,7,8]. Also, inflammatory mediators have been shown to promote increased vascular permeability, leukocyte adhesion and retinal cell death [9,10]. In fact, elevated levels of proinflammatory cytokines have been detected in the vitreous of diabetic patients with retinopathy [11] and in diabetic rat retinas with increased vascular permeability [12,13].

Although good glycemic control can reduce the risk for the development of diabetic retinopathy, even in patients with good glycemic control the disease can progress to more advanced stages. Therefore, it becomes imperative to implement new and effective therapeutic strategies capable of preventing or attenuating the progression of diabetic retinopathy, preferably during the earlier stages of the disease.

Sitagliptin, a dipeptidyl peptidase IV (DPP-IV, also known as CD26; EC 3.4.14.5) inhibitor, has been widely used as a clinical approach for

Abbreviations: AMC, aminomethylcoumarin; BRB, blood–retinal barrier; DPP-IV, dipeptidyl peptidase-IV; GLP-1, glucagon-like peptide-1; GLP-1R, glucagon-like peptide-1 receptor; ICAM-1, intercellular adhesion molecule-1; PFA, paraformaldehyde; STZ, streptozotocin; TJ, tight junction; ZDF, Zucker diabetic fatty; ZO-1, zonula occludens-1

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the management of poor glycemic control in type 2 diabetic patients. The inhibition of DPP-IV stabilizes the glucagon-like peptide (GLP-1), which stimulates its receptor thus enhancing the insulin production in response to chronic hyperglycemia. Diabetic patients without decrease in glucose levels through diet or oral medications have been shown to improve glycemic control with sitagliptin therapy [14]. Its clinical effectiveness seemed to occur mainly through an increase in the levels of the incretin hormone GLP-1, mediated by DPP-IV enzyme inhibition, exerting a number of actions that improve glucose homeostasis, including the enhancement of glucose-stimulated insulin secretion, promotion of beta-cell proliferation and survival, and inhibition of glucagon secretion [15]. DPP-IV is expressed in several cell types, including neuronal cells and brain capillary endothelial cells [16], being also found in the plasma and its inhibition increases GLP-1 plasma concentration [17]. Besides the insulinotropic effects of GLP-1 receptor (GLP-1R) activation in pancreatic cells, this receptor was shown to be expressed in a wide range of tissues, including the retina [18].

Recent studies have demonstrated beneficial effects of incretin-based therapies in the vasculature [19,20], kidney [21], heart [22] and brain [23]. Regarding the retina, it was reported that intravitreal injection of a GLP-1 analog (Exendin-4), could reverse changes in electroretinograms, prevent retinal cell death and maintain normal retinal thickness in diabetic rats [24]. Recently, we have demonstrated that sitagliptin can exert beneficial and protective effects in the BRB, inhibit apoptosis and inflammation, and positively modulate EPC in a type 2 diabetes animal model [25]. In this work, we showed, for the first time, that the beneficial effects of DPP IV inhibition on diabetic retina can be explained, at least partially, by a mechanism independent of increased insulin secretion. Our findings show that sitagliptin has protective effects in the early stages of diabetic retinopathy in a type 1 diabetic animal model independent of insulin secretion and normalization of glycemia levels, by a mechanism involving the regulation of TJ proteins and vascular repair. Moreover, its effects on inflammation and cell death were also addressed.

2. Material and methods

2.1. Animal model

All procedures involving animals were performed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research 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).

Male Wistar rats (8 weeks old) were housed at approximately 22 °C, 60% relative humidity, and a 12-h light, 12-h dark cycle was maintained. Throughout the study the animals had access to water and standard rat diet (SAFE A04 Augy, France) ad libitum. Diabetes was induced with a single intraperitoneal injection of streptozotocin (STZ, Sigma-Aldrich, St. Louis, MO, USA; 65 mg/kg in 10 mM citrate buffer, pH 4.5). After 48 h, animals with blood glucose levels above 13.9 mM were considered diabetic.

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 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 (data not shown).

2.2. Measurement of serum glucose, insulin and glycosylated hemoglobin (HbA_{1c}) levels

Rats 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 from the jugular vein were collected. Serum glucose and insulin levels were measured using commercial kits (Sigma-Aldrich and Mercodia, Uppsala, Sweden, respectively) and HbA_{1c} levels by using the DCA 2000 + analyzer (Bayer Diagnostics, Barcelona, Spain), according to the instructions of the manufacturer.

2.3. Western blot analysis

Retinal extracts were prepared as previously described [25]. For the Western blot analysis, 40 μg of protein from the retinal extracts or 100 μg from serum samples were loaded per lane, separated by electrophoresis on a SDS 7.5 or 10% polyacrylamide gel and transferred to polyvinylidene difluoride membranes (Boehringer Mannheim, Mannheim, Germany). Membranes were probed with rabbit polyclonal anti-DPP-IV/CD26 (1:4000) from Abcam (Cambridge, UK), rabbit polyclonal anti-intercellular adhesion molecule-1 (ICAM-1; 1:200) and rabbit polyclonal anti-Bax (1:200) from Santa Cruz Biotechnology (Santa Cruz, CA, USA), goat polyclonal anti-serum albumin (1:5000) from Bethyl Laboratories, Inc. (Montgomery, TX, USA) and mouse monoclonal anti-β-actin (1:10,000) antibody from Sigma-Aldrich. After washing, the membranes were probed with a secondary anti-rabbit or 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).

2.4. DPP-IV enzyme assay in serum

To measure the activity of DPP-IV in the serum, a fluorometric assay was employed, using H-Gly-Pro-AMC.HBr (BACHEM, Bubendorf, Switzerland). Gly-Pro-AMC is cleaved by DPP-IV to release the fluorescent aminomethylcoumarin (AMC). Briefly, 20 μL of serum sample was mixed with the assay buffer (50 mM glycine, 1 mM EDTA, pH 8.7) at room temperature. The reaction was initiated by the addition of the fluorogenic substrate to a final concentration of 200 μM. The final reaction volume for each well was 100 μL. Liberation of AMC was monitored, using an excitation wavelength of 360 nm and an emission wavelength of 460 nm (microplate reader Synergy HT, BioTek, Winooski, VT, USA), every 5 min for a total of 60 min.

For comparison of DPP-IV activity between samples, data was plotted as Relative Fluorescence Units versus time for each sample. The time range over which the reaction was linear was determined. A trend line for these data points was obtained and the slopes determined.

