Andreia Fernandes Dâmaso Gonçalves

CAN DPP-IV INHIBITORS OR GLP-1 ANALOGS BE TOMORROW'S THERAPY FOR DIABETIC RETINOPATHY?

Doctoral Thesis in Biomedical Engineering,
supervised by Doctor Rosa Cristina Simões Fernandes and Doctor António Francisco Ambrósio,
presented to the Faculty of Sciences and Technology of the University of Coimbra.

February 2016

Universidade de Coimbra

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Inibidores da DPP-IV e Análogos do GLP-1: Futuros Agentes Terapêuticos para a Retinopatia Diabética?

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Cover: Rat retinal whole-mount immunostained for the tight junction protein claudin-5, obtained by confocal microscopy. 100 x magnification.

Support

This work was conducted at the Institute for Biomedical Imaging and Life Sciences (IBILI), Faculty of Medicine, University of Coimbra, Portugal and at the Kellogg Eye Center - University of Michigan, Ann Arbor, Michigan, USA.

The presented work was supported by a Fulbright Research Fellowship and a PhD fellowship (SFRH/BD/103936/2014) from the Portuguese Foundation for Science and Technology (FCT) awarded to the author; by the European Foundation for the Study of Diabetes (EFSD)/ Glaxo Smith Kline (GSK) Programme and GIFT/Portuguese Society of Diabetology research grants awarded to R. Fernandes; by the FCT research grant PTDC/NEU-OSD/1113/2012 awarded to A.F. Ambrósio and by the FCT Strategic Project (PEst-C/SAU/UI3282/2011-2013 and UID/NEU/04539/2013), COMPETE-FEDER.

Agradecimentos/Acknowledgments

À Doutora Rosa Fernandes pela orientação, discussões e críticas construtivas cruciais à realização deste trabalho. Obrigada pelo voto de confiança, incentivo e apoio constante ao longo destes anos.

Ao Doutor Francisco Ambrósio pela co-orientação, disponibilidade e interesse manifestados na elaboração deste trabalho. Obrigada pelas críticas e sugestões que contribuíram para o enriquecimento deste trabalho.

To Dr. David Antonetti, thank you for promptly accepting me in your group, for all your help and support. Thank you for the scientific discussions that greatly contributed to improve the quality of this work.

A todos os colegas e amigos de laboratório, obrigada pela partilha dos bons e maus momentos, pelo incentivo, amizade e boa disposição constante.

Aos meus pais e avós, pelo apoio incondicional e palavras de encorajamento em todos os momentos desta caminhada.

A todos aqueles que de alguma forma contribuíram com a sua generosidade, amizade e disponibilidade para a realização deste trabalho.

Publications

The results presented in this dissertation have been published or submitted for publication in international peer-reviewed scientific journal:

Gonçalves, A., Marques, C., Leal, E., Ribeiro, C.F., Reis, F., Ambrósio, A.F. and Fernandes, R. (2014), Sitagliptin prevents blood-retinal barrier breakdown, inflammation and neuronal cell death in the retina of type 1 diabetic rats. Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease, 1842: 1454–1463. doi: 10.1016/j.bbadis.2014.04.013

Gonçalves, A., Lin, C.M., Muthusamy, A., Fontes-Ribeiro, C., Ambrósio, A.F., Abcouwer, S.F., Fernandes, R. and Antonetti, D.A. Protective effect of a GLP-1 analog on ischemia-reperfusion induced blood-retinal barrier breakdown and inflammation. (Under revision in Investigative Ophthalmology & Visual Science)

The following publications were under the scope of the present dissertation:

Fernandes, R., **Gonçalves, A.,** and Cunha-Vaz, J. (2012), Chapter 6: Blood-Retinal Barrier. The Fundamentals in Ocular drug delivery: Barriers and application of nanoparticulate systems. Eds Deepak Thassu and Gerald Chader; 111-132. doi: 10.1201/b12950-9

Gonçalves, A., Ambrósio, A., Fernandes, R. (2013), Regulation of claudins in blood-tissue barriers under physiological and pathological states. Tissue Barriers; 1:e24782. doi: 10.4161/tisb.24782

Note: The results presented in this dissertation, included in Chapter 2 and 3, are formatted according to the style of the journal where the papers were published or submitted for publication, with minor modifications.

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Abbreviations

AGE	advanced glycation end products
AMC	aminomethylcoumarin
BBB	blood-brain barrier
BRB	blood-retinal barrier
BREC	bovine retinal endothelial cells
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CCL2	C-C motif chemokine ligand 2
CNS	central nervous system
COX-2	cyclooxygenase-*2*
CREB	cAMP-response element binding protein
DME	diabetic macular edema
DMEM	Dulbecco's modified Eagle's medium
DPP-IV	dipeptidyl peptidase-IV
DTT	dithiothreitol
ECL	enhanced chemiluminiscence
Ex-4	Exendin-4
FBS	fetal bovine serum
GCL	ganglion cell layer
GFAP	glial fibrillary acid protein
GIP	gastric inhibitory polypeptide
GLP-1	glucagon-like peptide-1
GLP-1R	glucagon-like peptide-1 receptor
HbA_{1c}	glycosylated hemoglobin
HMGB1	high-mobility group box 1
HUVEC	human umbilical vein endothelial cells
IB4	isolectin B4
ICAM-1	intercellular adhesion molecule-1
IL	interleukin

INL	inner nuclear layer
IOP	intraocular pressure
IPL	inner plexiform layer
IR	ischemia-reperfusion
JAM	junctional adhesion molecule
MCM	microglia conditioned medium
MCP-1	monocyte chemotactic protein 1
MMP	matrix metalloproteinases
NF-κB	nuclear factor-κB
NFL	nerve fibre layer
NO	nitric oxide
NOS2	nitric oxide synthase 2
ONL	outer nuclear layer
OPL	outer plexiform layer
PBS	phosphate-buffered saline
PDR	proliferative diabetic retinopathy
PFA	paraformaldehyde
PKC	protein kinase C
PRL	photoreceptor layer
qRT-PCR	quantitative real-time polymerase chain reaction
RAGE	advanced glycation end products receptor
ROS	reactive species of oxygen
RPE	retinal pigment epithelium
SDS	sodium dodecyl sulfate
STZ	streptozotocin
TJ	tight junctions
TNF	tumor necrosis factor
Tx-100	Triton X-100
VCAM-1	vascular adhesion molecule-1
VEGF	vascular endothelial growth factor
ZDF	Zucker diabetic fatty
ZO	zonula occludens

Resumo

A retinopatia diabética é uma das complicações associadas à diabetes e uma das principais causas de perda de visão na população em idade ativa em todo o mundo. No entanto, os tratamentos disponíveis para esta doença são escassos, invasivos, não eficazes para todos os doentes, apresentam alguns riscos adversos e são normalmente administrados nas fases tardias da retinopatia diabética, quando a visão já se encontra significativamente comprometida. Desta forma, novas estratégias terapêuticas instituídas nos estadios iniciais da retinopatia diabética poderão ser efetivas para retardar ou atenuar a progressão da doença, prevenindo a perda de visão.

Terapias antidiabéticas baseadas nas incretinas, os inibidores da dipeptidil-peptidase-IV (DPP-IV) e os agonistas do recetor do peptídeo semelhante ao glucagão (GLP-1), melhoram o controlo glicémico em doentes com diabetes do tipo 2 através do aumento da secreção de insulina. Estudos anteriores do nosso laboratório demonstraram que a sitagliptina (um inibidor da DPP-IV) previne o stresse nitrosativo, a inflamação e a apoptose das células da retina, exercendo ainda efeitos protetores ao nível da barreira hemato-retiniana (BHR) e melhorando o controlo glicémico num modelo animal de diabetes do tipo 2.

Até ao início deste trabalho a informação existente sobre os efeitos diretos das terapias baseadas na modulação das incretinas na retina diabética era escassa. Nesse contexto, o principal objetivo da primeira parte deste trabalho foi investigar os efeitos da sitagliptina, independentes do seu efeito insulinotrópico, na retina diabética. Para tal foi usado um modelo animal de diabetes do tipo 1 induzida por estreptozotocina. O tratamento com sitagliptina (5 mg/kg/dia) foi iniciado após duas semanas de diabetes e prolongou-se por mais duas semanas, não se tendo observado qualquer efeito nos níveis de glicemia ou de insulina nos animais diabéticos. A sitagliptina preveniu o aumento da atividade e dos níveis da enzima DPP-IV no soro e na retina, induzido pela diabetes. A sitagliptina preveniu o aumento da permeabilidade da BHR, bem como alterações das proteínas das junções oclusivas induzidas pela diabetes. Além disso, a sitagliptina preveniu o aumento dos níveis da citocina pró-inflamatória interleucina-1β e da molécula de adesão intercelular-1 nas retinas diabéticas. A sitagliptina preveniu ainda o

aumento dos níveis da proteína pró-apoptótica Bax, o aumento do número de células positivas a TUNEL e a degenerescência de células neuronais nas retinas diabéticas.

Os resultados obtidos demonstram que a sitagliptina previne eficazmente a rutura da BHR e algumas das alterações que contribuem para a disfunção da BHR nas fases iniciais da retinopatia diabética, tais como a inflamação e a morte celular.

Uma vez que o mecanismo de ação proposto para a sitagliptina baseia-se no aumento dos níveis endógenos do GLP-1, pretendeu-se esclarecer se os efeitos benéficos da sitagliptina nas retinas diabéticas foram, de alguma forma, mediados pelo GLP-1. Com a finalidade de esclarecer essa questão, na segunda parte deste estudo, foram avaliados os potenciais efeitos benéficos de um agonista do recetor do GLP-1, a Exendina-4 (Ex-4), contra a rutura da BHR e inflamação. Foi usado um modelo animal de isquemia-reperfusão (IR) que apresenta alterações semelhantes às observadas na retinopatia diabética.

A IR ocular foi induzida em ratos através do aumento da pressão intra-ocular durante 45 min seguido de 48 h de reperfusão. O tratamento com Ex-4 (10 μg/kg) (duas administrações iniciais antes da isquemia e depois a cada 12 h durante o período de reperfusão) preveniu o aumento da permeabilidade vascular da retina induzido por IR. No entanto, estudos *in vitro* utilizando culturas primárias de células endoteliais da retina mostraram que o Ex-4 não preveniu o aumento da permeabilidade induzido por agentes permeabilizantes. O Ex-4 inibiu ainda a expressão de vários genes inflamatórios nas retinas de ratos sujeitos a IR. Em cultura de células da microglia, o Ex-4 reduziu a resposta inflamatória ao lipopolissacarídeo, muito provavelmente devido à inibição do fator nuclear-kB e à ativação do CREB. Além disso, a inibição da ativação das células da microglia foi capaz de proteger contra a disfunção da barreira de células endoteliais.

Este estudo sugere que o Ex-4 previne a rutura da BHR e a inflamação induzidas por IR através da inibição da produção de citocinas inflamatórias pelas células da microglia.

Em resumo, estes resultados oferecem uma nova perspetiva sobre os efeitos protetores das terapias baseadas nas incretinas contra a disfunção da BHR, inflamação e apoptose das células da retina. Com este trabalho foi ainda possível desvendar um novo mecanismo que ajuda a explicar os benefícios destas terapias na retinopatia diabética, isto é, a modulação da resposta pró-inflamatória nas células da microglia. Este trabalho contribui ainda para ajudar a criar condições para a utilização destas terapias não só

para a retinopatia diabética, mas como para outras patologias oculares caraterizadas por um aumento da permeabilidade vascular e inflamação.

Palavras-chave: barreira hemato-retiniana; exendina-4; inflamação; retinopatia diabética; sitagliptina.

Abstract

Diabetic retinopathy, a major complication of diabetes, is a leading cause of vision loss and blindness in working-age population worldwide. However, the treatments available are scarce, invasive, inefficient to a high percentage of patients, present some adverse risks, and are typically targeted for the later stages of diabetic retinopathy, when vision has already been significantly affected. Therefore, novel therapeutic approaches targeting the early stages of diabetic retinopathy could slow down the progression of the disease, being protective against vision loss.

Incretin-based antidiabetic agents, the dipeptidyl peptidase-IV (DPP-IV) inhibitors and glucagon-like peptide-1 (GLP-1) receptor agonists, improve glycemic control by increasing insulin secretion in patients with type 2 diabetes. We have previously demonstrated that the DPP-IV inhibitor sitagliptin prevents nitrosative stress, inflammation and apoptosis in retinal cells and exerts beneficial effects on the blood-retinal barrier (BRB), while improving glycemic control in an animal model of type 2 diabetes.

When this work started, the available information about the direct effects of incretin-based therapies in the diabetic retina was scarce. In this context, the main purpose of the first part of this work was to investigate the insulinotropic-independent effects of sitagliptin in the diabetic retina, by using an experimental model of streptozotocin-induced type 1 diabetes. Treatment with sitagliptin (5 mg/kg/day) started after two weeks of diabetes and lasted for two additional weeks. Sitagliptin treatment did not affect glycemia or insulin levels in diabetic animals. Sitagliptin prevented the diabetes-induced increase in both DPP-IV activity and DPP-IV levels in serum and retina. Sitagliptin prevented the increase of BRB permeability as well as alterations in tight junction proteins induced by diabetes. Furthermore, sitagliptin was able to prevent the upregulation of the pro-inflammatory cytokine interleukin-1β and the intercellular adhesion molecule-1 in the diabetic retinas. Sitagliptin also prevented the upregulation of the pro-apoptotic protein Bax, the increase in the number of TUNEL-positive cells and degenerating neuronal cells in the retina of diabetic animals.

Altogether, these data show that sitagliptin is efficient in preventing BRB breakdown and associated features, such as inflammation and cell death, which are known to underlie and contribute to BRB dysfunction in the early stages of diabetic retinopathy.

Since the proposed mechanism of action of sitagliptin lies in increasing the endogenous levels of GLP-1, we further aimed to elucidate whether the beneficial effects of sitagliptin in the diabetic retinas were somehow mediated by GLP-1. To clarify this issue, in the second part of this study, the potential beneficial effects of a GLP-1 receptor agonist, Exendin-4 (Ex-4), against BRB breakdown and inflammation were evaluated. For this purpose, an animal model of ischemia-reperfusion (IR) injury, presenting alterations known to occur in diabetic retinopathy, was used.

Ocular IR injury was induced in rats by increasing the intraocular pressure for 45 min followed by 48 h of reperfusion. Ex-4 (10 μg/kg) treatment (two initial administrations prior to ischemia and then every 12 h for the next 48 h during the reperfusion period) was able to prevent the increase in retinal vascular permeability triggered by IR injury. However, *in vitro* studies, using primary cultures of retinal endothelial cells, showed that Ex-4 fails to prevent the increase in permeability triggered by permeabilizing agents. Ex-4 also inhibited the expression of IR-responsive inflammatory genes in the rat retinas. In cultured microglial cells, Ex-4 decreased the inflammatory response to lipopolysaccharide, most likely due to inhibition of nuclear factor-κB and CREB activation. Furthermore, inhibition of microglia activation was able to protect against endothelial cell barrier dysfunction *in vitro*.

This study suggests that Ex-4 can prevent IR injury induced-BRB breakdown and inflammation through inhibition of inflammatory cytokine production by activated microglia.

The results presented herein provide a new insight into the protective effects of incretin-based therapies against BRB breakdown, inflammation and retinal cell apoptosis. Additionally, this work reveals a new mechanism that helps explaining the benefits of the incretin-based therapies in diabetic retinopathy, i.e., : the modulation of the pro-inflammatory response in microglial cells. Moreover, this work helps to envisage these therapies for diabetic retinopathy, as well as for other ocular pathologies characterized by increased vascular permeability and inflammation.

Keywords: blood-retinal barrier; diabetic retinopathy; exendin-4; inflammation; sitagliptin.

Chapter 1
Introduction

1.1 The retina

1.1.1 Anatomy of the retina

The retina is a light sensitive tissue located at the posterior part of the eye, and its main function is to convert light signals into electrical impulses that are sent through the optic pathway to the visual cortex for further processing. Due to its peripheral location and easy access for examination, the retina is the most extensively studied part of the central nervous system (CNS) (Hildebrand and Fielder, 2011). It is in the retina that around 80% of all sensory information in humans are thought to be originated (Sharma et al., 2003; Hildebrand and Fielder, 2011), which reveals the importance of a healthy retinal function.

The anatomic structure of the retina is composed of two main layers: an inner neurosensory layer adjacent to the vitreous of the eye and an outer layer composed of epithelial cells called the retinal pigment epithelium (RPE), which are tightly attached to the choroid. The neurosensory retina is divided into seven distinct layers, including (from the vitreous), the nerve fibre layer (NFL), the ganglion cell layer (GCL), the inner plexiform layer (IPL), the inner nuclear layer (INL), the outer plexiform layer (OPL), the outer nuclear layer (ONL) and the photoreceptor layer (PRL) (Figure 1.1). There are four major types of retinal cells: neurons; glial cells (Müller cells, astrocytes and microglia cells); retinal pigment epithelial cells; and the vascular cells (endothelial cells and pericytes) (Figure 1.1) (Hildebrand and Fielder, 2011).