2.5. Immunohistochemistry in retinal sections

Retinal sections (10 μm) were fixed in cold acetone for 10 min. The sections were then washed with PBS, permeabilized for 30 min with 0.25% Tx-100 in PBS with 0.02% BSA (PBS/BSA) and blocked with 10% normal goat serum or 5% BSA before incubation overnight at 4 °C with primary antibodies: goat polyclonal anti-IL-1β (1:100; R&D Systems, Minneapolis, MN, USA), rabbit polyclonal anti-DPP-IV/CD26 (1:200, Abcam) and rabbit polyclonal anti-Bax (1:50, Santa Cruz Biotechnology). Sections were then rinsed with PBS and incubated with DAPI for nuclear staining and the secondary fluorescent antibodies for 1 h at room temperature.

Anti-DPP-IV/CD26 immunostaining samples were imaged using a confocal microscope (LSM 710, Carl Zeiss, Gottingen, Germany). Anti-IL-1β and anti-Bax immunostaining samples were imaged using a fluorescence microscope (Leica DFC350 FX, Leica Microsystems, Bannockburn, IL, USA). Fluorescence intensity of 5 fields per retinal section from four animals of each group was quantified by two independent observers in a masked fashion.

2.6. Measurement of BRB permeability

Blood–retinal barrier permeability was quantified using the Evans blue dye, which binds irreversibly to serum albumin, according to the

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procedure previously described by our group [4]. Briefly, under anesthesia, the rats were administered with Evans blue (100 mg/kg; Sigma-Aldrich) via tail vein. After 2 h, the animals were perfused with citrate-buffered (0.05 M, pH 4.2) 1% paraformaldehyde (PFA) for 2 min. The eyes were enucleated and the retinas isolated and weighted. The Evans blue dye was extracted from the retinas with formamide for 18 h at 70 °C. The extract was then centrifuged at 70,000 *g* for 45 min at 4 °C. The absorbance of the supernatant was measured at 620 nm (maximum absorbance) and 720 nm (minimum absorbance). The concentration of the dye in the extracts was calculated from a standard curve of Evans blue in formamide and normalized to the retina weight.

2.7. Visualization of retinal vessel leakage

Evans blue dye was also used to qualitatively assess the retinal vascular leakage. The Evans blue (100 mg/kg in PBS) was administered via tail vein to the anesthetized rats. After 30 min, the eyes were enucleated and immediately immersed in 2% PFA for 2 h. The retinas were isolated and flat-mounted with the vitreous side up for visualization under a fluorescence microscope (Leica DFC350 FX, Leica Microsystems). All of the images were acquired in a masked fashion.

2.8. Whole-mount staining

Retina whole-mounts were prepared according to the procedure previously described by our group [3]. The retina whole-mounts were immunostained with mouse monoclonal anti-occludin (1:100), rabbit polyclonal anti-claudin-5 (1:100), and rabbit polyclonal anti-zonula occludens 1 (ZO-1; 1:100) from Zymed Laboratories (San Francisco, CA, USA). After washing, the retinas were incubated with the secondary antibody Alexa Fluor 488-conjugated goat anti-rabbit IgG or Alexa Fluor 568-conjugated goat anti-mouse IgG (Life Technologies, Paisley, UK) and then mounted with the vitreous side up for visualization under a confocal microscope (LSM 510, Carl Zeiss). From each retina, 10 images were used to analyze occludin, claudin-5 and ZO-immunoreactivity. The fluorescence intensity for the three tight junction proteins was measured in 30–40 retinal vessels of each experimental group.

2.9. Elisa

Retinal tissue was homogenized in 20 mM imidazole HCl (pH 6.8), 100 mM KCl 1 mM $MgCl_2$, 1% Triton X-100, 1 mM EGTA, 1 mM EDTA, supplemented with 10 mM NaF, 1 mM Na_3VO_4 and 1 × protease inhibitor cocktail (Roche, Indianapolis, IN, USA). The samples were centrifuged at 4 °C for 5 min at 10,000 *g*, and IL-1β was assayed in the supernatant using an ELISA kit (Peprotech, Rocky Hill, NJ, USA), according to the manufacturer's instructions.

2.10. Apoptosis assay

Apoptotic cell death was detected by TUNEL using the DeadEnd Fluorometric TUNEL System (Promega, Madison, WI, USA) and degenerating neurons were assessed by Fluoro-Jade B staining (Chemicon, Temecula, CA, USA), according to the instructions of the manufacturers. Slides were then analyzed under a confocal microscope (LSM 710, Carl Zeiss).

2.11. Statistical analysis

Data are expressed as mean ± SEM. Significance was determined using ANOVA followed by Bonferroni's post hoc test (GraphPad Prism 5.0 software, La Jolla, CA, USA), as indicated in figure legends. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Sitagliptin has no effect on body weight, and blood glucose, HbA_{1c} or insulin levels in diabetic animals

Diabetic animals presented impaired gain weight throughout the study, with 29% ($P < 0.001$) less body weight than age-matched control animals at 12 weeks of age (Table 1).

The average blood glucose levels of diabetic animals (40.22 ± 3.07 mM; $P < 0.001$) were significantly higher than those of control animals (9.24 ± 0.60 mM). Accordingly, diabetic animals also presented increased levels of HbA_{1c} (9.68 ± 0.09%; $P < 0.001$) when compared to controls (3.83 ± 0.06%) (Table 1).

As expected, STZ-induced diabetes reduced significantly the insulin levels in serum when compared to control animals (19.14 ± 3.48 pM and 584.64 ± 76.56 pM, respectively; $P < 0.001$) (Table 1).

Treatment with sitagliptin during the last 2 weeks of diabetes did not affect significantly body weight (230.70 ± 5.65 g), and blood glucose (41.02 ± 3.32 mM), HbA_{1c} (9.16 ± 0.25%) or insulin levels (22.62 ± 6.96 pM) when compared to untreated diabetic animals (Table 1).

3.2. Sitagliptin decreases the activity and protein levels of DPP-IV in diabetic animals

To investigate the effect of sitagliptin on DPP-IV, its activity and protein levels were evaluated in the serum, and in the retina, the protein levels and distribution were assessed by Western blotting and immunohistochemistry, respectively.

The activity of soluble DPP-IV was significantly increased in the serum of diabetic animals (129.9 ± 4.3% of control; $P < 0.001$). In diabetic animals, sitagliptin decreased the activity of DPP-IV to 39.3 ± 4.521% of control ($P < 0.001$), corresponding to a 70% decrease when compared to untreated diabetic animals (Fig. 1A).

The protein levels of soluble DPP-IV were assessed in the serum by Western blotting using a specific antibody against DPP-IV/CD26. Diabetic animals presented increased DPP-IV levels (182.9 ± 24.7% of control; $P < 0.01$) (Fig. 1B). Sitagliptin significantly reduced DPP-IV protein levels in the serum of diabetic animals (101.7 ± 14.5% of control; $P < 0.01$), compared to untreated animals (Fig. 1B).

DPP-IV protein levels were also assessed in the retina by Western blotting. Diabetes led to increased DPP-IV levels in total retinal extracts (128.7 ± 10.5% of control; $P < 0.05$) (Fig. 1C). The administration of sitagliptin to diabetic rats prevented the increase in DPP-IV protein levels in the retina, compared to diabetic animals without treatment (98.8 ± 8.5% of control; $P < 0.05$) (Fig. 1C). Since the increase in CD26 immunostaining could be due to increased leakage into the retinal parenchyma of diabetic animals, a Western blot to detect serum albumin was performed, with no staining detected (Fig. 1C). Immunohistochemistry experiments performed in retinal frozen sections confirmed these results (Fig. 1D, E). Diabetes promoted a significant increase in DPP-IV immunoreactivity (119.6 ± 2.2% of control; $P < 0.001$), particularly in the ganglion cell layer. Treatment with sitagliptin markedly decreased the immunoreactivity for DPP-IV

Table 1
Body weight, blood glucose, glycated hemoglobin and insulin levels in control and diabetic Wistar rats nontreated or treated with 5 mg/kg/day sitagliptin for 2 weeks.

	Control	Diabetic	Diabetic + Sita
Body weight (g)	319.00 ± 7.26	229.30 ± 5.37[a]	230.70 ± 5.65
Glucose (mM)	9.24 ± 0.60	40.22 ± 3.07[a]	41.02 ± 3.32
HbA_{1c} (%)	3.83 ± 0.06	9.68 ± 0.09[a]	9.16 ± 0.25
Insulin (pM)	584.64 ± 76.56	19.14 ± 3.48[a]	22.62 ± 6.96

Data are expressed as mean ± SEM of 10–12 animals per group.
[a] $P < 0.001$ vs. control rats. ANOVA followed by Bonferroni's post hoc test.

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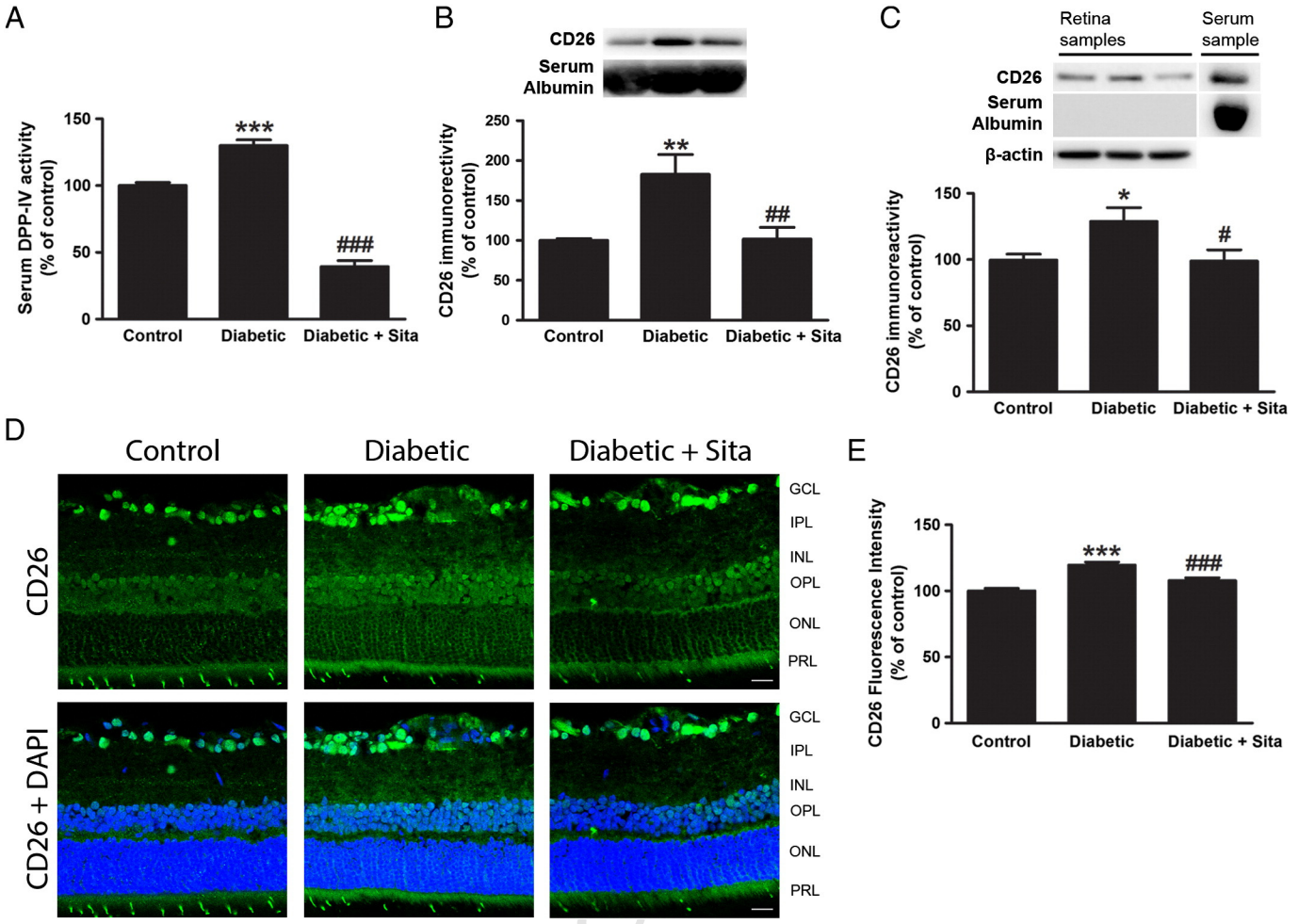

Fig. 1. Sitagliptin prevents the upregulation of DPP-IV activity and content induced by diabetes. (A) DPP-IV activity was determined in the serum using the fluorogenic substrate Gly-Pro-AMC. The protein levels of DPP-IV/CD26 (110–120 kDa) and serum albumin (65 kDa) were assessed by Western blotting in serum samples (B) and retinal lysates (C). The Western blots presented are representative of each group of animals. Data are presented as percentage of control and represent the mean ± SEM of 7–8 animals. (D) Representative confocal images for each group of animals, showing DPP-IV/CD26 immunoreactivity (green) and nuclear staining with DAPI (blue) in retinal sections. Magnification 400×. Bar: 20 μm. (E) Quantification of fluorescence intensity for DPP-IV/CD26 immunoreactivity in retinal frozen sections (10 μm). Data are presented as percentage of control and represent the mean ± SEM of 5 fields per section from 4 animals; ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ vs. control rat; #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$ vs. diabetic rats. ANOVA followed by Bonferroni's post hoc test. Legend: GCL — ganglion cell layer; IPL — inner plexiform layer; INL — inner nuclear layer; ONL — outer nuclear layer; PRL — photoreceptor layer.

in the retinas of diabetic rats (107.8 ± 2.2% of control; $P < 0.001$) (Fig. 1D, E).