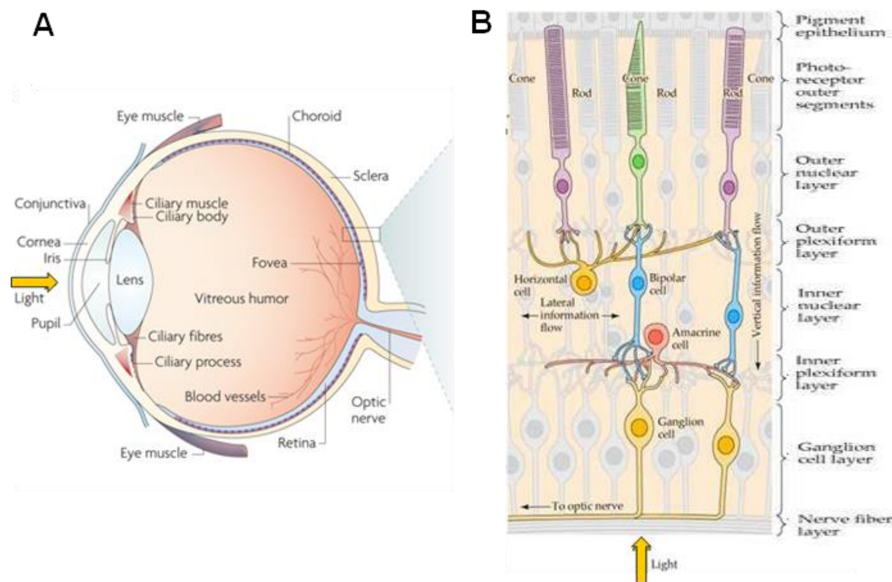

Figure 1.1. Anatomy of the retina. (A) Schematic representation of the human eye with focus on retina localization (adapted from Wright *et al.*, 2010) **(B)** Schematic representation of a retinal cross section showing the different layers and cell types of the retina (adapted from Purves and Williams, 2001).

1.1.1.1 The neuronal retina

Although constituted by several other types of cells, the retina is predominantly enriched in neurons that, as mentioned above, are allocated into five major types: photoreceptors, bipolar cells, horizontal cells, amacrine cells and ganglion cells. The retinal layer adjacent to the RPE is composed exclusively by photoreceptors. They are sensitive to light and are responsible for phototransduction process, converting light photons into electrical signals (Levin, 2003). These pulses are then processed by layers of neighboring neurons, and then transmitted along the axons of ganglion cells which converge to form the optic nerve, this pathway is known as vertical or direct pathway (Figure 1.2). On the other hand, signals from the photoreceptors may follow a lateral pathway, where the horizontal and amacrine cells provide the integration of visual signals (Figure 1.2). The three major types of neural cells involved in the direct pathway, which transmits impulses generated by light to the brain are then: photoreceptors (first-order neurons), bipolar cells (second-order neurons) and ganglion cells (third-order

neurons). The activity of these cells is modulated by other cell types including horizontal cells and amacrine cells, thereby indirectly affecting the transmission of visual information for the eye to the brain (Forrester *et al.*, 2016).

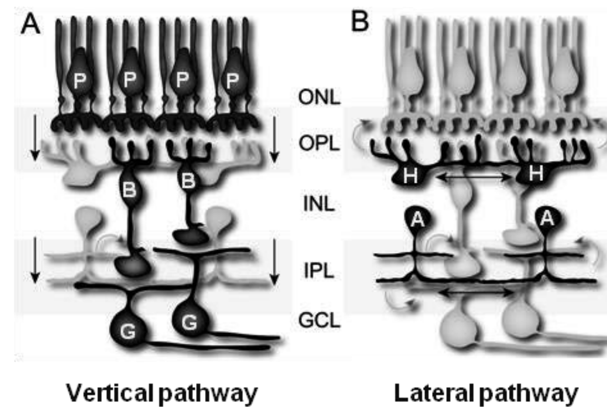

Figure 1.2. Visual information flow in the retina. (A) Vertical pathway. Signals flow from photoreceptors to bipolar cells and from these to ganglion cells. **(B)** Lateral pathway. In the outer retina, horizontal cells integrate visual signals laterally over large areas by sharing their membrane potential. In the inner retina, amacrine cells also perform lateral integration via either electrical or conventional synapses (adapted from Joselevitch, 2008). Legend: P, photoreceptor; B, bipolar cell; G, ganglion cell; H, horizontal cell; A, amacrine cell; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer.

Photoreceptor cells

Photoreceptors are the first-order neurons, lying on the outermost layer of the retina perpendicularly to the RPE layer. There are two main types of photoreceptors: rods and cones. The human retina contains approximately 77-107 million rods and 4-5 million cones (Hildebrand and Fielder, 2011). Rods are responsible for sensing motion, contrast and brightness, while cones are required for colour vision, perception of detail and spatial resolution (Kolb *et al.*, 2001). Each individual photoreceptor is organized in an outer segment, inner segment, a nucleus, an inner fiber and the synaptic terminal (Hildebrand and Fielder, 2011).

The outer segments facing the apical microvilli of RPE cells contain membranous disks with the photo-capturing photopigments and proteins responsible for light absorption

and initiation of neuronal signals. In the inner segment of the photoreceptor are present the nucleus and all the cellular machinery necessary for the highly metabolic demand of these cells and the biosynthesis of the membranous disks, such as mitochondria and smooth and rough endoplasmatic reticulum. As the new disks are formed at the base of the outer segment, the oldest disks at the top are shed into the subretinal space between the neural retina and the RPE (Young, 1976). The inner fiber acts as the axon of the photoreceptor cell and transmits the signals to the OPL via its synaptic terminals (Hildebrand and Fielder, 2011).

Bipolar and ganglion cells

Bipolar cells are the second-order neurons, responsible for transmitting signals from the photoreceptors and horizontal cells to the ganglion cells (Kolb *et al.*, 2001). They are located in the middle layer of the retina, with their dendrites placed in the OPL, their nuclei and cell body in the INL and their axons in the IPL. Bipolar cells form synapses with either rods or cones, but never with both, designating them by rod bipolar or cone bipolar cells. Cone bipolar cells may make contact with as few as just one cone, while in the peripheral retina one rod bipolar cell may receive input from up to 70 rods (Hildebrand and Fielder, 2011).

The ganglion cells are the third-order neurons, responsible for transmitting the integrated signal from the retina to the visual cortex (Usui *et al.*, 2015) and up to 20 different types of ganglion cells have been described in the human retina (Hildebrand and Fielder, 2011). The large cell bodies of the ganglion cells are located within the innermost nucleated layer of the retina, the GCL, with their dendrites located in the IPL. Dendrites from these cells can receive impulses from bipolar and amacrine cells. Ganglion cell axons form the optic nerve and establish synapses in the lateral geniculate nucleus and the superior colliculus, brain areas involved in visual perception (Dacey, 1999; Forrester *et al.*, 2016).

Horizontal and amacrine cells

Horizontal and amacrine cells are both interneurons present in the INL. Localized at opposite margins of the INL, they form homotypic and heterotypic connections within

the IPL and OPL, respectively (Usui *et al.*, 2015). Horizontal cells are characterized by wide horizontal extensions of their processes and their cell bodies are located in the outer portion of the INL. They help integrate and regulate the input from multiple photoreceptor cells into bipolar cells at the OPL and are responsible for allowing the retina to adjust in different light intensity situations.

Amacrine cells, known by their large size and oval shape, are mostly inhibitory neurons with their cell bodies located in the INL and their dendritic arbores to the IPL where they interact with the RGC and/or bipolar cells (Balasubramanian and Gan, 2014). Likewise horizontal cells, amacrine cells also modulate the output of bipolar cells, enhancing motion perception and sensitivity to different light intensities (Forrester *et al.*, 2016).

1.1.1.2 The retinal glial cells (neuroglia and microglia)

In the human retina there are two main types of glial cells: the macroglia, constituted by astrocytes and Müller cells (also referred as neuroglia), and microglia (Figure 1.3). Due to their organization and spatial arrangement in the retina, glial cells are considered to be the bridge between the neurons and the retinal vasculature, providing immunosurveillance, nutritional and regulatory support.

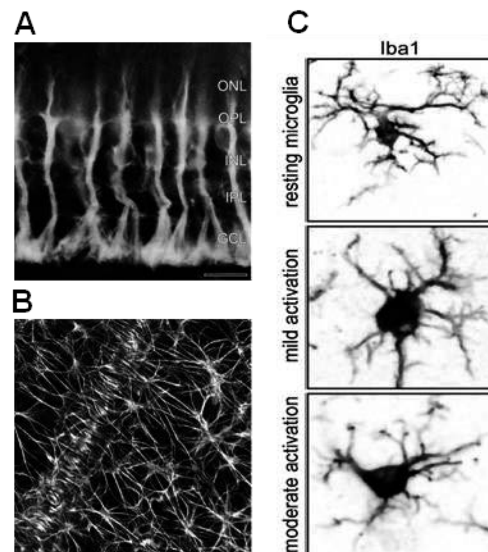

Figure 1.3. Retinal glial cells. (A) Immunostaining of a rabbit retina for vimentin, a filament protein abundant in Müller cells. These glial cells are evenly spaced throughout the tissue and span the whole retinal thickness. Scale bar: 20 µm (adapted from Robinson and Dreher, 1990). **(B)** Astrocytes immunoreactive for glial fibrillary acid protein (GFAP) in a whole-mounted retina. Astrocyte end-feet ensheathe a blood vessel (adapted from Barber *et al.*, 2000). **(C)** Microglia immunoreactivity for Iba1. High-magnification visualization of resting/surveilling, or nonactivated, and activated microglia reveals differences in both immunoreactivity and morphology (adapted from VanGuilder *et al.*, 2011). Legend: GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer.

Astrocytes

Found predominately in the GCL and NFL, but also found in the IPL and INL, astrocytes are thought to derive from stem cells in the optic nerve (Provis, 2001). Morphological hallmarks include flattened cell body and a fibrous series of long unbranched cellular processes, giving a star shape appearance to these cells. They are mainly found around the retinal blood vessels and their pedicles are believed to contribute to the maintenance and function of the blood-retinal barrier (BRB). Recent research suggests that they also play an important role in retinal inflammation and injury (Wang *et al.*, 2014). Several animal studies have demonstrated the close relation between the BRB and astrocytes as avascular retinas lack astrocytes and their appearance only in vascularized parts of the retina (Stone and Dreher, 1987; Schnitzer, 1988).

Müller cells

Müller cells are the main glial cells of the retina (Saint-Geniez and D'Amore, 2004; Hildebrand and Fielder, 2011) and their origin is the same as the retinal neurons (Fischer and Reh, 2003). With a radial orientation across the retina to form both the outer and inner limiting membranes, Müller cells may be considered one of the most crucial retinal cells. They play a vital role in maintaining the local environment that allows the visual process to function normally (Kolb *et al.*, 2001). Together with astrocytes, Müller cells provide the essential substrates from the blood circulation to neurons and contribute also to the properties of barrier in the retinal capillaries (Gardner *et al.*, 1997) and synaptic function (Newman, 2003). Additionally, Müller cells regulate ionic and osmotic balance (Reichenbach *et al.*, 2007), are involved in glutamate/glutamine cycle which maintains a balanced retinal homeostasis and have an important role in controlling neurotransmission as well as in protecting neurons against glutamate excitotoxicity (Lieth *et al.*, 2001).

Microglia

Microglial cells are known as the resident macrophages of the CNS and play importante roles in tissue surveillance and intercellular communication (Lee *et al.*, 2008; Karlstetter *et al.*, 2014) due to their dynamic behavior. Microglial cells enter the retina from the retinal blood vessels during development and have a phagocytic function as part of the reticuloendothelial system (Provis, 2001; Hildebrand and Fielder, 2011). Under normal conditions, resting retinal microglial cells, characterized by a highly ramified morphology, are present in the IPL and OPL and are absent from the ONL (Grigsby *et al.*, 2014). They respond to various insults, including oxidative stress, ischemia, inflammation, trauma and retinal detachment by releasing proinflammatory cytokines (Krady *et al.*, 2005) and phagocytic clearance of necrotic or apoptotic cells (Elward and Gasque, 2003). In most of the cases, when activated in response to any of the situations mentioned above, microglia change to an ameboid, and are rapidly recruited to the site of injury, where they can proliferate and release cytokines, nitric oxide and reactive species of oxygen (ROS). When activated, they may also play a role in the retinal

immune response. Nevertheless, in unhealthy situations where the prolonged and chronic activation of microglia occurs, there can be an exacerbation of microglia response which can lead to retinal damage and ultimately increase the inflammatory state (Fetler and Amigorena, 2005; Nimmerjahn A, 2005; Karlstetter *et al.*, 2015; Li *et al.*, 2015).

1.1.1.3 Retinal pigment epithelium

The RPE is composed of a single layer of retinal pigment epithelial cells that are joined laterally toward their apices by tight junctions (TJ) between adjacent lateral cell walls, forming the outer BRB. The RPE resting upon the underlying Bruch's membrane separates the neural retina from the fenestrated choriocapillaries and plays a fundamental role in regulating access of nutrients from the blood to the photoreceptors as well as eliminating waste products and maintaining retinal adhesion. The metabolic relationship of the RPE apical microvilli and the photoreceptors is considered to be critical for the maintenance of visual function (Young, 1967).

1.1.1.4 Vascular cells (endothelial cells and pericytes)

In the retinal capillaries the endothelial cells form a continuous monolayer surrounded by pericytes, constituting the primary barrier between the blood and the retinal parenchyma. Two capillary networks arising from the central retinal artery circulation provide nutritional support and waste product removal for the inner retina. One is located in the NFL and GCL and the other in the INL. Endothelial cells have secretory functions, present receptors for hormones and neurotransmitters and are responsive to vasoactive substances, thus regulating blood flow. Pericytes, also known as vascular smooth muscle cells due to their rich content of α smooth muscle actin, are located around the endothelial cells, occurring at a ratio of 1:1 with these cells in the human retina. Indeed retinal microvasculature contains a unique feature that relies on the pericytes: the endothelial cells ratio that is greater than those observed in brain capillaries (Frank *et al.*, 1990). Pericytes have contractile properties and are involved in control of blood flow through capillaries (Chakravarthy and Gardiner, 1999; Rucker *et al.*, 2000; Bandopadhyay *et al.*, 2001).

1.1.2 The blood-retinal barrier

1.1.2.1 Anatomy and function of the blood-retinal barrier

The retina is known for having the higher oxygen consumption per unit weight of tissue than any other tissue, which makes the active neural retina a highly metabolically demanding system (Arden et al., 2005). To achieve all the oxygen and nutrients needed the retina contains a complex dual vascular system, the inner two thirds are nourished by retinal circulation, formed by branches of the central retinal blood vessels and the outer third (photoreceptors) is nourished by the choroidal circulation (Figure 1.4) (Usui et al., 2015).

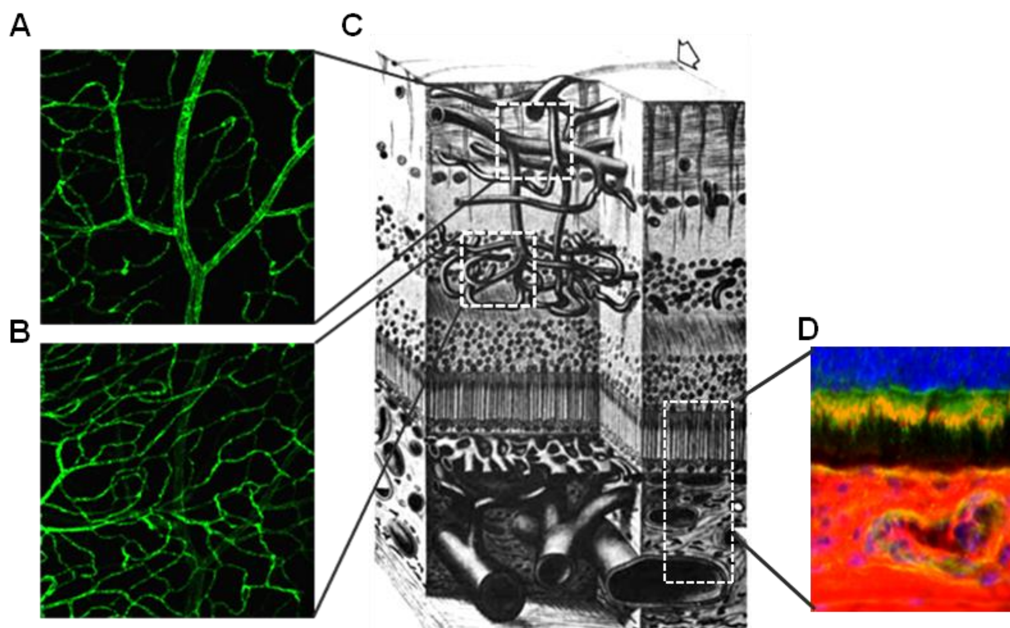

Figure 1.4. Retinal blood-supply. Retinal whole-mount immunostained for the tight-junction protein claudin-5 showing **(A)** the inner capillary network in the ganglion cell layer and **(B)** the lower capillary bed in the inner nuclear and outer plexiform layers. **(C)** Distribution of the dual separate vascular system of the retina (adapted from Bargmann, 1967). **(D)** Cross-section of a rat retina showing a large choroidal vessel, below the retinal pigment epithelium layer.