3.3. Sitagliptin prevents the increase in BRB permeability induced by diabetes

The breakdown of the BRB induced by diabetes was assessed by Evans blue extravasation from retinal vessels. As a first approach, the retinal blood vessel integrity was analyzed in flat mount retinas. Evans blue was shown to be confined to the retinal blood vessels, without any leakage occurring, in control rats (Fig. 2A). After 1 month of diabetes, the dye was shown to leak from the capillaries and larger vessels to the surrounding tissue. The administration of sitagliptin to diabetic animals was able to prevent this effect (Fig. 2A). The quantitative measure of Evans blue dye, from the retinal tissue, confirmed the data obtained by fluorescence microscopy. Diabetes increased the BRB permeability in diabetic rats (15.8 ± 1.4 μg Evans blue per g wet weight retina; $P < 0.01$) when compared to control rats (7.2 ± 1.0 μg Evans blue per g wet weight retina) (Fig. 2B). Treatment with sitagliptin significantly prevented BRB breakdown in diabetic rats (9.7 ± 1.7 μg Evans blue per g wet weight retina; $P < 0.05$) when compared to untreated diabetic animals (Fig. 2B).

3.4. Sitagliptin prevents the alterations in the distribution of TJ proteins in retinal vessels induced by diabetes

In order to establish a correlation between the effects observed on the BRB permeability and TJ organization, whole retinas were immunostained for the three main constituents of these junctions, zonula occludens (ZO)-1, occludin and claudin-5. In control animals, the immunoreactivity for all three proteins was preferentially localized at the plasma membrane of retinal endothelial cells. In some retinal vessels of diabetic animals, there were pronounced alterations in the subcellular distribution of the three TJ proteins. A quantitative analysis revealed that there was a significant decrease in ZO-1 (58.8 ± 10.7% of control; $P < 0.05$) and claudin-5 (45.4 ± 3.6% of control; $P < 0.001$) immunoreactivity at endothelial cell borders, as well as intracellular accumulation of occludin in retinal vascular endothelial cells when compared to control animals (Fig. 2C, D). Treatment with sitagliptin was able to significantly prevent the decrease in claudin-5 (91.8 ± 4.3% of control; $P < 0.001$) and occludin (106.9 ± 11.2% of control; $P < 0.05$) immunoreactivity at the cell membranes, as well as the redistribution and intracellular accumulation of occludin in the endothelial retinal cells. Although a recovery of ZO-1 staining at the cell borders was noticed in the retinas of