These particular and specific properties of the retinal blood supply provide the basis of the BRB function. The BRB is a highly specialized blood-neural-barrier similar to the blood-brain-barrier (BBB) that serves several functions to maintain the retinal neural

environment. It functions as a selective barrier, restricting the flux of nutrients, ions, metabolites, and harmful toxins to and from the retina.

The BRB is formed by two barriers: the inner and outer BRB. The former is a unit formed by the two beds of capillary endothelia, pericytes, supporting glial cells (Müller cells and astrocytes) and neural cells (Figure 1.5) (Antonetti *et al.*, 1999). The latter is formed by the RPE and lies on the outer surface of the photoreceptor layer. It regulates the movement of solutes between the fenestrated capillaries of the choroid and the photoreceptor layer of the retina (Antonetti *et al.*, 2006; Kaur *et al.*, 2008; Runkle and Antonetti, 2011; Klaassen *et al.*, 2013).

TJ, first described as "kissing-points" between endothelial cells of the inner BRB and between adjacent epithelial cells of the RPE provide the physical seal and define the selective barrier of the BRB (Antonetti *et al.*, 2006).

Transport across the BRB is highly regulated and exists in two main forms, the paracellular pathway which is regulated by dynamic opening and closing of intercellular junctions, and the transcellular pathway which involves specialized transport vesicles (caveolae) and receptor-mediated transport. The paracellular flow is mainly governed by TJ, present in the intercellular junction complex. This complex also includes adherens junctions, desmosomes and gap junctions (Fernandes *et al.*, 2012; Klaassen *et al.*, 2013) (Figure 1.6).

The BRB is understood today as playing a fundamental role in retinal function in both health and disease. The major diseases that affect visual function, diabetic macular edema, and "wet" age-related macular degeneration are characterized by a breakdown of the inner and outer BRB, respectively. The BRB is now at the core of our understanding of retinal disease and the development of new therapies such as steroid and anti-vascular endothelial growth factor (anti-VEGF) treatments.

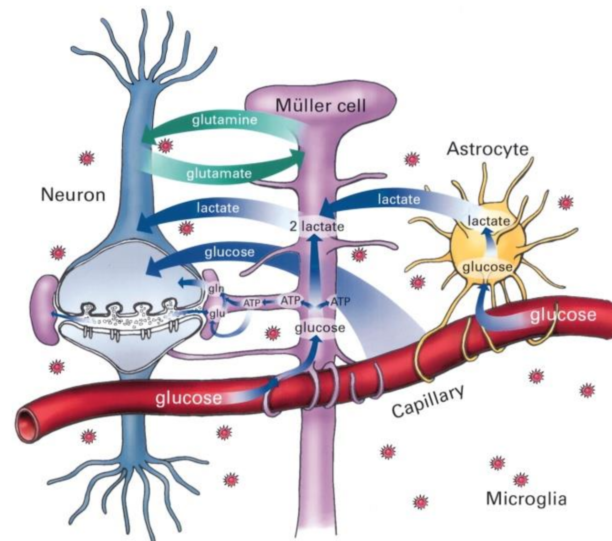

Figure 1.5. The neurovascular unit. Schematic representation of the metabolic interactions at the inner BRB, between blood vessels, astrocytes, Müller cells, and glutamatergic neurons (Antonetti *et al.*, 2006).

1.1.2.2 Tight junctions

The molecular biology of the TJ is complex as they have three major functions: barrier, fence, and a polarity function (Anderson and Van Itallie, 2009). The barrier function serves to selectively regulate the movement of ions, water, and solutes between epithelial and endothelial cells, the aforementioned paracellular flux. The fence function regulates lateral diffusion of proteins in the plasma membrane throughout the surface of the cell helping to define cellular boundaries. The third function of TJ is to aid in the establishment of cellular polarity.

TJ are located in the most apical region or in the most luminal side of epithelial or endothelial cells and are composed by a complex mixture of transmembrane proteins including occludin, claudins and junctional adhesion molecule (JAM). In addition, the transmembrane proteins embedded in the plasma membrane are attached to several cytoskeleton and cytoplasmic scaffold proteins. The major cytoplasmatic proteins are zonula occludens (ZO), ZO-1, ZO-2, ZO-3, that are part of the membrane-associated guanylate kinase proteins family, cingulin, symplekin and 7H6 (Figure 1.6) (Gardner *et al.*, 2002; Kaur *et al.*, 2008; Fernandes *et al.*, 2012). The transmembrane proteins, occludin and claudins, seal adjacent cells and form the paracellular diffusion barrier,

restricting the flow of macromelules ions and polar solutes between adjacent endothelial and epithelial cells (Furuse *et al.*, 2002). Occludin is well known to be expressed in epithelial and endothelial cells, and the level correlates with the degree of permeability of the barrier (Mitic and Anderson, 1998). Claudin-5 is a protein member of the claudin family, a group of TJ proteins, expressed on endothelial cells of the brain and retina (Antonetti *et al.*, 1998). The cytosolic proteins, ZO-1/2/3, are proteins with multiple domains, required for structural support of the TJ by establishing interactions with the transmembrane proteins and cytoskeletal components (Fanning *et al.*, 1999; Wittchen *et al.*, 1999).

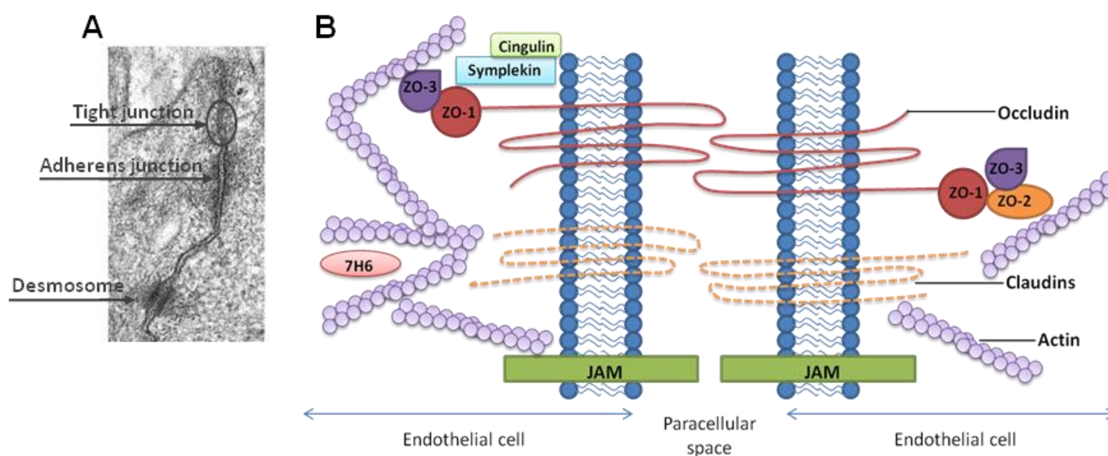

Figure 1.6. Intercellular junctions. (A) Electron micrograph showing the main types of intercellular junction in epithelial and endothelial cells (adapted from Kobielak and Fuchs, 2004) **(B)** Schematic representation of the basic structural components of TJ. TJ are composed of transmembrane proteins (occludin, claudins, and JAMs) and several cytosolic accessory proteins such as ZO proteins, cingulin, symplekin and 7H6. Transmembrane proteins are linked to the actin cytoskeleton via cytoplasmic ZO proteins.

1.2 Diabetes mellitus

Diabetes mellitus, commonly called diabetes, is a group of metabolic diseases characterized by chronic high blood glucose levels – hyperglycemia. Diabetes is characterized by low insulin production or by the lack of a proper response by the cells

to the insulin that is produced. According to the International Diabetes Federation, the current number of individuals with diabetes worldwide is about 415 million, accounting for 6-9 % of the population of Western countries, and it is estimated that by the year 2040 it will be increased to 642 million (IDF, 2015). The fact that diabetes is a disease of prolonged duration and that its incidence is rising, both in industrialized and in developing countries, highlights its contribution to morbidity and mortality in general, to the high cost for the public health system and the importance of implementation of effective and preventive therapeutic strategies (Bandello *et al.*, 2013).

According to the World Health Organization, the vast majority of cases of diabetes fall into two categories, type 1 diabetes and type 2 diabetes. Type 1 diabetes is generally diagnosed in children and young adults and accounts for approximately 5-10% of diabetes cases. Type 1 diabetes is characterized by a lack of insulin production due to autoimmune destruction of the pancreatic β cells, which requires daily administration of exogenous insulin in patients to achieve normal blood glucose levels. On the other hand, type 2 diabetes accounts for 90-95% of all diagnosed diabetes cases and is associated with lifestyle, often a consequence in obese individuals and in those with a sedentary lifestyle. In this category there is an initial insulin resistant state where the cells do not respond appropriately to insulin, leading to hyperglycemia. As hyperglycemia develops, the pancreas increases insulin production in an effort to achieve normal glucose levels, creating a state of hyperinsulinemia and developing a phenomenon called insulin resistance (Zimmet *et al.*, 2001).

The metabolic alterations associated with diabetes are closely related with the long-term damage that the chronic levels of glucose can cause, resulting, amongst others, in chronic vascular complications. In particular, macrovascular complications of diabetes result in atherosclerosis, hypertension, increased risk of myocardial infarction and stroke. Microvascular dysfunction is responsible for a number of co-morbidities associated with prolonged diabetes, such as retinopathy, neuropathy and nephropathy (Aiello, 2002). In fact, diabetic retinopathy is one of the most common microvascular complications associated with diabetes, and despite the efforts in research it remains the leading cause of blindness among working-age population (Fong *et al.*, 2004).

1.2.1 Diabetic retinopathy

Diabetic retinopathy remains one of the most common complication of diabetes and a leading cause of visual loss in industrialized nations (Fong *et al.*, 2004). Its prevalence is strongly related with the duration of the disease and epidemiological studies have demonstrated that in a follow up of 20 years nearly all patients with type 1 diabetes, and more than 60% of type 2 diabetes individuals, had some degree of retinopathy (Klein *et al.*, 1989; Fong *et al.*, 2004; Romero-Aroca *et al.*, 2010).

Diabetic retinopathy is a multifactorial condition and its pathogenesis is extremely complex, involving biochemical and metabolic abnormalities occurring in all cells of the retina (Bandello *et al.*, 2013; Stitt *et al.*, 2013). It is characterized by intraretinal specific lesions throughout the progression of the disease, and can be broadly divided into two clinical stages, non-proliferative and proliferative diabetic retinopathy (PDR) (Figure 1.7). The alterations observed in the non-proliferative stage of the disease are due to increased vascular permeability, vessel occlusion and loss of pericytes, leading to the formation of microaneurysms, hemorrhages, hard exudates, cotton wool spots and macular edema. The PDR stage progressively affects the integrity of the retinal microvessels, resulting in abnormal permeability, non-perfusion of capillaries and formation of microaneurysms (Cheung *et al.*, 2010). PDR occurs when the occlusion of retinal capillaries leads to retinal ischemia and promotes the development of new dysfunctional vessels. Typically these new blood vessels are fragile and leaky and, if left untreated, can become enveloped by fibrous connective tissue. Diabetic macular edema (DME) happens when fluid builds up and affects the macula, threatening central visual acuity, and it can result in irreversible severe vision loss or even blindness (Stitt *et al.*, 2013).

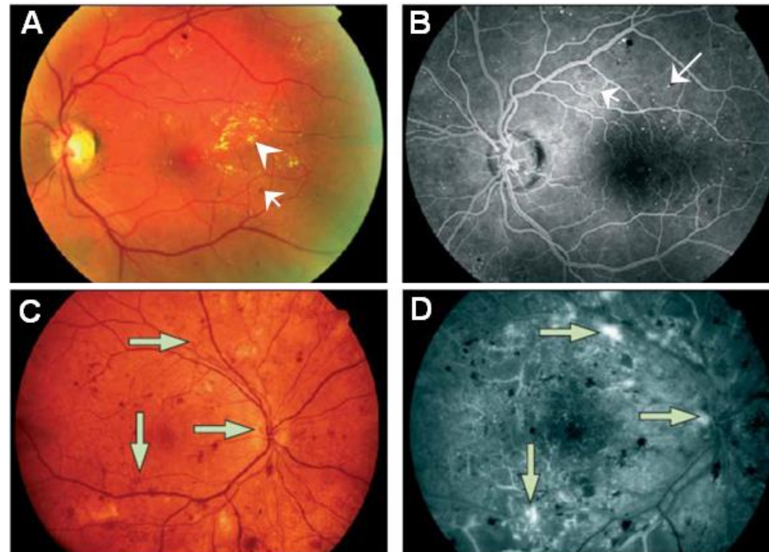

Figure 1.7. (A and C) Eye fundus photograph and **(B and D)** fluorescein angiogram from a diabetic patient with non-PDR and PDR, respectively, showing microaneurysms (long arrow, B), hemorrhages (short arrow, A and B), hard exudates (arrowhead, A), leakage (arrows, C and D) and neovascularization (adapted from Cheung *et al.*, 2010).

Several mechanisms have been described to contribute to the pathophysiology of diabetic retinopathy, such as hyperglycemia, inflammation, and neuronal dysfunction (Bandello *et al.*, 2013). Hyperglycemia is the main factor implicated with the structural and functional changes associated to diabetic retinopathy. The activation of several metabolic pathways after the onset of hyperglycemia, including increased polyol flux, increased intracellular formation of advanced glycation end products (AGE), protein kinase C (PKC) activation, oxidative stress and the increased secretion of various inflammatory cytokines have been linked to development of retinal microvascular injury (He and King, 2004; Bandello *et al.*, 2013).

In diabetic retinopathy, AGEs and their receptor (RAGE) are responsible for increasing inflammation and neurodegenerative processes (Zong *et al.*, 2011). In addition, chronic levels of glucose also activate the aldose-reductase metabolic pathway, which leads to an accumulation of polyol that stimulates oxidative stress and cellular damage (Lorenzi, 2007; Bandello *et al.*, 2013). Moreover, AGEs are also implicated in the activation of PKC (Geraldes and King, 2010; Bandello *et al.*, 2013), which is known to play a role in the increased vasopermeability and capillary occlusion, release pro-inflammatory proteins

and oxidative stress (Coral *et al.*, 2009). The stimulation of oxidative stress caused by hyperglycemia is a result of increased production of ROS and/or failure of antioxidant defense and it has been associated with diabetic retinopathy progression by damaging retinal cells (Madsen-Bouterse and Kowluru, 2008). Inflammation is another important player in the pathogenesis of diabetic retinopathy, contributing to BRB disruption, which will be discussed in more detail in the next sections.

1.2.1.1 Inflammation in diabetic retinopathy

It is well established that diabetic retinopathy has many features of a low-grade chronic inflammation such as increased vascular permeability, infiltration of immune cells, increased expression of cytokines and chemokines and microglia activation (Antonetti *et al.*, 2006). In the early stages, inflammatory mediators promote increased vascular permeability and leukocyte adhesion (Miyamoto *et al.*, 1999; Rungger–Brändle *et al.*, 2000; Joussen *et al.*, 2009). As inflammation persists certain cytokines can exacerbate the vascular lesions. Several proinflammatory mediators have been associated with the pathology, such as VEGF, nitric oxide (NO), eicosanoid, lipids, cytokines, chemokines, angiotensin II, and the renin–angiotensin system (Adamis and Berman, 2008; Abu El-Asrar *et al.*, 2013). VEGF is actually the most potent vasoactive factor inducing angiogenesis and increasing vascular permeability (Aiello *et al.*, 1997; Zhang *et al.*, 2009). VEGF has been shown to induce conformational changes in the TJ of endothelial cells and has been associated with the upregulation of an endothelial adhesion molecule, intercellular adhesion molecule-1 (ICAM-1), leading to further endothelial cell damage (Bandello *et al.*, 2013).

Elevated levels of proinflammatory cytokines, such as interleukin (IL) -1β and tumor necrosis factor alpha (TNF-α), have been also detected in the vitreous of diabetic patients with diabetic retinopathy and in diabetic rat retinas (Abu El Asrar *et al.*, 1992; McLeod *et al.*, 1995; Joussen *et al.*, 2002; Gonçalves *et al.*, 2012). The increase of IL-1β has been correlated with increase vascular permeability, possibly mediated by leukocyte adhesion, nuclear factor-κB (NF-κB) activation and retinal capillary cell death (Bamforth *et al.*, 1997; Kowluru and Odenbach, 2004). In addition, TNF-α has been implicated with diabetic retinopathy as it increases the adhesion of leukocytes to the retinal

endothelium and the permeability of BRB (Joussen *et al.*, 2002; Saishin *et al.*, 2003; Ben-Mahmud *et al.*, 2004).

It is clear that multiple retinal cells are involved in the pathogenesis of diabetic retinopathy. Retinal microglia are one of the major cells involved in inflammation seen in diabetic retinopathy. In fact, as a consequence of diabetes, microglia are activated to secrete proinflammatory cytokines that contribute to neuronal loss and BRB breakdown (Ibrahim *et al.*, 2011). Moreover, these cells also become activated in response to cytokines derived from other cell types such as astrocytes and pericytes (Romeo *et al.*, 2002). Indeed, the role of microglia in initiating chronic neuroinflammation in the diabetic retina has been extensively studied. Human and animal studies have elucidated that activated microglia stimulates a cycle of inflammation that leads to the recruitment of leukocytes, causes vascular permeability, induces glial dysfunction and neuronal cell death through the release of cytotoxic substances (Yang *et al.*, 2009; Grigsby *et al.*, 2014). Although microglia is obviously altered suggesting their activation in experimental models of diabetic retinopathy, the precise nature of the activation has not been defined (Abcouwer, 2011). In animal studies, microglia becomes activated soon after the onset of hyperglycemia and some data indicates that inhibition of microglia activation correlates with neuronal protection in the diabetic retina (Rungger–Brändle *et al.*, 2000; Zeng *et al.*, 2000; Krady *et al.*, 2005). At the early stage of diabetic retinopathy in the inner retina, microglia shifts from resting to an activated amoeboid state, with few or no processes, corresponding to an increase in retinal expression of IL-1β and TNF-α mRNA (Krady *et al.*, 2005). Retinal microglial activation in diabetic retinopathy is thought to be a normal adaptive response to retinal neurodegeneration or trigged by extravasated plasma proteins due to BRB dysfunction (Milner *et al.*, 2007). Moreover, microglia activation might also be linked to hyperglycemia and to the production of AGEs and/or other protein glycation products within the retina as the disease progresses (Zong *et al.*, 2011; Grigsby *et al.*, 2014). Once activated, microglia will also release several molecules such as glutamate, proteases, leukotrienes, IL-1β, IL-3, IL-6, TNF-α, VEGF, lymphotoxin, monocyte chemotactic protein 1 (MCP-1), matrix metalloproteinases (MMPs) and ROS, which lead to a vicious cycle causing more inflammation, enhancing microglial activation and leukocyte recruitment (Grigsby *et al.*, 2014). Understanding the role of microglia and inflammation in the progression of diabetic retinopathy is of extreme importance as

it may allow therapeutic manipulation of their activation state and ultimately prevent or delay the damage effects of retinal microglial activation and exacerbated inflammation (Grigsby *et al.*, 2014).

1.2.1.2 Blood-retinal barrier breakdown in diabetic retinopathy

The fundamental cause and the precise mechanisms behind the development of diabetic retinopathy are still unclear. However, retinal microvascular dysfunction is one of the earliest detectable changes in this disease, and BRB breakdown is considered the hallmark of diabetic retinopathy (Cunha-Vaz *et al.*, 1975). As the disease progresses, the increase in vascular permeability can lead to retinal edema, as previously mentioned, which is the main cause for vision loss (Klein *et al.*, 1995).

Both inner BRB and outer BRB are affected in diabetic retinopathy; however, the retinal endothelium seems to be a major target of diabetes (Vinores *et al.*, 1989; Carmo *et al.*, 1998). In fact, increased BRB permeability has been demonstrated to occur in diabetic patients as well as in diabetic rodent models (Cunha-Vaz *et al.*, 1975; Leal *et al.*, 2007; Leal *et al.*, 2010). As previously described, the structural and functional integrity of the BRB is mainly due to the presence of organized and dynamic structures, the junctional complexes present between endothelial cells. It is then expected that disruption of the TJ complexes will have a direct effect on BRB function. Indeed, studies have shown that chronic hyperglycemia induces changes in both levels and subcellular distribution of TJ proteins within the retinal vascular endothelium, which seem to directly contribute to increased vascular permeability (Antonetti *et al.*, 1999; Barber *et al.*, 2000; Leal *et al.*, 2007; Leal *et al.*, 2010). The most known diabetes-induced changes in the TJ complex involve the transmembrane protein occludin. Reduction in occludin content and its subcellular redistribution (translocation from the plasma membrane to intracellular compartments of endothelial cells) has been correlated with a concomitant increase in vascular permeability in animal models of diabetic retinopathy (Antonetti *et al.*, 1998; Barber *et al.*, 2000; Leal *et al.*, 2010; Gonçalves *et al.*, 2012). Such changes in vascular permeability are due to increased occludin phosphorylation, promoting its degradation (Antonetti *et al.*, 1998), and altering the interaction between occludin and ZO-1 and ZO-2, which compromises the integrity of the TJ (Kale *et al.*, 2003).

Alterations in the levels of ZO-1, in particular decreased protein levels and changes in subcellular localization have also been linked with increased endothelial permeability (Antonetti *et al.*, 1999; Leal *et al.*, 2007; Leal *et al.*, 2010). Another TJ protein that is known to be decreased in diabetic retinopathy is claudin-5, where its reduction has also been correlated with increased vascular permeability (Leal *et al.*, 2010).

In addition to the changes that occur in TJ proteins complex, other factors such as microvascular cell death, leukocyte adhesion, retinal glial cell dysfunction (astrocytes and Müller cells) and activation of microglia cells also contribute to the disruption of the BRB (Antonetti *et al.*, 2006). Moreover, inflammatory mediators have shown to promote vascular permeability, leukocyte adhesion and retinal cell death (Miyamoto *et al.*, 1999; Krady *et al.*, 2005).

1.2.1.3 Oxidative stress in the pathogenesis of diabetic retinopathy

The retina is extremely prone to oxidative stress damage due to the high oxygen consumption, elevated rate of glucose oxidation and its constant exposure to light (Anderson *et al.*, 1984).

Increasing evidence shows that increased ROS production results in the activation of several biochemical pathways (glucose autooxidation, increased flux through the polyol pathway, and increased protein glycation), which are known to be involved in the pathogenesis of diabetic retinopathy (Kowluru and Chan, 2007). It has now been established that all these biochemical processes are interconnected or might stem from the overproduction of superoxide by the mitochondrial electron-transport chain, which results in the establishment of a unifying mechanism for diabetic complications, such as diabetic retinopathy (Brownlee, 2005). In addition, the increased superoxide production seems to be the major oxidant mediator of diabetes tissue damage (Brownlee, 2005). The generation of ROS such as superoxide anion, hydroxyl radical, nitric oxide and hydrogen peroxide can cause peroxidation of lipids, upregulation of adhesion molecules and retinal vascular damage (Giugliano *et al.*, 1996). Elevated levels of superoxide anion were found in both diabetic rat retinas and endothelial cells exposed to high glucose, probably due to mitochondrial activation (Nishikawa *et al.*, 2000; Du *et al.*, 2003; Kowluru and Abbas, 2003).

Increased oxidative stress might result not only from increased generation of free radicals but also from an impairment of the antioxidant defense system, responsible for scavenging free radicals and maintaining redox homeostasis. The levels of reduced glutathione and the activity of glutathione reductase, glutathione peroxidase, superoxide dismutase and catalase are markedly diminished in the retinas of diabetic animals (Kern *et al.*, 1994; Kowluru *et al.*, 1997; Kowluru *et al.*, 2001). In diabetic rats, increased oxidative stress has been shown to promote capillary cell loss by apoptosis and an increase in the number of acellular capillaries in the retina (Ansari *et al.*, 1998; Kowluru and Odenbach, 2004).

1.2.1.4 Current therapies for diabetic retinopathy

Prevention, such as regular retinal exams with dilation of the pupil, is the key for diabetic patients to detect sight-threatening changes in a timely manner and enable ophthalmologists to carry out treatment (Nentwich and Ulbig, 2015). It has been reported that average healthcare costs increase significantly with the severity of diabetic retinopathy, suggesting that the development of novel therapeutic strategies are of benefit in addition to preventing or slowing the progression of the disease (Heintz *et al.*, 2010). Therefore, the way of preventing or arresting the development of diabetic retinopathy is to treat the classic risk factors, such as hyperglycemia and hypertension.

Although a 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. Current treatments target the later stages of diabetic retinopathy, when vision has already been significantly affected. At this stage laser photocoagulation is the main treatment, being generally indicated in PDR or in clinically significant diabetic macular edema. This intervention prevents further vision deterioration when applied earlier as possible; however, it does not restore lost vision and has potentially severe adverse effects (Simó and Hernández, 2015).

Intravitreal injections of corticosteroids or anti-VEGF agents and vitreoretinal surgery have emerged as more recent treatments for the later stages of diabetic retinopathy. VEGF is known to cause BRB leakage and retinal edema (Qaum *et al.*, 2001), therefore intravitreal injection of anti-VEGF drugs may restore or diminish BRB breakdown.

Moreover, this route of administration enables high local concentrations in the vitreous and a low systemic exposure. However, repeated intravitreal injections of anti-VEGF medications are necessary in patients with DME (Nentwich and Ulbig, 2015).

In the past few years major clinical studies have used anti-VEGF therapy for DME (Simó and Hernández, 2015). These studies have demonstrated that intraocular administration of anti-VEGF agents is better than laser therapy both in preserving and in improving vision for patients with DME. Moreover, there is growing evidence that VEGF can also have neuroprotective and neurotrophic effects (Jin *et al.*, 2000; Zachary, 2005; Saint-Geniez *et al.*, 2008). Although intravitreous anti-VEGF therapy is the first treatment for DR patients without previous surgical intervention, it is still an invasive procedure, which may cause postoperative infection such as endophthalmitis, a case of severe infection of the inner eye and its internal structures, and it may also cause retinal detachment (Nentwich and Ulbig, 2015; Simó and Hernández, 2015).

Apart from anti-VEGF, intravitreal corticosteroids have also been mainly used for DME treatment. Corticosteroids have anti-inflammatory effects but recent studies have suggested additional benefits such as neuroprotection (Zhang *et al.*, 2013). Nevertheless, corticosteroid injection is also associated with adverse complications, the most common being glaucoma and cataract formation (Gunther and Ip, 2009). Nowadays, several different classes of intravitreal corticosteroids which differ in biological properties and duration of action are available (Bandello *et al.*, 2014; Cunha-Vaz *et al.*, 2014). At the moment two implants are available for the treatment of DME, a sustained-release low-dose delivery of dexamethasone and a non-absorbable fluocinolone acetonide implant, which both may limit the frequent intravitreal injection (Nentwich and Ulbig, 2015; Simó and Hernández, 2015).

Ultimately, in extreme complicated situations of diabetic retinopathy, vitreoretinal surgery is suggested, although it is an expensive and complicated treatment (Newman, 2010). All these treatments are expensive, require a vitreoretinal specialist and have a significant number of secondary effects. Therefore, new treatments for the early stages of diabetic retinopathy are needed and recent studies have focus on targeting the molecular mechanisms that are triggered by chronic hyperglycemia, such as AGEs, oxidative stress and inflammation.

Trying to inhibit the accumulation and formation of AGEs, the AGE-RAGE interaction blockage or attenuation of the downstream signaling pathways are potential therapeutic strategies in diabetic retinopathy, however the first AGE inhibitor (aminoguanidine) tested in diabetic patients was suspended due to its adverse effects (Jakuš and Rietbrock, 2004).

As the retina has a highly metabolic demand, it is extremely susceptible to oxidative stress. Hyperglycemia contributes to the formation of ROS and increases oxidative damage. Therefore, antioxidant treatment could potentially reverse this effect. Indeed, several experimental approaches using the systemic administration of antioxidants such as green tea, cocoa, superoxide dismutase mimetics, vitamin E or nutritional supplements containing zeaxanthin, lutein, lipoic acid, or omega-3 fatty acids have been effective in preventing retinal neurodegeneration and early microvascular impairment in animal studies (Yulek *et al.*, 2007; Rosales *et al.*, 2010; Kowluru *et al.*, 2014). Antioxidant therapy has also been put in study in clinical trials with diabetic patients, however these studies have been limited, and the results have been inconclusive (Mayer-Davis *et al.*, 1998; Haritoglou *et al.*, 2009).

Finally, anti-inflammatory therapies might be important in the therapy of diabetic retinopathy as the disease is characterized by chronic low-grade inflammation. Recent studies have demonstrated that mice lacking the IL-1β receptor there was a reduction in retinal capillaries degeneration induced by diabetes (Vincent and Mohr, 2007). This evidence suggests that the inhibition of IL-1β production or its receptor could be an important approach for treating diabetic retinopathy. Data has also demonstrated that systemic administration of eternacept, a soluble TNF-α receptor that acts as a competitive inhibitor to block TNF-α, reduced leukostasis (Joussen *et al.*, 2002), BRB breakdown and NF-κB activation in the retina (Joussen *et al.*, 2009), while another TNF-α inhibitor, pegsunercept, led to reduction in pericyte loss and capillary degeneration in the retina of diabetic rats (Behl *et al.*, 2008; Behl *et al.*, 2009). Apart from these therapeutic approaches, several other have been studied, such as the administration of aspirine and salicilates, and the inhibition of several mediators involved in inflammatory response such as cyclooxygenase-*2* (COX-2), which have all proved useful in reducing retinal microvascular abnormalities induced by diabetes in experimental models,

nevertheless such evidences in clinical studies remain unknown (Simó and Hernández, 2015).

Although there are numerous ongoing studies showing promising results for treating diabetic retinopathy there is the need to implement new and effective therapeutic strategies capable of preventing or attenuating the progression of the disease with little adverse effects associated, preferably during the earlier stages of the disease.

1.3 Incretin-based therapies

1.3.1 The incretin system and the incretin defect in diabetes

Incretins are hormones secreted from endocrine cells in the gastrointestinal tract (so called enteroendocrine cells) into the blood stream within minutes of the ingestion of food (Baggio and Drucker, 2007). The incretin hormones have a physiological role in the modulation of the insulin secretory response to food nutrients (Baggio and Drucker, 2007). The "incretin effect" was described as "the higher insulin release in response to an oral glucose challenge compared to an equal intraveneous glucose load" (Elrick *et al.*, 1964). This phenomenon that accounts for 50 - 70% of the total insulin secreted after oral glucose administration in healthy subjects can only be explained by an intrinsic gastrointestinal sensing mechanism followed by an efferent β-cell response (Nauck *et al.*, 1986; Deacon and Ahren, 2011).

The two hormones found to mediate the incretin effect are glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like-peptide-1 (GLP-1) (Mortensen *et al.*, 2003). GIP was the first incretin hormone described. It has the ability to stimulate insulin secretion at physiological doses in a glucose-dependent manner (Ding and Gromada, 1997). However, more than 50% of the incretin effect remains after removal of GIP (Ebert *et al.*, 1983). Other peptides were then identified including GLP-1, which has considerable insulinotropic activities at physiological levels (Kreymann *et al.*, 1987; Mojsov *et al.*, 1987). GLP-1 is positively coupled to a specific receptor (GLP-1R) in order to achieve its insulinotropic effects by increasing intracellular cAMP and Ca2+ levels in β-cells (Figure 1.8). GLP-1 not only stimulates insulin release from pancreatic β-cells in a

glucose-dependent manner, but also promotes the transcription of proinsulin and insulin biosynthesis. It also shows both anti-apoptotic and pro-proliferative effects on β-cells (Farilla *et al.*, 2002; Farilla *et al.*, 2003). In addition, other anti-diabetic effects include the inhibition of glucagon secretion from pancreatic α-cells, suppression of gastric emptying and appetite reduction (Garber, 2010).

However, in patients with type 2 diabetes, the incretin effect is largely impaired (Bagger *et al.*, 2011), since signals from gut-derived factors are attenuated by a defective secretion or degradation of GLP-1 and /or specific defect in GIP action. Indeed, in diabetic patients, the incretin effect is reduced due to a reduction in GLP-1 but not GIP secretion (Vilsboll *et al.*, 2001). Furthermore, the insulintropic effects of GLP-1 are largely preserved in type 2 diabetes, despite its decreased levels. In fact, exogenous GLP-1 has been shown to exert potent glucose-lowering activity in patients with the disease. In contrast, the glucoregulatory effects of GIP are severely impaired although its levels are normal or even higher compared to healthy controls and this impairment cannot be offset even by raising GIP levels to supra-physiological concentrations (Meier and Nauck, 2010). On the basis of such reasoning, considerable effort has been made to develop GLP-1-based therapies in order to restore GLP-1 physiologic function in patients with type 2 diabetes.

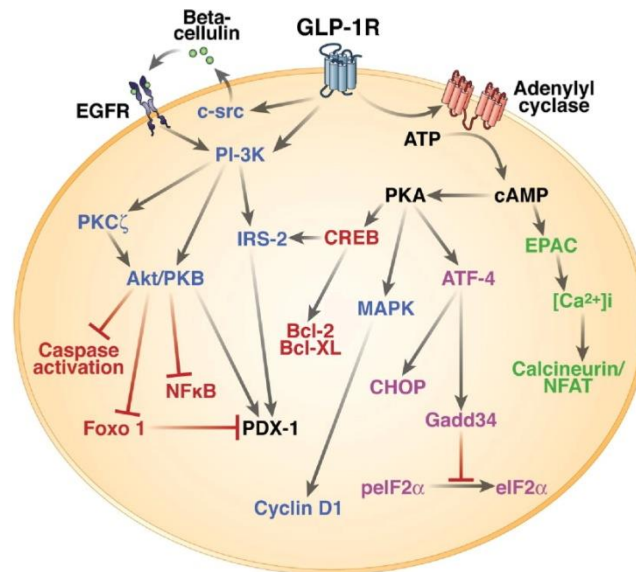

Figure 1.8. GLP-1R–dependent intracellular signaling pathways in the pancreatic β-cell. GLP-1R activation leads to insulin secretion and biosynthesis (green), β-cell proliferation and neogenesis (blue), inhibition of apoptosis (red), and endoplasmic reticulum stress reduction (purple) (Baggio and Drucker, 2007).