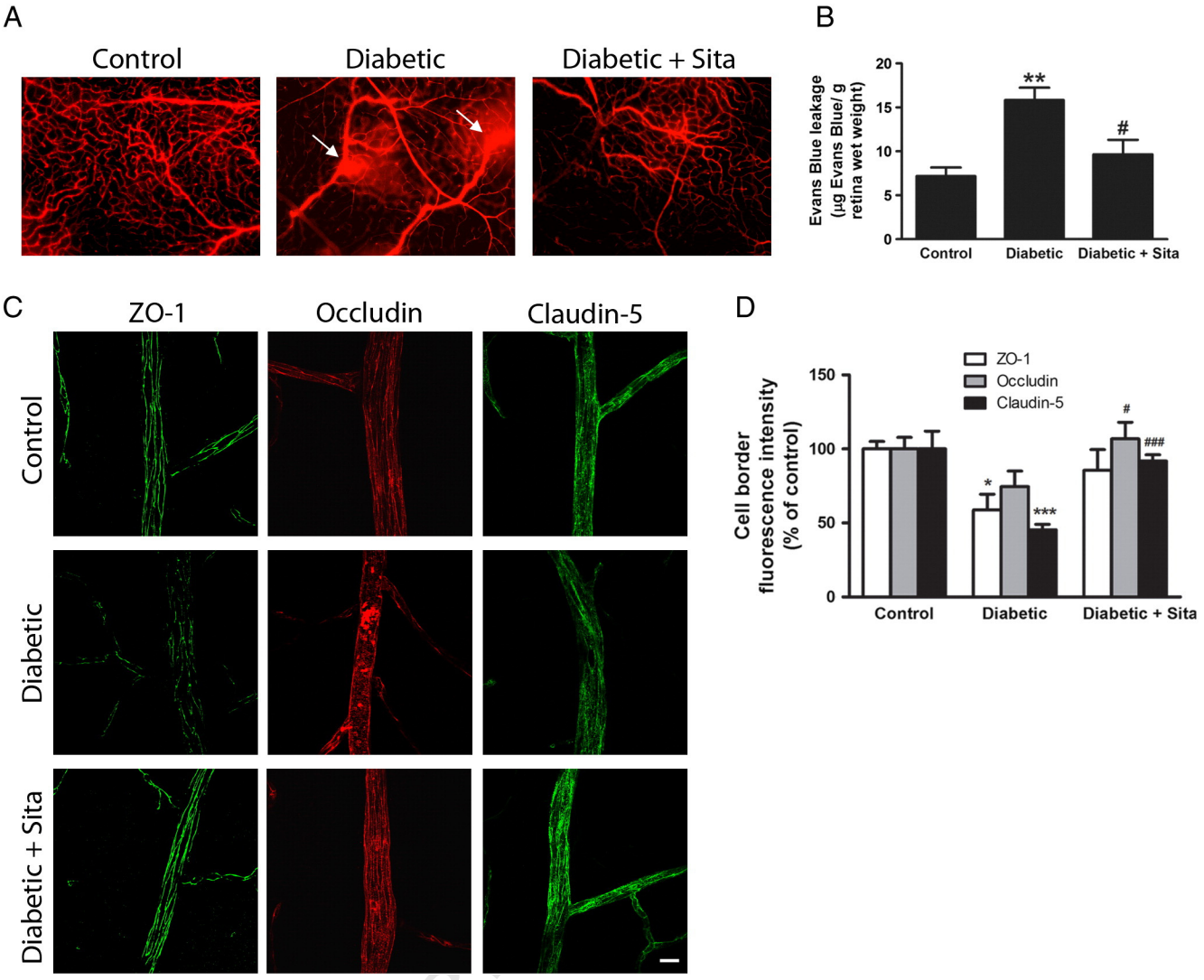

Fig. 2. Sitagliptin protects against the increase in BRB permeability and tight junction disassembly triggered by diabetes. (A) Representative images, from 3 animals per group, showing Evans blue fluorescence in the retina. Evans blue, which binds to blood albumin, allows the detection of leakage sites (arrows) in the retinal vessels. Magnification: 100×. (B) Quantitative measurement of BRB permeability by quantification of extravasated Evans blue to retinal parenchyma. Data are presented as µg of Evans blue per retina wet weight (g) and represent the mean ± SEM of 5–6 animals. (C) Sitagliptin prevents the decrease in ZO-1 and claudin-5 immunoreactivity, and the redistribution and accumulation of occludin in rat retinal vessels induced by diabetes. Whole mount preparations of the retinas were imaged by fluorescence confocal microscopy, and images are representative of each group of animals. Magnification 400×. Bar: 20 µm. (D) Quantification of cell border immunostaining for the tight junction proteins (ZO-1, occludin and claudin-5). Data are presented as percentage of control and represent the mean ± SEM of at least 10 fields per retina from 4 animals. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control rats; #$P < 0.05$, ###$P < 0.001$ vs. diabetic rats. ANOVA followed by Bonferroni's post hoc test.

diabetic animals treated with sitagliptin, no significant difference was reached when comparing to ZO-1 staining in the retinas of diabetic animals (Fig. 2C, D).

3.5. Sitagliptin is able to decrease inflammation in the retina of diabetic animals

Inflammation has been implicated in the pathogenesis of diabetic retinopathy, and IL-1β, a proinflammatory cytokine, has been correlated with BRB breakdown [13,26]. As expected, the results obtained by immunohistochemistry revealed that the retinas of diabetic animals presented an overall increase in IL-1β (191.8 ± 7.1% of control; $P < 0.001$) (Fig. 3A, B) immunoreactivity. Treatment with sitagliptin significantly decreased the immunoreactivity for IL-1β (117.4 ± 3.4% of control; $P < 0.001$) in the retinas of diabetic animals, particularly in the ganglion cell layer and inner plexiform layer (Fig. 3A, B). As shown in Fig. 3C, IL-1β levels in the retina of diabetic animals were higher (2550 ± 80.66 pg/mL) compared to control (1849 ± 151.2 pg/mL), as assessed by ELISA. Treatment with sitagliptin was able to prevent this increase (1981 ± 138.2 pg/mL; $P < 0.05$), when compared to untreated animals (Fig. 3C).

As the inflammatory process develops, the increase of local cytokine levels will promote the leukocyte adhesion to retinal vessels mediated by ICAM-1, which is expressed by endothelial cells [27]. ICAM-1 protein levels were significantly increased in the diabetic retinas (138.2 ± 9.6% of control; $P < 0.05$) (Fig. 3D). Sitagliptin treatment prevented the increase of this adhesion molecule induced by diabetes (102.4 ± 6.8% of control; $P < 0.01$) (Fig. 3D).

3.6. Sitagliptin prevents neuronal cell death induced by diabetes

The death of pericytes and acellular capillary formation are common features of the early stages of diabetic retinopathy and impaired angiogenic response to increased vascular permeability, may contribute to the breakdown of BRB [28]. Moreover, it has been shown that translocation of Bax, a pro-apoptotic protein, into the mitochondria triggers a caspase-dependent apoptosis in retinal cells exposed to chronic hyperglycemia [29].

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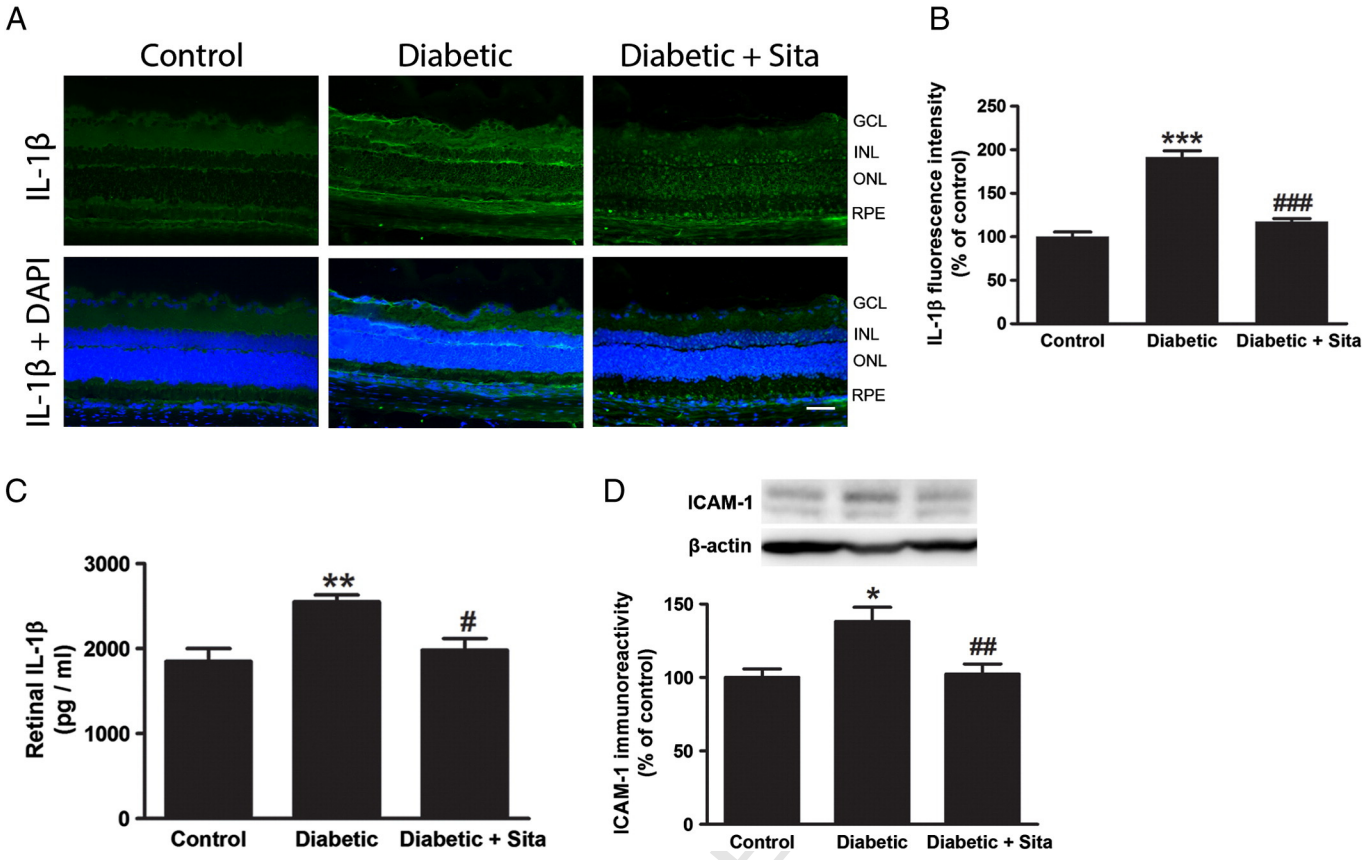

Fig. 3. Sitagliptin inhibits the increase in IL-1β and ICAM-1 levels in the retina of diabetic animals. Representative fluorescence images for each group of animals, showing IL-1β (A) immunoreactivity (green) and nuclear staining with DAPI (blue) in retinal sections (10 μm). Magnification 200×. Bar: 40 μm. Legend: GCL — ganglion cell layer; IPL — inner plexiform layer; INL — inner nuclear layer; OPL — outer plexiform layer; ONL — outer nuclear layer; PRL — photoreceptor layer; RPE — retinal pigment epithelium. Quantification of fluorescence intensity for IL-1β (B) immunoreactivity in retinal sections. Data are presented as percentage of control and represent the mean ± SEM of 5 fields per section from 4 animals per group. The levels of IL-1β were quantified in the supernatant of total retina homogenates, by ELISA (C). Data are presented as pg/mL of IL-1β and represent the mean ± SEM of 5–6 animals. The protein levels of ICAM-1 (110 kDa) were assessed by Western blotting in total retinal extracts (D). The Western blot presented is representative of each group of animals. Data are presented as percentage of control and represent the mean ± SEM of 7–8 animals. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ vs. control rats; ###$P < 0.001$, ##$P < 0.01$, #$P < 0.05$ vs. diabetic rats. ANOVA followed by Bonferroni's post hoc test.

A significant increase (123.6 ± 5.9% of control; $P < 0.05$) in Bax protein levels was detected in the retinas of diabetic rats, when compared to the control animals (Fig. 4A). The administration of sitagliptin significantly decreased the pro-apoptotic state (90.4 ± 5.8% of control; $P < 0.01$) induced by diabetes (Fig. 4A). These observations were confirmed by immunohistochemistry experiments. Diabetes induced an increase in Bax immunoreactivity in the retina (137.1 ± 7.2% of control; $P < 0.001$), especially at the plexiform and photoreceptor layers, indicating a pro-apoptotic state. Oral treatment with sitagliptin for 2 weeks was able to prevent the increase in Bax immunoreactivity in the diabetic retinas (114.8 ± 5.2% of control; $P < 0.05$) comparing to untreated animals (Fig. 4B, C). These results were confirmed by TUNEL assay. The number of TUNEL-positive cells (cells undergoing apoptosis) was increased in the diabetic retinas (2.3 ± 0.4 TUNEL-positive cells per 100 μm horizontal length; $P < 0.001$), when compared to control animals (0.3 ± 0.1 TUNEL-positive cells per 100 μm horizontal length). Besides the TUNEL-positive cells at the outer nuclear layer, we also observed TUNEL-positive staining at the ganglion cell layer in some of the retinal sections analyzed (Fig. 4D). Treatment with sitagliptin significantly decreased the number of TUNEL-positive cells in the diabetic retinas (1.2 ± 0.2 TUNEL-positive cells per 100 μm horizontal length; $P < 0.05$) (Fig. 4D, E).

To further investigate the potential protective effects of sitagliptin against retinal cell death, retinal sections were stained with Fluoro-Jade B, which is a well-established marker of degenerating neurons in the brain and retina [30,31]. In control retinas, we could not detect any staining indicative of cell death (Fig. 4F). In diabetic retinas, degenerating cell bodies were observed mainly in the ganglion cell and inner nuclear layers (arrows). Furthermore, some astroglial processes were also stained, extending from the ganglion cell layer to the inner plexiform layer (Fig. 4F). In the retinas of diabetic animals treated with sitagliptin, no specific staining of cell bodies indicative of cell death was found (Fig. 4F).

4. Discusssion

The present study is the first providing evidence that DPP-IV inhibition with sitagliptin has protective effects in the retina of diabetic animals by a mechanism independent of enhanced insulin secretion. Most research on sitagliptin has been focused on type 2 diabetes with normalization of blood glucose [17,25,32,33]. In this work, we show that sitagliptin was able to prevent several alterations occurring in the retina in a type 1 diabetes animal model, during the early stages of the disease. Sitagliptin prevented BRB breakdown, TJ complexes disassembly/disorganization, inflammation, retinal cell apoptosis, and the impaired mobilization and adhesion ability of circulating cells with vasculogenic potential, despite continued hyperglycemia and hypoinsulinemia. These results indicate that sitagliptin has direct effects on the retina that are independent of its antihyperglycemic effects.

Elevated serum DPP-IV activity has been described in both type 2 and type 1 diabetic patients [34,35]. We observed an increased serum DPP-IV activity after one month of diabetes induced by STZ (type 1 diabetes). In other study, using the same animal model, DPP-IV activity in the plasma is increased 1 week after STZ injection and treatment with