After release into the circulation, GLP-1 and GIP ($t_{1/2}$ of approximately 2 min) suffer rapid cleavage and inactivation by the enzyme dipeptidyl peptidase-IV (DPP-IV, also known as adenosine deaminase complexing 2 or CD26; EC 3.4.14.5) (Kieffer *et al.*, 1995; Gorrell *et al.*, 2001).

DPP-IV is a ubiquitously expressed peptidase which exists in a soluble (sDPP-4) and a membrane-bound form (Figure 1.9). The former which lacks the cytoplasmic domain and the transmembrane region comes from a shedding process driven by matrix metalloproteinases, namely from adipocytes and smooth muscle cells (Rohrborn *et al.*, 2015). Alternatively, sDPP-4 can be released into circulation via vesicles in the form of exosomes, ectosomes and apoptotic bodies (Zhong *et al.*, 2015). The latter can be found on the surface of mostly epithelial and endothelial cells, but also activated immune cells (Augustyns *et al.*, 1999; Balkan *et al.*, 1999; Ludwig *et al.*, 2002).

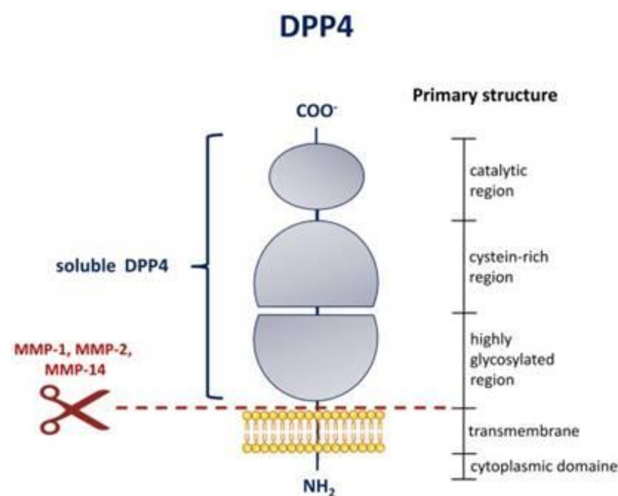

Figure 1.9. Structure of DPP-IV. Schematic representation of the membrane-bound DPP-IV monomer. The soluble form of DPP-IV is illustrated on the left in blue. Shedding of DPP4 from the membrane is driven by matrix metalloproteinases (scissor symbol in red). On the right are represented the primary structure with the delineation of the different regions (adapted from Rohrborn *et al.*, 2015).

DPP-IV is a serine aminopeptidase that cleaves GLP-1 and GIP via dipeptide cleavages with alanine, proline, or hydroxyproline in the N-terminal amino acids (Drucker, 2007). It has been reported that *in vitro*, DPP-IV cleaves many chemokines and peptide hormones, accounting for incretin-independent effects. However, *in vivo*, there are comparatively fewer peptides that have been identified as endogenous physiological substrates for DPP-IV. Both GIP and GLP-1 are endogenous physiological substrates for DPP-IV. DPP-IV knockout mice studies showed improved glucose tolerance concomitant with increased bioavailability of GIP and GLP-1 and enhanced secretion of insulin subsequent to oral glucose challenge. This was accompanied by decreased accumulation of fat and diminished intake of food as well as increased resistance to diet-induced obesity (Marguet *et al.*, 2000; Conarello *et al.*, 2003). *In vivo* studies on mice which had their incretin receptors genetically inactivated showed that the mechanisms transducing the glucoregulatory effects of DPP-IV inhibitors were dominated by GIP and GLP-1 receptor-dependent pathways (Marguet *et al.*, 2000). Thus, the compounds that inhibit the biological action of DPP-IV enzyme such as DPP-IV inhibitors will prolong the half-life and increase the bioavailability of the incretin hormones and consequently improve

their biological effect to maintain glucose homeostasis in individuals with type 2 diabetes.

1.3.2 GLP-1-based therapies to diabetes

GLP-1-based therapies have been approved by the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) for the treatment of patients with type 2 diabetes. Currently approved by EMA, these therapies include GLP-1 receptor agonists: exenatide, liraglutide, lixisenatide and albiglutide; and DPP-IV inhibitors: sitagliptin, vildagliptin, saxagliptin, linagliptin and alogliptin. Exenatide and sitagliptin were the first to be approved in each drug class and will be the focus of this dissertation.

Exenatide is the synthetic version of Exendin-4 (Ex-4), a 39 residue peptide with 53 % sequence identity with GLP-1 firstly isolated from the saliva of the Gila monster *H suspectum* (Eng et al., 1992). Regardless of its relatively low sequence identity with GLP-1, Ex-4 is a high affinity potent agonist of the GLP-1R (Raufman *et al.*, 1992) exerting its glucoregulatory activities in a similar manner to GLP-1 (Kolterman *et al.*, 2003).

In Ex-4, the alanine at position 2 of the peptide in GLP-1 is replaced by a glycine residue in Ex-4 (Figure 1.9), thereby making it less susceptible to DPP-IV mediated cleavage and consequent inactivation, resulting in longer half-life *in vivo* compared to endogenous GLP-1. Due to its longer pharmacological half-life, Exenatide can be administered twice daily subcutaneously (Al-Sabah and Donnelly, 2003). Besides its anti-hyperglycemic effects it has also shown to significantly reduce body weight and waist circumference in patients with type 2 diabetes (Bunck *et al.*, 2010).

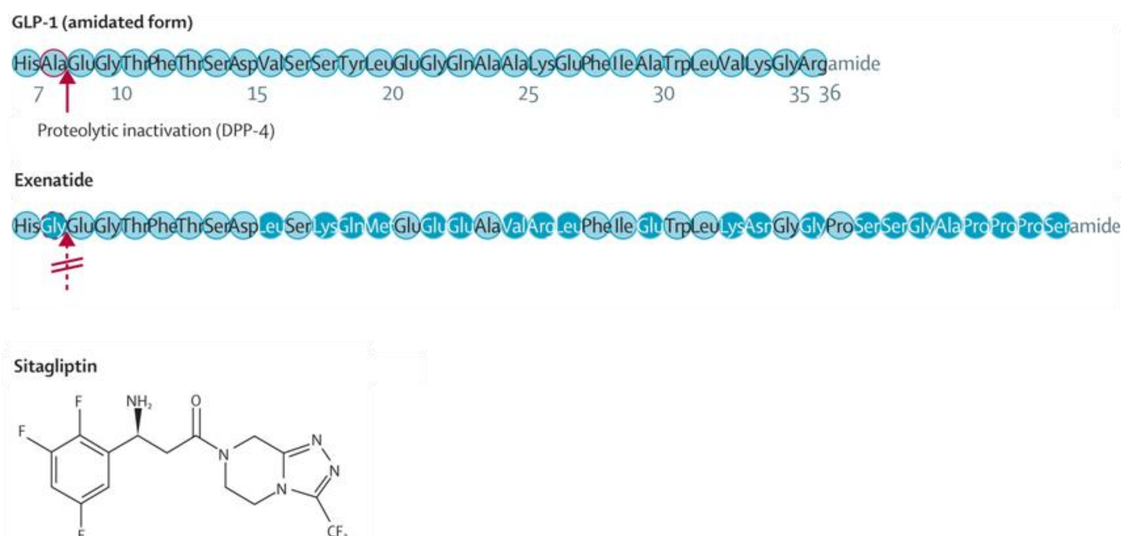

Figure 1.10. Structure of GLP-1, GLP-1R agonist exenatide and DPP-IV inhibitor sitagliptin (adapted from Drucker and Nauck, 2006)**.**

Sitagliptin ((R)-4-oxo-4- [3-(trifluoromethyl)-5,6-dihydro[1,2,4]triazolo[4,3-a]pyrazin-7 (8H)-yl]-1-(2,4,5-trifluorophenyl)butan-2-amine), a DPP-IV inhibitor) is a low molecular weight molecule (523.32Da) and its chemical structure is depicted in Figure 1.10. *In vitro* studies revealed that sitagliptin was selective to DPP-IV and did not inhibit DPP-VIII and DPP-IX activity, at least at concentrations similar to those achieved at therapeutic doses (Kim *et al.*, 2005). A single sitagliptin dose of 50 mg or higher was associated with >80% inhibition of plasma DPP-IV activity for at least 12 hours, leading to an approximately 2- to 3-fold increase in post-meal active GLP-1 levels, efficiently reducing Hba1c levels and effectively limiting postprandial hyperglycemia (Herman *et al.*, 2005; Aschner *et al.*, 2006). Sitagliptin was reported to have a neutral effect on body weight, well tolerated and not associated with hypoglycemia (Deacon, 2011). The elimination for sitagliptin is almost exclusively renal, occurring clearance of the active inhibitor molecule (Deacon and Lebovitz, 2015). As DPP-IV inhibitors are orally active, they are much more convenient for clinical use.

In recent years, research involving GLP-1R stimulation, whether with GLP-1R agonists or increasing the levels of endogenous GLP-1, has shifted from type 2 diabetes to focus upon diabetes-associated complications and also cardiovascular and neurodegenerative diseases (Campbell and Drucker, 2013; Gallwitz, 2014; Mulvihill and Drucker, 2014).

The increased attention given to the extrapancreatic effects of GLP-1R was mostly due to its expression in a wide array of tissues e.g. heart, kidney, brain, lungs (Baggio and Drucker, 2007). GLP-1R is also expressed in retinas from rats (Zhang *et al.*, 2009) chickens (Fischer *et al.*, 2008), in ARPE-19 cells (a cell line of human retinal pigment epithelium) (Puddu *et al.*, 2013) and more recently it was shown to be present in the human retina (Figure 1.11).

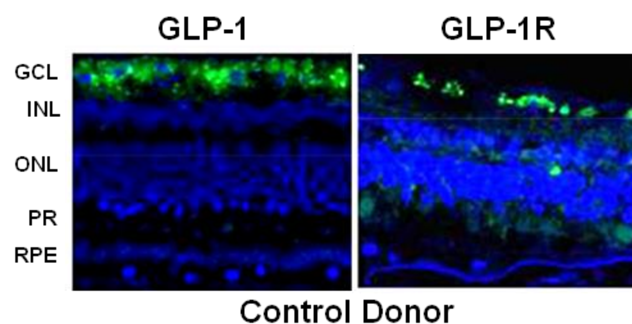

Figure 1.11. Expression of GLP-1 and GLP-1R in the human retina. Retinal cross-sections from control donors immunostained for GLP-1 and its receptor (adapted from Hernandez *et al.*, 2016). Legend: GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; PR, photoreceptor layer; RPE, retinal pigment epithelium.

Recently, incretin-based therapies have been reported to induce protective effects against diabetic retinopathy. We previously reported that sitagliptin (10 mg/kg) can exert beneficial and protective effects in the retina of a type 2 diabetes animal model. Sitagliptin inhibited changes in the TJ as well as nitrosative stress, inflammation and apoptosis, which are known to underlie the breakdown of the BRB. These effects were correlated with a potential increase in vascular repair by endothelial progenitor cells in diabetic retinal damaged vessels (Gonçalves *et al.*, 2012). Other researchers have demonstrated that in an animal model of type 1 diabetes, sitagliptin (40 mg/kg) was able to reduce Hba1c and blood glucose levels. This was accompanied by a reduction in AGE production in the diabetic retina, reduced severity of lenticular and retinal degeneration, and prolonged onset of cataract formation (Pandit *et al.*, 2013). In the same animal model the GLP-1R agonist Ex-4 administered subcutaneously or intravitreally was able to reverse changes in electroretinograms, prevent retinal cell death and maintain normal

retinal thickness in diabetic rats (Zhang *et al.*, 2009; Zhang *et al.*, 2011). In addition, Ex-4 has demonstrated to regulate apoptosis, leading to neuroprotective effects and prevention of hyperglycemia-induced injury to the retinal ganglion cells *in vitro* (Fu *et al.*, 2012). This drug also demonstrated anti-inflammatory properties in human RPE, decreasing levels of AGE and adhesion molecules ICAM-1 and vascular adhesion molecule-1 (VCAM-1) after stimulation with TNF-α and glycated albumin (Dorecka *et al.*, 2013). Besides its neuroprotective effects, intravitreal Ex-4 was also shown to ameliorate early microvascular impairment in type 2 diabetic Goto-Kakizaki rats, preventing the decrease in TJ proteins and the increased vascular permeability (Fan *et al.*, 2014). Hernandez and colleagues (Hernandez *et al.*, 2016) recently proposed a topical route of administration for GLP-1R agonists, including Ex-4, as they observed similar neuroprotective effects in diabetic rat retinas after topical or systemic administration of these drugs. Furthermore, they observed a downregulation of GLP-1 in human diabetic retinas, whereas GLP-1R expression was maintained, indicating that the administration of GLP-1R agonists can be envisaged as a replacement therapy of a natural neurotrophic factor that is downregulated in the diabetic retina.

1.4 Objectives

Diabetic retinopathy is one of the most common complications of diabetes and is a leading cause of vision loss and blindness among working-age adults worldwide. Presently, the treatments available are scarce and are mainly targeted to the later stages of the disease. In fact, there are no effective treatments to avoid the progression of diabetic retinopathy, and it is assumed that the possibility to delay its progression resides mainly in the early stages of the disease.

Recent studies have highlighted the extrapancreatic effects of incretin-based therapies, particularly in diabetic-related complications. Two of the most recently approved classes of therapeutic agents for the treatment of type 2 diabetes, GLP-1 receptor agonists and DPP-IV inhibitors, exert their actions through the potentiation of incretin receptor

signaling, mainly via GLP-1. GLP-1 has an important physiologic role in controlling glucose homeostasis, by enhancing glucose-stimulated insulin secretion and inhibiting glucagon secretion (Baggio and Drucker, 2007). However, beyond its acute insulinotropic effect, GLP-1 seems to have protective effects on various extra-pancreatic tissues.

We have previously reported that the DPP-IV inhibitor sitagliptin is able to prevent retinal alterations induced by chronic hyperglycemia in type 2 diabetic animals. In addition to improved glycemic control, as expected, sitagliptin prevented nitrosative stress, inflammation and apoptosis in retinal cells and exerted beneficial effects on the blood-retinal barrier integrity in diabetic rat retinas (Gonçalves *et al.*, 2012).

In Chapter 2, our main goal was to investigate whether sitagliptin has beneficial effects in the diabetic retinas beyond the mechanisms dependent on increased insulin secretion and normalization of hyperglycemia. This was accomplished by using a type 1 diabetes animal model, where the insulin secretion is residual due to the destruction of pancreatic β-cells by the toxin streptozotocin (STZ). We assessed the effects of sitagliptin on BRB breakdown, including vascular permeability and tight-junction complex organization. We also investigated the effects of sitagliptin on some of the mechanisms known to underlie BRB dysfunction, such as inflammation and cell death.

Given that the mechanism of action of sitagliptin relies on increasing the levels of endogenous GLP-1, thus enhancing its protective actions, in Chapter 3 the aim was to clarify the beneficial effects of GLP-1R activation on retinal vascular permeability, by using the GLP-1R agonist Ex-4. In order to clarify the protective effects on the BRB, an animal model of ischemia-reperfusion (IR) injury was used, as this model recapitulates several features of various retinal pathologies, such as diabetic retinopathy, which include increased BRB permeability, neuroinflammation and neurodegeneration, and does not present metabolic alterations, as the diabetic models do. Furthermore, we aimed to investigate the mechanisms underlying Ex-4 protection against BRB breakdown. Given that neuroinflammation and microglia are key mediators of BRB breakdown, we intended to elucidate whether Ex-4 could affect microglia reactivity and microglia-mediated neuroinflammation.

Chapter 2
DPP-IV inhibition prevents blood-retinal barrier breakdown, inflammation and neuronal cell death in the retina of type 1 diabetic rats

2.1 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 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.

2.2 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 (Cai and Boulton, 2002). In streptozotocin-induced diabetic mice and rats, as well as in diabetic humans, it has been demonstrated an increase in BRB permeability, which is the hallmark of the early stages of diabetic retinopathy progression (Cunha-Vaz *et al.*, 1975; Leal *et al.*, 2007; Leal *et al.*, 2010). Diabetes-induced vascular permeability seems to be correlated with the disruption of TJ, which form a complex network structure between the endothelial cells, comprising the inner BRB (Fernandes *et al.*, 2013; Goncalves *et al.*, 2013). 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 (Antonetti *et al.*, 1999; Barber *et al.*, 2000; Leal *et al.*, 2007; Leal *et al.*, 2010). Also, inflammatory mediators have been shown to promote increased vascular permeability, leukocyte adhesion and retinal cell death (Miyamoto *et al.*, 1999; Krady *et al.*, 2005). In fact, elevated levels of proinflammatory cytokines have been detected in the vitreous of diabetic patients with retinopathy (Abu el Asrar *et al.*, 1992) and in diabetic rat retinas with increased vascular permeability (Carmo *et al.*, 2000; Joussen *et al.*, 2002).