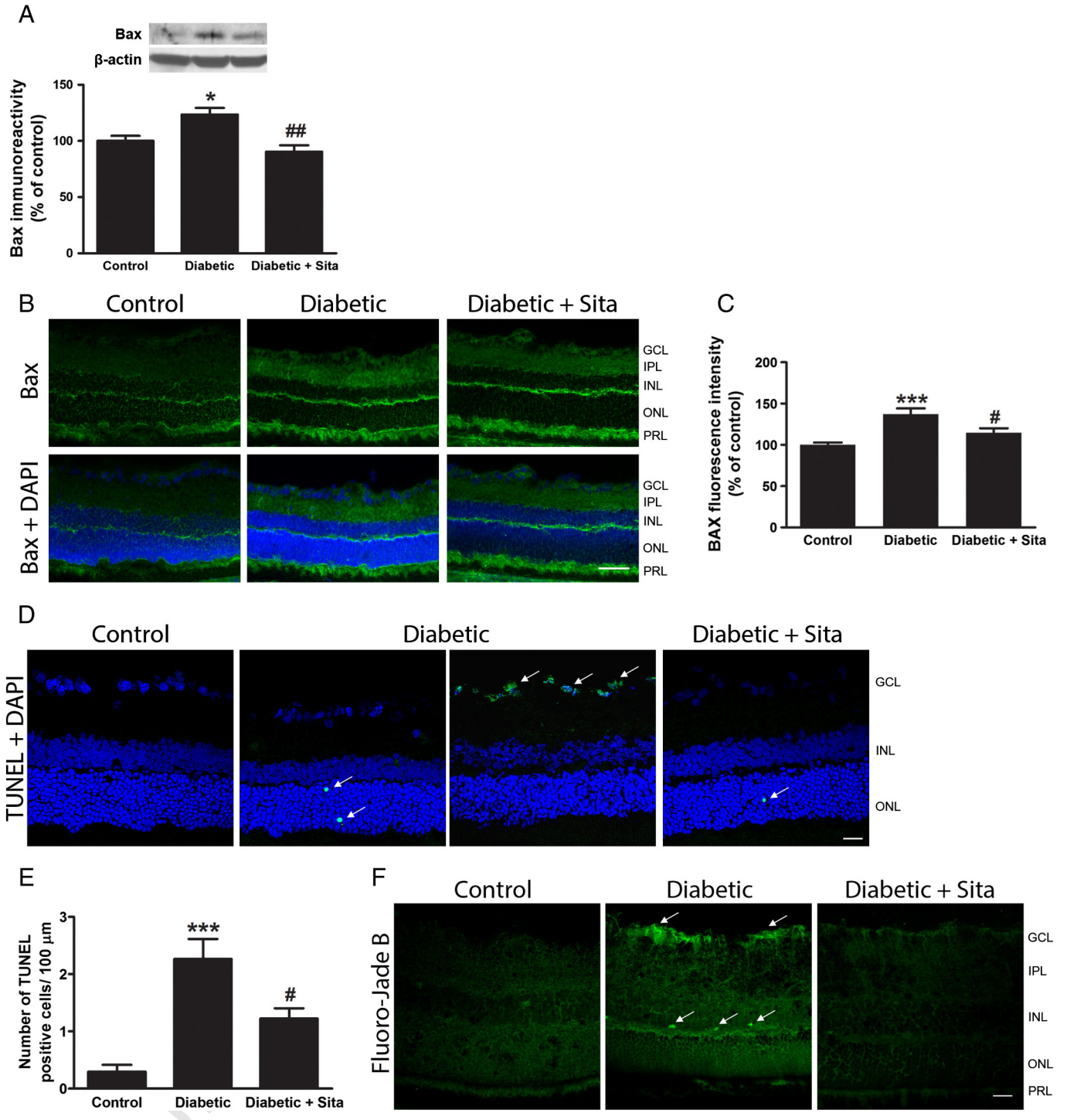

Fig. 4. Sitagliptin prevents neuronal cell death induced by diabetes in the retina. (A) The protein levels of Bax were assessed by Western blotting in retinal lysates. The Western blots presented are representative of each experimental group. Data are presented as percentage of control and represent the mean ± SEM of 7–8 animals. (B) Representative fluorescence images of Bax immunoreactivity (green) and nuclear staining with DAPI (blue) in 10 µm retina sections. Magnification 200×. Bar: 40 µm. (C) Quantification of fluorescence intensity of Bax immunoreactivity in retinal frozen sections. Data are presented as percentage of control and represent the mean ± SEM of 5 fields per section from 4 animals; (D) Representative confocal images for each group of animals showing TUNEL-positive cells (green, arrows) and nuclear counterstaining with DAPI (blue) in retinal sections. Magnification 400×. Bar: 20 µm. Legend: GCL — ganglion cell layer; IPL — inner plexiform layer; INL — inner nuclear layer; ONL — outer nuclear layer; PRL — photoreceptor layer. (E) Quantification of the number of TUNEL-positive cells in retinal sections. Data are presented as number of TUNEL-positive cells per 100 µm horizontal length and represent the mean ± SEM of 5 fields per section from 4 animals; $^{*}P < 0.05$, $^{***}P < 0.001$ vs. control rats; $\#P < 0.05$, $\#\#P < 0.01$ vs. diabetic rats. ANOVA followed by Bonferroni's post hoc test. (F) Representative confocal images of each experimental group showing degenerating cell bodies (arrows) stained with Fluoro-Jade B. Magnification 400×. Bar: 20 µm. Legend: GCL — ganglion cell layer; IPL — inner plexiform layer; INL — inner nuclear layer; ONL — outer nuclear layer; PRL — photoreceptor layer.

sitagliptin for a month promoted a strong inhibition of DPP-IV activity and concomitantly increased levels of active plasma GLP-1 levels [36]. In our study, 2 weeks of treatment with sitagliptin was able to inhibit by 70% the activity of DPP-IV in the serum of diabetic animals, compared to untreated animals. Furthermore, we observed a positive correlation between serum DPP-IV activity and its serum protein levels in diabetic animals. The same correlation has also been described for type 2 diabetes and other diseases, like rheumatoid arthritis [32,37], suggesting that

increased DPP-IV activity in serum may reflect the increase in DPP-IV levels. Indeed, we found that the inhibition of DPP-IV activity also promoted a decrease in its serum levels. In the retina, sitagliptin was also able to prevent the increase in DPP-IV levels induced by diabetes. It remains to be clarified if the positive correlation between activity and DPP-IV levels is maintained for DPP-IV in the retina.

Increased retinal vascular permeability, which may cause macular edema, is a well-established consequence of diabetes, and is one of the first detectable signs of the development of diabetic retinopathy [2]. In our animal model of diabetes, increased BRB permeability was observed one month after STZ injection. Consistently, it has been shown that within 1 week after STZ induction of diabetes, diabetic rodents demonstrate increased BRB permeability to high-molecular weight molecules [38]. In the present study, sitagliptin effectively inhibited the increased permeability of retinal vessels induced by diabetes.