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 the management of poor glycemic control in type 2 diabetic patients. The inhibition of DPP-IV stabilizes 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

(Drucker and Nauck, 2006). 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 (Brubaker and Drucker, 2004). DPP-IV is expressed in several cell types, including neuronal cells and brain capillary endothelial cells (Ludwig *et al.*, 2002), being also found in the plasma and its inhibition increases GLP-1 plasma concentration (Balkan *et al.*, 1999). Besides the insulinotropic effects of GLP-1R activation in pancreatic cells, this receptor was shown to be expressed in a wide range of tissues, including the retina (Zhang *et al.*, 2009).

Recent studies have demonstrated beneficial effects of incretin-based therapies in the vasculature (Shah *et al.*, 2011; Shiraki *et al.*, 2012), kidney (Mega *et al.*, 2011), heart (Bose *et al.*, 2005) and brain (Gaspari *et al.*, 2011). 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 (Zhang *et al.*, 2011). Recently, we have demonstrated that sitagliptin can exert beneficial and protective effects in the BRB, inhibit apoptosis, inflammation, and positively modulate endothelial progenitor cells in a type 2 diabetes animal model (Goncalves *et al.*, 2012). 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.

2.3 Materials and Methods

2.3.1 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 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).

2.3.2 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.

2.3.3 Western blot analysis

The intact retinas were homogenized by pipetting in 150 mM NaCl, 50 mM Tris (pH 7.5), 5 mM EGTA, 1% Triton X-100 (Tx-100), 0.5% sodium deoxycholate and 0.1% SDS, supplemented with 2 mM phenylmethylsulfonyl fluoride, 2 mM iodoacetamide and 1x protease inhibitor cocktail (Roche, Indianapolis, IN, USA). After centrifugation at 16,000 x *g*, for 10 min, at 4°C, supernatants were used to determine protein concentration by bicinchoninic acid reagent (Pierce, Rockford, IL, USA) and then were denatured with Laemmli buffer (0.5 M Tris, 30% glycerol, 10% SDS, 0.6 M dithiothreitol (DTT), 0.012 bromophenol blue) at 37ºC for 1h.

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 blocked with 5% non-fat milk or 5% bovine serum albumin (BSA) for 1h at room temperature and then probed overnight at 4°C with the primary antibodies: anti-DPP-IV/CD26 anti-ICAM-1, anti-Bax, anti-albumin and anti-β-actin (Table 2.1). After washing, the membranes were probed with a secondary anti-rabbit, anti-mouse or anti-goat IgG-HRP-linked antibody (1:10,000; Bio-Rad, Hercules, CA, USA) for 1 h at room temperature. Immunoreactive bands were detected by enhanced chemiluminiscence (ECL) substrate using an imaging system (VersaDoc 4000 MP, Bio-Rad). Densitometric analyses were performed using the ImageJ 1.47 software (NIH, Bethesda, MD, USA).

Table 2.1. List of primary antibodies used in Western blot or immunofluorescence.

Primary Antibody	Dilution		Catalog #	Manufacturer
	WB	IF		
Rabbit anti-DPP-IV/CD26	1:4000	1:200	ab28340	Abcam
Rabbit anti-ICAM-1	1:200	------	sc-1511	Santa Cruz Biotech.
Rabbit anti-Bax	1:200	1:50	sc-6236	Santa Cruz Biotech.
Goat anti-Albumin	1:5000	------	A90-134A	Bethyl Laboratories
Mouse anti-β-Actin	1:10000	------	A5441	Sigma-Aldrich
Goat anti-IL-1β	------	1:100	AF-501-NA	R&D Systems
Mouse anti-occludin	------	1:100	33-1500	Zymed Laboratories
Rabbit anti-claudin-5	------	1:100	34-1600	Zymed Laboratories
Rabbit anti-ZO-1	------	1:100	61-7300	Zymed Laboratories

WB: Western blot; IF: immunofluorescence

2.3.4 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.

2.3.5 Immunohistochemistry in retinal sections

Retinal sections (10 μm) were obtained in a cryostat and collected on SuperFrost Plus glass slides (Menzel-Glaser, Thermo Fisher Scientific) and stored at -80°C until use. Retinal sections were air dried for at least 45 min at room temperature and then fixed in cold acetone for 10 min. The sections were 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 with 10% normal goat serum or 5% BSA before incubation overnight at 4°C with primary antibodies: anti-IL-1β, anti-DPP-IV/CD26 and anti-Bax (Table 2.1). Sections were then rinsed with PBS and incubated with DAPI (2.5 μg/ml; Molecular Probes, Life Technologies, Grand Island, NY, USA) for nuclear staining and the secondary fluorescent antibody Alexa Fluor 488-conjugated goat anti-rabbit IgG or Alexa Fluor 488-conjugated rabbit anti-goat IgG (1:200; Life Technologies, Paisley, UK) for 1h at room temperature. After washing with PBS the sections were coverslipped using Glycergel™ mounting medium (Dako, Agilent Technologies, Santa Clara, CA, USA).

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.

2.3.6 Measurement of BRB permeability

Blood-retinal barrier permeability was quantified using the Evans blue dye, which binds irreversibly to serum albumin, according to the procedure previously described by our group (Leal *et al.*, 2010). Under anesthesia, the rats were administered 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 x *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.

2.3.7 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 animals were sacrificed and 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 images were acquired in a masked fashion.

2.3.8 Whole-mount staining

Retina whole-mounts were prepared according to the procedure previously described by our group (Leal *et al.*, 2007), with minor modifications. Retinas were isolated and immersed in 2% paraformaldehyde for 2 × 5 min at room temperature. After washing 2 × 5 min in PBS with 0.3% Tx-100 (PBS-T), the retinas were blocked with 5% BSA in PBS-T for 1 h, and then incubated for 3 days at 4°C with primary antibodies: anti-occludin, anti-claudin-5 and anti-ZO-1 (Table 2.1). After incubation, the retinas were washed with PBS-T for 24 h and 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) for another 24h at 4°C. After washing for 24 h with PBS the retinas were 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 TJ proteins was measured in 30-40 retinal vessels of each experimental group.

2.3.9 ELISA

Retinal tissue was homogenized in 20 mM imidazole HCl (pH 6.8), 100 mM KCl 1 mM $MgCl_2$, 1% Tx-100, 1 mM EGTA, 1 mM EDTA, supplemented with 10 mM NaF, 1 mM

Na_3VO_4 and 1x protease inhibitor cocktail (Roche). The samples were centrifuged at 4ºC 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. Briefly, 96-well microplate was coated with capture antibody (2 µg/ml), sealed and left overnight at room temperature. Then, wells were washed with 0.05% Tween-20 in PBS and blocked with 1% BSA for 1 h at room temperature. Retinal samples and biotin-conjugated antibody (0.5µg/ml) were added to the wells, and plate was incubated at room temperature for 2h. After washing, avidin-HRP (1:2000) was added and then kept once again at room temperature for 30 min. After washing, the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) substrate solution was added to each well for 20 min at room temperature. The absorbance was measured at 405 nm with wavelength correction set at 650 nm, using a microplate reader (Biotek, Synergy HT). A standard curve was used to calculate IL-1β protein levels (pg/mL of total protein levels).

2.3.10 Apoptosis assay

Apoptotic cell death was detected by TUNEL, which allows the detection of fragmented DNA of apoptotic cells, using the DeadEnd Fluorometric TUNEL System (Promega, Madison, WI, USA), according to the instructions of the manufacturers. Retinal sections (10 µm) were fixed in 4% PFA for 15 min, washed with PBS and then permeabilized with 20 µg/ml proteinase K in PBS for 10 min at room temperature. After washing in PBS for 5 min, the sections were incubated with equilibration buffer (200 mM potassium cacodylate, 25 mM Tris, 0.2 mM dithiothreitol, 2.5 mM $CoCl_2$, and 0.25 mg/ml BSA, pH 6.6) for 10 min at room temperature. Next, sections were incubated with 600 U/ml recombinant TdT enzyme and nucleotide mix containing 5 µM fluorescein-12-dUTPs, 10 µM dATP, and 0.1 mM EDTA, diluted in equilibration buffer at 37 °C for 60 min. The reaction was stopped by immersing the slides in saline-citrate buffer (300 mM NaCl, 30 mM sodium citrate, pH 7.0) for 15 min at room temperature. After washing three times in PBS, the nuclei were stained with DAPI (2.5 µg/ml, Molecular Probes) for 10 min at room temperature. After washing three times in PBS sections were mounted with Glycergel^{TM} mounting medium (Dako). Images of the retinal sections were acquired in a confocal microscope (LSM 710, Carl Zeiss).

Degenerating neurons were assessed by Fluoro-Jade B staining (Chemicon, Temecula, CA, USA), according to the instructions of the manufacturers. Briefly, retinal sections (10 μm) were allowed to air dry for 45 min and then they were first immersed in a basic alcohol solution consisting of 1% sodium hydroxide in 80% ethanol for 5 min. They were then rinsed for 2 min in 70% ethanol, for 2 min in distilled water, and then incubated in 0.06% potassium permanganate solution for 15 min. Following a 1-2 min water rinse, the slides were then transferred for 1 h to a 0.0001% solution of Fluoro-Jade dissolved in 0.1% acetic acid. The slides were then rinsed through three changes of distilled water for 1 min per change. The slides were then air dried on a slide warmer at 50°C for at least 5 min. The air dried slides were then cleared in xylene for at least 1 min and then coverslipped with Di-n-butyl phthalate in Xylene (DPX) (Fluka) nonfluorescent mounting media. Slides were then analyzed under a confocal microscope (LSM 710, Carl Zeiss).

2.3.11 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.

2.4 Results

2.4.1 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 2.2).

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 2.2).

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 2.2).

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 2.2).

Table 2.2. 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.

2.4.2 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 (Figure 2.1A).

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; $P < 0.01$) (Figure 2.1B). 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 (Figure 2.1B).

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; $P < 0.05$) (Figure 2.1C). 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; $P < 0.05$) (Figure 2.1C). 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 (Figure 2.1C). Immunohistochemistry experiments performed in retinal frozen sections confirmed these results (Figure 2.1D and 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 in the retinas of diabetic rats (107.8 ± 2.2 % of control; $P < 0.001$) (Figure 2.1D and E).

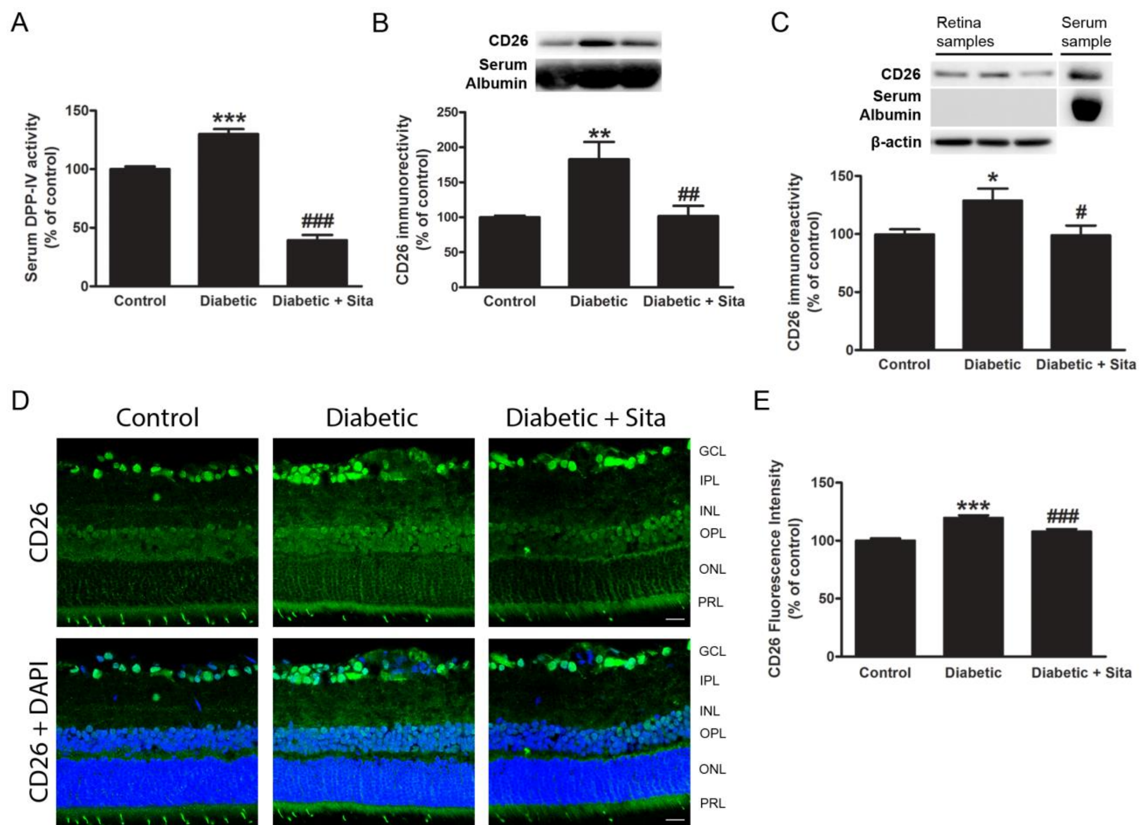

Figure 2.1. Sitagliptin prevents the upregulation of DPP-IV activity and content induced by diabetes. (A) DPP-IV activity was determined in the serum using the fluorogenic substrate Gly-Pro-AMC. The protein levels of DPP-IV/CD26 (110-120 kDa) and serum albumin (65 kDa) were assessed by Western blotting 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 400x. 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.

2.4.3 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 vessels integrity

was analysed in flat mount retinas. Evans blue was shown to be confined to the retinal blood vessels, without any leakage occurring, in control rats (Figure 2.2A). 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 (Figure 2.2A). 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) (Figure 2.2B). 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 (Figure 2.2B).

2.4.4 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, 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 (Figure 2.2C and 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 diabetic animals treated with sitagliptin, no significant difference was reached when comparing to ZO-1 staining in the retinas of diabetic animals (Figure 2.2C and D).

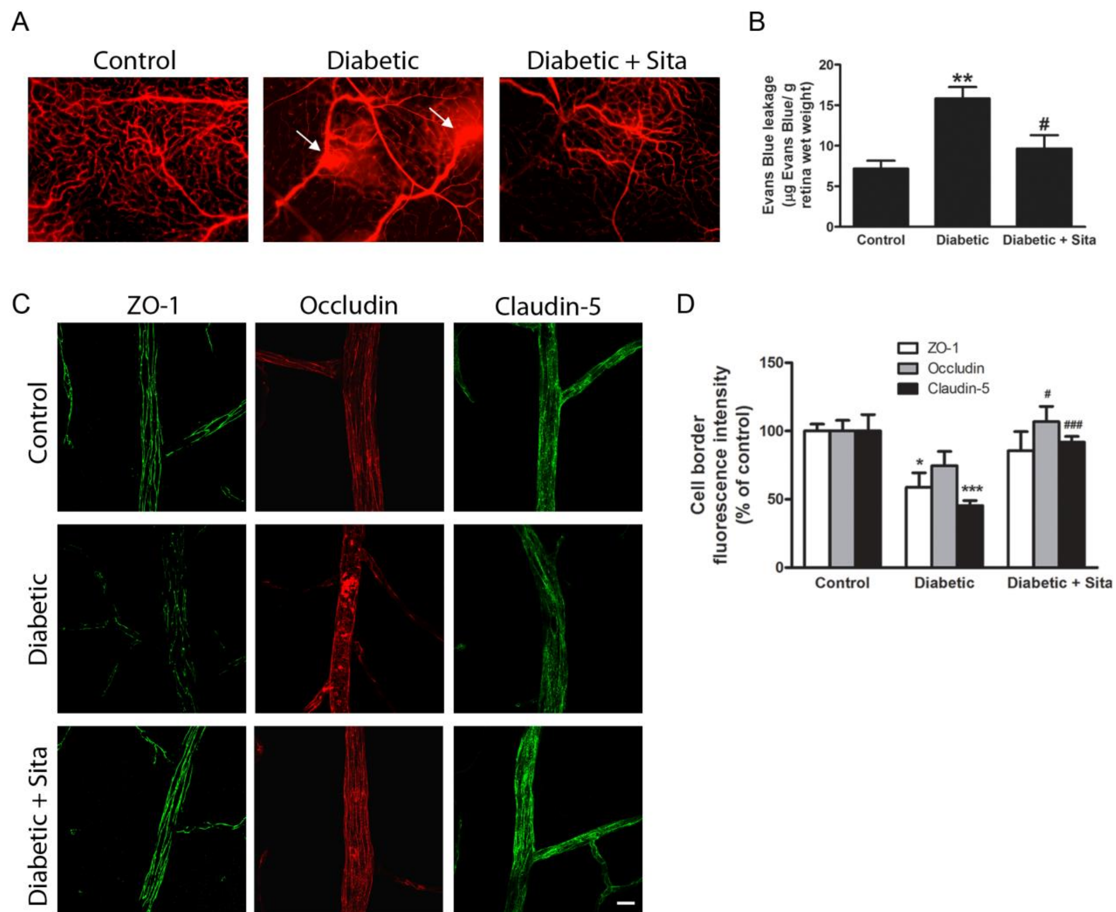

Figure 2.2. Sitagliptin protects against the increase in BRB permeability and TJ disassembly triggered by diabetes. (A) Representative images, from 3 animals per group, showing Evans blue fluorescence in the retina. Evans blue, which binds to blood albumin, allows the detection of leakage sites (arrows) in the retinal vessels. Magnification 100x. **(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 400x. Bar: 20 µm. **(D)** Quantification of cell border immunostaining for the TJ 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.

2.4.5 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 (Carmo *et al.*, 2000; Joussen *et al.*, 2004). 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; $P < 0.001$) (Figure 2.3A and 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 (Figure 2.3A and B). As shown in Figure 2.3C, 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 (Figure 2.3C).

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 (Collins *et al.*, 1995). ICAM-1 protein levels were significantly increased in the diabetic retinas (138.2 ± 9.6 % of control; $P < 0.05$) (Figure 2.3D). Sitagliptin treatment prevented the increase of this adhesion molecule induced by diabetes (102.4 ± 6.8 % of control; $P < 0.01$) (Figure 2.3D).

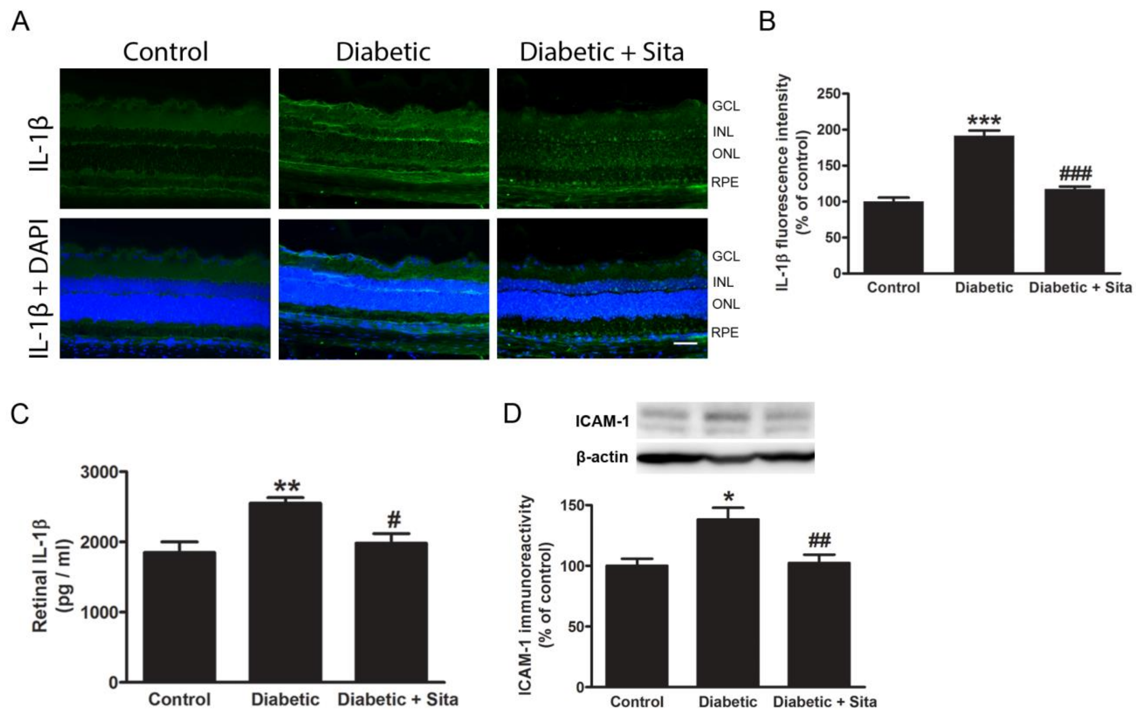

Figure 2.3. Sitagliptin inhibits the increase in IL-1β and ICAM-1 levels in the retina of diabetic animals. Representative fluorescence images for each group of animals, showing IL-1β **(A)** immunoreactivity (green) and nuclear staining with DAPI (blue) in retinal sections (10 μm). Magnification 200x. 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.

2.4.6 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 (Mizutani *et al.*, 1996). 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 (Gao *et al.*, 2009).

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 (Figure 2.4A). The administration of sitagliptin significantly decreased the pro-apoptotic state (90.4 ± 5.8 % of control; $P < 0.01$) induced by diabetes (Figure 2.4A). 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 (Figure 2.4B and 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 (Figure 2.4D). 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; $P < 0.05$) (Figure 2.4D and 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 (Schmued *et al.*, 1997; Chidlow *et al.*, 2009). In control retinas, we could not detect any staining indicative of cell death (Figure 2.4F). 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 (Figure 2.4F). In the retinas of diabetic animals treated with sitagliptin, no specific staining of cell bodies indicative of cell death was found (Figure 2.4F).

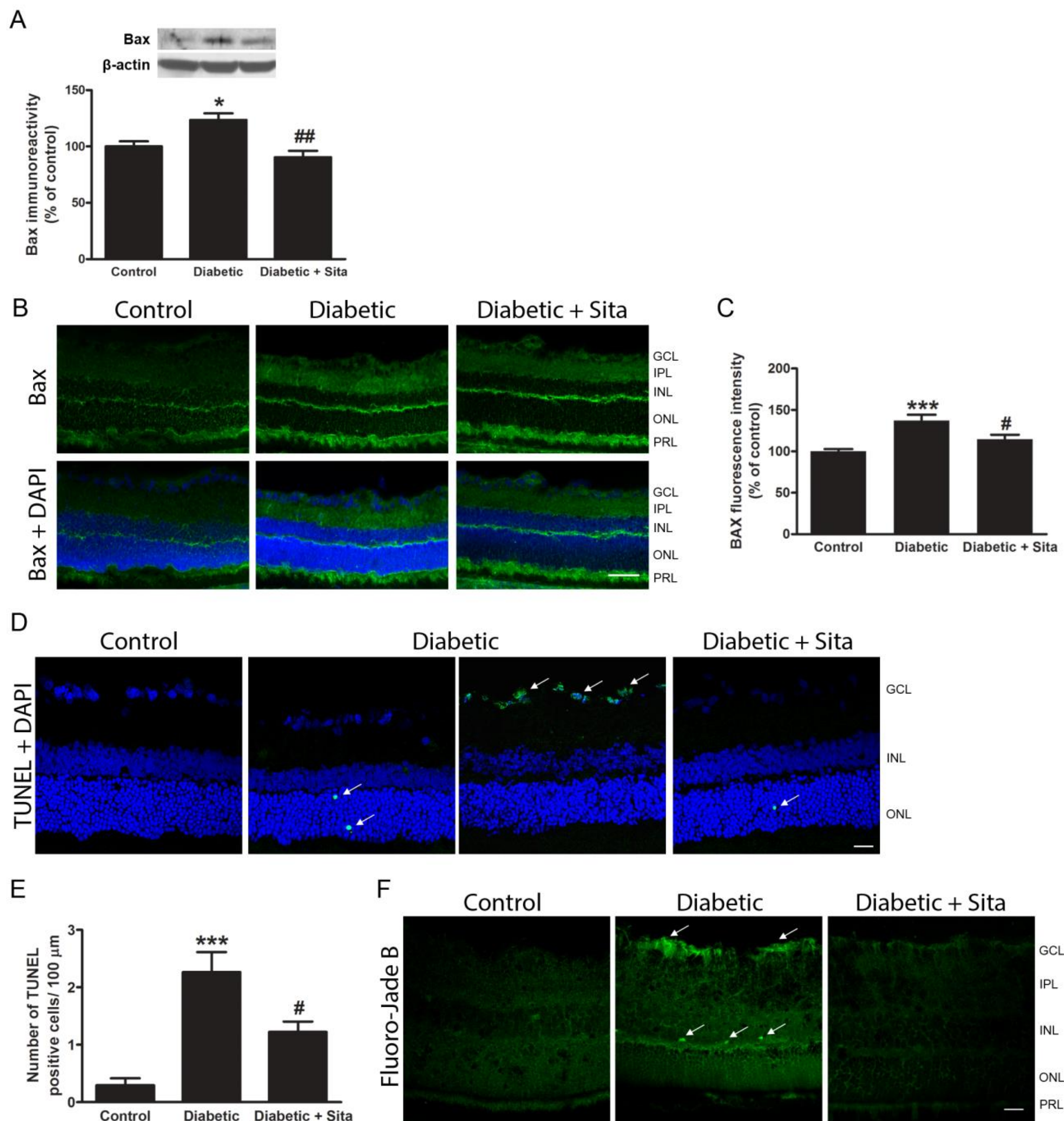

Figure 2.4. Sitagliptin prevents neuronal cell death induced by diabetes in the retina. (A) The protein levels of Bax were assessed by Western blotting in retinal lysates. The western blots presented are representative of each experimental group. Data are presented as percentage of control and represent 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 200x. 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 400x. 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 400x. Bar: 20 µm. Legend: GCL – ganglion cell layer; IPL – inner plexiform layer; INL – inner nuclear layer; ONL – outer nuclear layer; PRL – photoreceptor layer.

2.5 Discussion

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 (Balkan *et al.*, 1999; Ferreira *et al.*, 2010; Aso *et al.*, 2012; Goncalves *et al.*, 2012). 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 (Varga *et al.*, 2011; Fadini *et al.*, 2012). 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 sitagliptin for a month promoted a strong inhibition of DPP-IV activity and concomitantly increased levels of active plasma GLP-1 levels (Kim *et al.*, 2008). 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 (Busso *et al.*, 2005; Aso *et al.*, 2012), 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 (Cunha-Vaz *et al.*, 1975). 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 (Xu *et al.*, 2001). 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 complexes disassembly (Antonetti *et al.*, 1999; Barber *et al.*, 2000; Leal *et al.*, 2007; Leal *et al.*, 2010). 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 cells 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 (Antonetti *et al.*, 1998; Barber *et al.*, 2000; Leal *et al.*, 2010; Goncalves *et al.*, 2012). These changes appear to be correlated with increased occludin phosphorylation, which may target this protein to degradation (Antonetti *et al.*, 1999) and also alter its interaction with ZO-1 and ZO-2, compromising the integrity of the TJ (Kale *et al.*, 2003). ZO-1 decreased levels and changes in its localization and phosphorylation state also appear to be associated with increased endothelial permeability (Antonetti *et al.*, 1999; Leal *et al.*, 2007; Leal *et al.*, 2010). Moreover, a decrease in the protein levels of claudin-5 has also been correlated with increased vascular permeability induced by diabetes (Leal *et al.*, 2010).

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 (Abu el Asrar *et al.*, 1992; McLeod *et al.*, 1995; Joussen *et al.*, 2002; Goncalves *et al.*, 2012). Moreover, increased levels of both IL-1β and ICAM-1 have been correlated with increased retinal vascular permeability (Miyamoto *et al.*, 1999; Carmo *et al.*, 2000; Leal *et al.*, 2010). 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 (Ferreira *et al.*, 2010; Goncalves *et al.*, 2012; Mega *et al.*, 2014), as well as in the serum of type 2 diabetic patients (Fadini *et al.*, 2010).

Studies have shown that both hyperglycemia and IL-1β are shown to activate NF-κB leading to the upregulation of cell surface expression of adhesive proteins, namely ICAM-1, in endothelial cells (Morigi *et al.*, 1998; Kowluru and Odenbach, 2004) 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 (Hu *et al.*, 2012). 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 (Schroder *et al.*, 1991). 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 (Barber *et al.*, 2011). 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 (Barber *et al.*, 1998; Kanamori *et al.*, 2004). 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 (Zhang *et al.*, 2008; Zhang *et al.*, 2011), 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 (Barber *et al.*, 1998; Kanamori *et al.*, 2004).

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 (Chidlow *et al.*, 2009; Leonelli *et al.*, 2010). 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 (Goncalves *et al.*, 2012). 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 (Zhang *et al.*, 2011). 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 inhibition of caspase-3, by increasing the expression of Bcl-2 and decreasing the expression of Bax (Wang and Brubaker, 2002; Kim *et al.*, 2005). Moreover, recent studies in rodents have also provided evidence of neuroprotective effects of GLP-1 in the brain (During *et al.*, 2003).

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 slowdown both the neuronal and vascular damage, as a result of prolonged hyperglycemia.

Since the biological activity of a large number of chemokines, adipokines, neuropeptides, and incretins are 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 envisaged as a strong candidate for further consideration as a therapeutic drug in reducing the retinal complications of diabetes.

Chapter 3

Protective effect of a GLP-1 analog on ischemia-reperfusion induced blood-retinal barrier breakdown and inflammation

3.1 Abstract

Inflammation associated with BRB breakdown is a common feature of several retinal diseases, and therefore development of novel non-steroidal anti-inflammatory approaches may provide important therapeutic options. Previous studies demonstrated that inhibition of dipeptidyl peptidase-IV, the enzyme responsible for the degradation of glucagon-like peptide-1 (GLP-1), led to insulin-independent prevention of diabetes-induced increases in BRB permeability, suggesting that incretin-based drugs may have beneficial pleiotropic effects in the retina. In the current study, the barrier protective and anti-inflammatory properties of Ex-4, an analog of GLP-1, was tested in a rat model of retinal IR injury. Ex-4 prevented the increase in BRB permeability induced by IR injury, which was associated with suppression of inflammatory gene expression. Moreover, *in vitro* studies using the BV-2 microglial cell line showed that Ex-4 also reduced the inflammatory response to LPS and inhibited NF-κB activation. Also, inhibition of pro-inflammatory cytokine production by Ex-4 prevented endothelial cell barrier dysfunction in response to BV2 conditioned media. These studies suggest that Ex-4 can modulate IR injury induced-inflammation through inhibition of inflammatory cytokine production by activated microglia and may provide a novel option for therapeutic intervention in diseases involving retinal inflammation.

3.2 Introduction

A number of ocular diseases have been associated with retinal IR injury, including retinal vascular occlusion, acute glaucoma, diabetic retinopathy, and retinopathy of prematurity (Osborne *et al.*, 2004). IR models have been widely used to study retinal neuronal cell damage and its associated mechanisms after ischemic injury, as well as for devising new therapeutic strategies. The intraocular pressure IR model consists of transient ischemia followed by natural reperfusion, which causes neural cell damage and an inflammatory response (Osborne *et al.*, 2004). Recently, it has been recognized that IR injury also induces alterations at the BRB, such as increased vascular permeability in a vascular endothelial growth factor dependent fashion (Abcouwer *et al.*, 2010) and vascular remodeling (Zheng *et al.*, 2007).

The BRB creates a selective barrier between the vascular lumen and neural retina, and helps maintaining the specific microenvironment necessary for proper neuronal function (Cunha-Vaz *et al.*, 1966). BRB breakdown associated with vascular hyperpermeability may lead to tissue edema with consequent vision loss, and is a common feature of numerous retinal pathologies, including diabetic retinopathy (Cunha-Vaz *et al.*, 1975). Sterile inflammation may contribute to BRB alterations in diabetic retinopathy, as a number of inflammatory cytokines are elevated in the vitreous of patients with active diabetic retinopathy including both non-proliferative and proliferative disease (Adamiec-Mroczek and Oficjalska-Mlynczak, 2008; Patel *et al.*, 2008; Schwartzman *et al.*, 2010). In fact, inflammatory mediators have been shown to promote increased vascular permeability, junctional deregulation, leukocyte adhesion and retinal cell death (Miyamoto *et al.*, 1999; Krady *et al.*, 2005; Tang and Kern, 2011). Pro-inflammatory cytokines such as TNF and IL-1β contribute to BRB breakdown as they induce redistribution or reduced expression of TJ proteins promoting an increase in endothelial permeability both *in vivo* and *in vitro* (Carmo *et al.*, 2000; Aveleira *et al.*, 2010).

GLP-1 is an incretin hormone secreted from enteroendocrine L-cells that enhances glucose-dependent insulin response via the G-protein-coupled GLP-1R (Drucker and Nauck, 2006). GLP-1 displays a very short half-life in circulation due to degradation by the enzyme DPP-IV, therefore long-acting GLP-1 analogs like Ex-4 have been developed to promote insulin secretion in type 2 diabetic patients.

GLP-1R is not only expressed in pancreatic ß-cells, where activation exerts insulinotropic effects, but also in a wide range of tissues, including the retina (Zhang *et al.*, 2009). Furthermore, due to its high lipophilicity, Ex-4 efficiently crosses the BBB (Kastin and Akerstrom, 2003), and therefore likely enters the retina as well. Beyond its acute insulinotropic effect, GLP-1 and its analogs have shown cardioprotective and improved cardiac function in ischemic heart disease, neuroprotective and neurotrophic effects in a number of neurodegenerative disease animal models, renoprotection in diabetic animal models and have also demonstrated antiatherosclerotic potential (Campbell and Drucker, 2013). Concerning the retina, Ex-4 has been shown to have neuroprotective effects in retinas of STZ-diabetic rats, preventing neuronal death, abnormalities in electroretinogram responses, and inner retinal layer thinning (Zhang *et al.*, 2011). Using the same animal model, we previously reported (Goncalves *et al.*, 2014) that a DPP-IV inhibitor (sitagliptin) prevents the diabetes-induced increase in BRB permeability, while exerting anti-inflammatory and anti-apoptotic effects, independent of increased insulin secretion. A recent study (Fan *et al.*, 2014) also showed that intravitreal administration of Ex-4 was able to reduce retinal vascular permeability in type 2 diabetic Goto-Kakizaki rats and inhibit placental growth factor and ICAM-1 expression. These studies collectively suggest that GLP-1R activation may have benefits in treating diabetic retinopathy in addition to its role as an incretin. However, these studies provide little mechanistic understanding of the action of Ex-4 on retinal vascular permeability.