It is well established that diabetes-induced BRB breakdown is mainly due to TJ complex disassembly [3,4,7,8]. We showed that sitagliptin protected the barrier function by preventing the downregulation or subcellular redistribution of the TJ proteins claudin-5, occludin, and ZO-1. Sitagliptin prevented the decreased ZO-1 and claudin-5 immunostaining in retinal endothelial cell plasma membrane, and the intracellular accumulation of occludin induced by diabetes. Consistently, we and others previously observed a reduction in occludin content and a subcellular redistribution, due to translocation from the plasma membrane to intracellular compartments of endothelial cells in response to diabetes, with concomitant increased vascular permeability [4,8,25,39]. These changes appear to be correlated with increased occludin phosphorylation, which may target this protein to degradation [7] and also alter its interaction with ZO-1 and ZO-2, compromising the integrity of the TJ [40]. ZO-1 decreased levels and changes in its localization and phosphorylation state also appear to be associated with increased endothelial permeability [3,4,7]. Moreover, a decrease in the protein levels of claudin-5 has also been correlated with increased vascular permeability induced by diabetes [4].

Several studies have implicated a chronic low-grade inflammation in the pathogenesis of diabetic retinopathy. Elevated levels of proinflammatory cytokines and adhesion molecules have been detected in the vitreous of diabetic patients with diabetic retinopathy and in diabetic rat retinas [11,12,25,41]. Moreover, increased levels of both IL-1β and ICAM-1 have been correlated with increased retinal vascular permeability [4,10,13]. Our results are consistent with these findings, since we observed increased IL-1β and ICAM-1 levels in the diabetic retinas, along with an increase in BRB permeability. Sitagliptin seems to have an anti-inflammatory effect, because it prevented the increase of both inflammatory mediators in the retinas of diabetic animals, which might contribute for the prevention of the BRB breakdown. In fact, we and others have previously reported that this DPP-IV inhibitor decreases IL-1β levels in the serum, pancreas and retina of Zucker diabetic fatty (ZDF) rats [25,33,42], as well as in the serum of type 2 diabetic patients [43].

Studies have shown that both hyperglycemia and IL-1β are shown to activate nuclear factor-κB (NF-κB) leading to the upregulation of cell surface expression of adhesive proteins, namely ICAM-1, in endothelial cells [44,45] A recent in vitro study also showed that sitagliptin promotes a dose dependent inhibition of tumor necrosis factor induction of ICAM-1, through an inhibition of NF-κB expression, and that this effect was both GLP-1-dependent and independent [46]. Furthermore, capillary occlusion by inflammatory mediators has been shown to contribute to the formation of acellular capillaries which are considered one of the early markers of diabetic retinopathy, leading to the progression of cell death and ischemia [47]. Thus, by inhibiting the inflammatory processes, sitagliptin could have cytoprotective effects and prevent some of the vascular alterations induced by diabetes.

It has been largely demonstrated that chronic hyperglycemia and inflammation can lead to the activation of cell death pathways in vascular and neuronal cells in diabetic retinopathy [48]. The present results indicate that diabetes increased DNA fragmentation as visualized by TUNEL labeling and increased levels of the pro-apoptotic protein Bax. In the majority of the previous studies, the quantification of TUNEL-positive cells was performed in retinal whole mounts, and the relative changes in the number of TUNEL-positive cells between diabetic retinas and control are about 8–9 fold [49,50]. Similar to those reports, our data showed an 8 fold-increase in the number of TUNEL-positive cells in the retinas of diabetic animals when compared to control. The apoptotic cells were detected in both outer and inner layers, mainly at the outer nuclear and ganglion cell layers in diabetic retinas. Although apoptotic cells and reduction of layer thickness have been detected in the outer nuclear layer [24,51], the majority of the studies claim that the most prevalent alterations occurring after 1 month of STZ-induced diabetes affect primarily the ganglion cell layer and inner nuclear layer [49,50].

It has been claimed that neuronal cell death can occur early in the retinas of diabetic animals, but this is still a controversial issue, namely regarding how early neuronal death can occur and which cell types can be mainly affected.

Additionally, we found that diabetic retinas presented an increased number of degenerative neurons, positively stained for Fluoro-Jade B. Fluoro-Jade appears to be a more ubiquitous labeling agent than was originally described. Indeed, a non-specific staining in astroglia processes was also observed, but only in diabetic retinas, indicating that Fluoro-Jade can stain reactive glial cells, as already described by others [31,52]. Nonetheless, reactive astroglia staining appeared to be decreased in the retinas of diabetic animals treated with sitagliptin.

We have previously reported, in ZDF rats, that sitagliptin reduced the pro-apoptotic state and cell death in the retina [25]. Similarly, in the present study, using a type 1 diabetes animal model, we found that sitagliptin was able to prevent the upregulation of the pro-apoptotic protein Bax, the increase in the number of TUNEL-positive cells and degenerating neuronal cells, suggesting that the inhibition of DPP-IV induces neuroprotective effects in the diabetic retinas.

Furthermore, it was described that intravitreal injection of exendin-4 (a GLP-1 analog), in STZ-induced diabetic animals, could prevent the reduction in retinal thickness and cell loss, especially in the outer nuclear layer [24]. This indicates that the anti-apoptotic effects observed in our model might be mediated trough the activation of GLP-1R present in the retina, since sitagliptin stabilizes GLP-1. In fact, it has been described that activation of incretin receptors in pancreatic β-cells can promote resistance to apoptosis through the activation of several pathways leading to the inhibition of caspase-3, by increasing the expression of Bcl-2 and decreasing the expression of Bax [53,54]. Moreover, recent studies in rodents have also provided evidence of neuroprotective effects of GLP-1 in the brain [55].

Although it remains to be clarified whether it is vascular or neuronal dysfunction that appears first in the development of diabetic retinopathy, there is no doubt that the neurovascular unit homeostasis is crucial to the structural and functional integrity of the retina. So, it is desirable to prevent or slow down both the neuronal and vascular damages, as a result of prolonged hyperglycemia.

Since the biological activity of a large number of chemokines, adipokines, neuropeptides, and incretins is altered by DPP-IV, the inhibition of this enzyme might have multiple pleiotrophic effects. Taking that into account, future studies are required to unravel the molecular mechanisms behind the protective effects of sitagliptin in the diabetic retina, and also to establish whether these effects are GLP-1 dependent or independent.

In the present report, we have found that sitagliptin prevents BRB breakdown, and TJ disassembly has anti-inflammatory and anti-apoptotic effects. Thus, for the first time, we provide evidence that sitagliptin can have protective effects in the diabetic retina by a mechanism independent of increased insulin secretion. Further studies are warranted in order to better understand the molecular mechanisms behind the observed beneficial effects, so that sitagliptin could be

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envisaged as a strong candidate for further consideration as a therapeutic drug in reducing the retinal complications of diabetes.

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Conflict of interest

The authors have no conflict of interest to declare.

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