Previous studies demonstrated that retinal IR injury in the rat induces a VEGF driven alteration in permeability and subsequent inflammatory response that maintains permeability at least 48 h after ischemic injury (Abcouwer *et al.*, 2010; Abcouwer *et al.*, 2013; Muthusamy *et al.*, 2014). The present work tested the effect of Ex-4 on retinal inflammation and vascular permeability in this IR model. Ex-4 prevents the increase in BRB permeability induced by IR injury at 48h. Ex-4 also exhibits anti-inflammatory effects by inhibiting the expression of markers of classical inflammation in IR retinas. *In vitro* studies using microglial and endothelial cell cultures revealed that Ex-4 has anti-inflammatory effects that prevent the ability of microglia to produce factors that are known to cause vascular permeability, but fails to directly inhibit the permeability response of primary endothelial cell cultures treated with cytokines or VEGF. These data

suggest that Ex-4 acts to suppress microglia activation in models of retinal inflammation reducing subsequent vascular permeability.

3.3 Materials and Methods

3.3.1 Retinal ischemia-reperfusion

Adult male Long-Evans rats (Charles River Laboratories, Wilmington, MA, USA) weighing 200 to 225 g were housed at approximately 22°C, 60% relative humidity and a 12-h light, 12-h dark cycle was maintained. The animals were given access to water and rodent maintenance chow *ad libitum*. Animals were maintained in specific pathogenic-free conditions, monitored by quarterly sentinel testing and treated in accordance with the University of Michigan Committee on Use and Care of Animals (UCUCA) and consistent with ARVO Statement for the Use of Animals in Ophthalmic and Visual Research. All animal procedures were approved by the University of Michigan Committee on the Use and Care of Animals. Animals were anesthetized with intramuscular injection of ketamine and xylazine (66.7 mg/kg and 6.7 mg/kg body weight, respectively). Ischemia was applied to the left eyes by increasing the intraocular pressure (IOP) to cut off the blood supply from the retinal artery as previously described (Abcouwer *et al.*, 2010; Abcouwer *et al.*, 2013; Muthusamy *et al.*, 2014). Constant intraocular pressure was achieved by inserting a 32-gauge needle into the anterior chamber of the eyes connected to a syringe pump with infusion of continuous flow (40 mL/min) of sterile saline. IOPs were monitored with a microtonometer designed for use on rodent eyes (TonoLab; Icare, Helsinki, Finland). Retinas were subjected to 45 min ischemia followed by natural reperfusion of blood for a period of 48 h. The contralateral eyes were subjected to needle puncture and served as sham controls. Ex-4 (Tocris Bioscience, Bristol, UK), dissolved in 0.9% saline solution (pH 6), was administered twice-daily as a subcutaneous injection of 10 µg/kg, with two initial administrations prior to ischemia and every 12 h for the next 48 h during the reperfusion period (Figure 3.1). Non-treated animals received saline vehicle injections. The last injection was given 1 h prior to injection of Evans blue dye for the permeability assay or 1 h prior to sacrificing the animals and collecting the retinas for the gene expression and whole-mount staining studies.

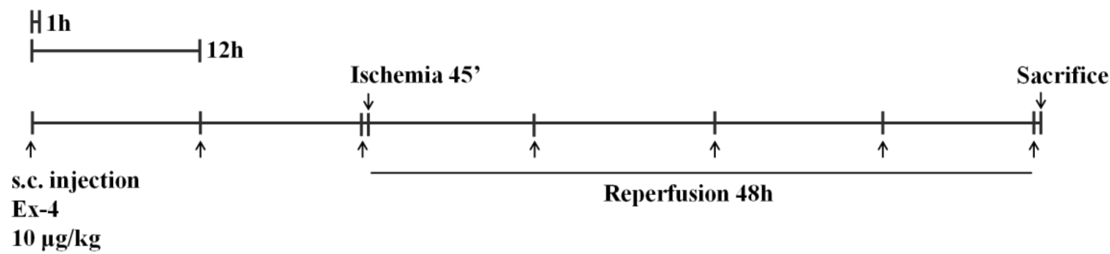

Figure 3.1. Scheme of the drug administration protocol used to evaluate the effect of Ex-4 on ischemia-reperfusion injury.

3.3.2 Evans Blue Assay

Retinal vascular permeability was measured by the accumulation of albumin-binding dye, Evans blue, according to the method described by Xu and colleagues (Xu *et al.*, 2001). The Evans Blue content in blood plasma after 2 h of circulation was used for normalization, and values expressed as microliters of plasma per gram of retina (dry weight) per hour of circulation.

3.3.3 Isolation of tissue and cell RNA and quantitative RT-PCR

Retinas and cell pellets were collected, flash-frozen in liquid nitrogen, and stored at -80°C until analysis. Total RNA was purified using RNeasy Plus™ Mini kit (Qiagen, Venlo, Limburg, Netherlands), using QiaShredders™ (Qiagen) for dissociation of retinal tissues. Quantitative real-time polymerase chain reaction (qRT-PCR) was performed by reverse transcription of 1.0 µg of total RNA using random hexamers and oligo-dT primers in the presence of RNase inhibitor (Omniscript™ RT kit, Qiagen). Duplex qPCRs were performed using the equivalent of 1 µl of RT reaction with gene-specific primers and FAM-labeled probes (Applied Biosystems Life Technologies, Carlsbad, CA, USA), along with β-actin-specific primers and VIC-labeled probes (primer limited formulation, Applied Biosystems Life Technologies) and TaqMan™ Universal PCR master mix (Applied Biosystems Life Technologies). Primer-probe assay information and gene information are provided in Table 3.1. Reactions were performed and monitored using a CFX384 real time PCR system (Bio-Rad, Hercules, CA, USA). Relative normalized mRNA levels were calculated using the $\Delta\Delta C_t$ method.

Table 3.1. Gene and primer-probe assay information

Gene Symbol	RefSeq accession		Applied Biosystems Assay #	
	Rat	Mouse	Rat	Mouse
CCL2	NM_031530.1	NM_011333.3	Rn00580555_m1	Mm00441242_m1
ICAM1	NM_012967.1	-------------	Rn00564227_m1	----------------
IL1B	NM_031512.2	NM_008361.3	Rn00676333_g1	Mm00434228_m1
IL6	NM_012589.1	NM_031168.1	Rn99999011_m1	Mm00446190_m1
PTGS2/COX2	NM_017232.3	-------------	Rn01483828_m1	----------------
TNF	NM_012675.3	NM_013693.3	Rn99999017_m1	Mm00443258_m1
NOS2	-------------	NM_010927.3	----------------	Mm00440502_m1
ACTB	NM_031144.3	-------------	Rn00667869_m1	Mm02619580_g1

CCL2: Chemokine (C-C Motif) Ligand 2; ICAM1: Intercellular Adhesion Molecule 1; IL1B: Interleukin 1, Beta; IL6: Interleukin 6 (Interferon, Beta 2); PTGS2/COX2: Prostaglandin-Endoperoxide Synthase 2 (Prostaglandin G/H Synthase And Cycloxygenase); TNF: Tumor Necrosis Factor; NOS2: Nitric Oxide Synthase 2, Inducible; ACTB: Actin, Beta

3.3.4 Retinal whole-mounts immunostaining

Retina whole-mounts were prepared according to the procedure previously described (Abcouwer *et al.*, 2013). Retinas were incubated with anti-rat CD45 (Table 3.2) for 3 days at 4°C. After washing, the retinas were incubated with the secondary antibody Alexa Fluor 568-labeled goat anti-mouse IgG (1:1,000, Life Technologies), Alexa Fluor 488-labeled isolectin B4 (IB4) from *Griffonia simplicifolia* (1:75, Life Technologies) and 10 µg/ml Hoechst-33342 DNA stain (Life Technologies) for 24 h at 4°C. Retinas were mounted with the vitreous side up for visualization under a confocal microscope (Leica TCS SP5 AOBS, Leica Microsystems, Wetzlar, Germany). Eight animals per group were used and from each retina 8 fields were averaged and used to determine the number of CD45-positive cells in a masked fashion.

Table 3.2. List of primary antibodies used in Western blot or immunofluorescence.

Primary Antibody	Dilution		Catalog #	Manufacturer
	WB	IF		
Mouse anti-rat CD45	------	1:50	550566	BD Biosciences
Rabbit anti-CREB	1:1000	------	9197	Cell Signaling Tech.
Rabbit anti-phosphoCREB	1:1000	------	9198	Cell Signaling Tech.
Rabbit anti-p65	1:1000	1:100	4764	Cell Signaling Tech.
Rabbit anti-GLP-1R	1:300	------	ab39072	Abcam
Goat anti-GAPDH	1:10000	------	AB0049-200	Sicgen
Rabbit anti-Lamin B	1:4000	------	ab16048	Abcam

WB: Western blot; IF: immunofluorescence

3.3.5 Measurement of bovine retinal endothelial cell permeability

Bovine retinal endothelial cells (BREC) were isolated as previously described (Antonetti and Wolpert, 2003). BREC were cultured in flasks coated with 1 µg/cm^2 fibronectin in MCDB-131 medium, supplemented with 10% fetal bovine serum (FBS), 22.5 µg/mL endothelial cell growth factor, 120 µg/mL heparin, 0.01 mL/mL antibiotic-antimycotic. For experimentation, BREC were used from passage 4 to passage 7. To measure cell monolayer permeability, BREC were grown to confluence on 0.4 µm pore transwell filters (Corning Costar, Acton, MA, USA) and then cell culture media was changed to MCDB-131 medium supplemented with 1% FBS, 0.01 mL/mL antibiotic/antimycotic, and 100 nmol/L hydrocortisone for 2 days before the experiment. BREC were pre-treated with 10 nmol/L Ex-4, a concentration described to prevent increased permeability in endothelial cells (Li *et al.*, 2015). After 1 h of incubation, BREC were then exposed to TNF (5 ng/mL for 1.5 h), VEGF (50 ng/mL for 30 min), or the combination of both. After the treatments, 10 µmol/L rhodamine B isothiocyanate-dextran (70 kDa) (Sigma-Aldrich, St. Louis, MO, USA) was added to the apical chamber of inserts. Aliquots were removed from the basolateral chamber every 30 min for 4 h for quantification, and an aliquot from the apical chamber was taken at the end of the experiment. The rate of diffusive flux (P_o) was calculated by the following formula,

$$P_o = [(F_A/\Delta t)V_A]/(F_L A)$$

where P_o is in centimeters per second; F_A is basolateral fluorescence; F_L is apical fluorescence; Δt is change in time; A is the surface area of the filter (in square centimeters); and V_A is the volume of the basolateral chamber (in cubic centimeters).

3.3.6 Murine microglial cell line BV2 culture and treatment

The immortalized murine BV2 microglial cell line (Blasi *et al.*, 1990) was cultured in Dulbecco's modified Eagle's medium (DMEM) containing Glutamax and supplemented with 10% FBS, 10,000 U/mL penicillin and 10 mg/mL streptomycin. For the experiments, BV2 cells were plated at a density of 1 x 10^5 cells/cm^2 in DMEM containing 1% FBS and then treated with 100 nmol/L Ex-4 1 h prior to the addition of LPS (from *Escherichia coli*, Sigma-Aldrich) at a concentration of 100 ng/mL for the times specified in the respective figure legends. The concentration of Ex-4 used for microglial cells was based on preliminary results showing a dose-dependent inhibition of IL-1β expression after treatment with LPS for 4 h (data not shown).

3.3.7 Total cell lysates

BV2 cells were washed twice with ice-cold PBS and then collected in RIPA buffer: 50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 5 mmol/L EGTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mmol/L DTT, supplemented with 2 mmol/L phenylmethylsulfonyl fluoride, 2 mmol/L iodoacetamide and 1x protease inhibitor cocktail (Roche, Basel, Switzerland). The lysates were incubated on ice for 30 min, sonicated six times (for 1 s each) and then centrifuged at 16,000 *g* for 10 min at 4°C. Supernatants were used to determine the protein concentration by the BCA colorimetric assay (Pierce, Rockford, IL, USA). Samples were then denaturated with 6x Laemmli buffer, boiled for 5 min at 95°C and stored at -20°C until use.

3.3.8 Measurement of cAMP levels

BV2 cells were incubated with Ex-4 for the periods of time indicated in the respective figure legend. Cells were lysed in 0.1 mol/L HCl and the intracellular levels of cAMP were

measured using a colorimetric competitive immunoassay kit according to the manufacturer instructions (Enzo Life Sciences, Farmingdale, NY, USA). Results were normalized to protein content.

3.3.9 Subcellular fractionation assay for nuclear extraction

BV2 cells were washed twice with ice-cold PBS and lysed with a hypotonic buffer (10 mmol/L HEPES, pH 7.9, 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.4% Nonidet P-40, 1 mmol/L DTT), containing protease inhibitors for 30 min on ice. After centrifugation at 18,000 *g* for 5 min at 4°C, the supernatant containing the cytoplasmic fraction was discarded. The pellet was resuspended in high-salt extraction buffer (20 mmol/L HEPES, pH 7.9, 420 mmol/L NaCl, 1 mmol/L EDTA, 10% glycerol, 1 mmol/L DTT), containing protease inhibitors. The nuclear suspension was agitated for 1 h at 4°C and centrifuged at 18,000 *g* for 5 min at 4°C. The resultant supernatants containing nuclear proteins were collected. Sample preparation and protein quantification were performed as for the total extracts.

3.3.10 Immunoblot analysis

For immunodetection of p65, p-CREB, CREB and GLP-1R, 30 µg of protein from nuclear or total cell extracts were separated using SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Boehringer Mannheim, Mannheim, Germany). Western blot was performed as previously described (Goncalves *et al.*, 2012). Membranes were probed with primary antibodies (Table 3.2) anti-p65, anti-CREB, anti-phosphoCREB and anti-GLP-1R. GAPDH and Lamin B (Table 3.2) were used as loading controls for total and nuclear extracts, respectively. Immunoreactive bands were detected by ECL substrate using an imaging system (VersaDoc 4000 MP, Bio-Rad) and quantification was performed using ImageJ 1.47 software (NIH, Bethesda, MD, USA).

3.3.11 Immunocytochemistry

BV2 cells cultured on cover glass were washed with pre-warmed PBS and fixed with ice-cold 4% paraformaldehyde for 15 min at room temperature. Cells were then washed

with PBS, blocked with 5% BSA in PBS containing 0.3% Triton X-100 (PBS-T) for 1 h and incubated with the primary antibody anti-p65 (Table 3.2) in PBS-T containing 1% BSA overnight at 4°C. After washing with PBS, cells were incubated at room temperature for 1 h (kept dark) with the secondary antibody (1:1,000 dilution in PBS-T) Alexa Fluor-488 goat anti-rabbit (Life Technologies) and 10 μg/ml Hoechst-33342 DNA stain (Life Technologies) for nuclear staining. The cover slips were mounted upside down on glass slides and visualized under a confocal microscope (Leica TCS SP5 AOBS). Nuclear p65 immunostaining was measured using ImageJ software by averaging the fluorescence intensity of delimitated nuclei from 4 fields per slide from 3 experiments in a masked fashion.

3.3.12 Microglia-Conditioned Medium Experiments

BV2 cells were seeded in 6-well plates at a density of 1 x 10^5 cells/cm^2 and treated with 100 nmol/L Ex-4 for 1 h and then exposed to 100 ng/mL LPS for 24 h. Microglia conditioned medium (MCM) was collected from each condition and then centrifuged at 500 *g* for 5 min at 4°C to discard cell debris. Supernatant was collected and confluent BREC were incubated with the MCMs for 1 h before starting the permeability assay as described above. BREC were also incubated with BV2 culture medium and that condition was used to normalize the permeability values obtained.

3.3.13 Statistical analysis

Results are expressed as mean ± SEM. Statistical differences between like-treated Sham and IR retina groups were analyzed by paired Student's *t*-test, with effects of treatments analyzed by unpaired Student's *t*-test. One-way analysis of variance (ANOVA) followed by Bonferroni's *post hoc* test was employed to calculate the statistical difference between three or more groups. Prism 4.0 (GraphPad Software, San Diego, CA, USA) was used and values of $P<0.05$ were considered statistically significant.

3.4 Results

3.4.1 Exendin-4 prevents the increase in BRB permeability induced by ischemia-reperfusion injury

It was previously described that the IR model used presents increased vascular permeability at 4 to 48 h of natural reperfusion following the 45 min ischemia period (Abcouwer *et al.*, 2010; Muthusamy *et al.*, 2014). In the present study, the effect of Ex-4 therapy on prevention of retinal vascular permeability induced by ischemia-reperfusion injury was assessed by a quantitative Evans Blue dye accumulation assay. Vascular permeability was significantly increased by 3.8-fold ($P<0.001$) in IR retinas at 48 h after reperfusion when compared to non-treated sham retinas, as revealed by the increased retinal Evans Blue dye accumulation. Treatment with Ex-4 by subcutaneous injection twice a day was able to significantly prevent the vascular permeability increase ($P<0.01$) (Figure 3.2).

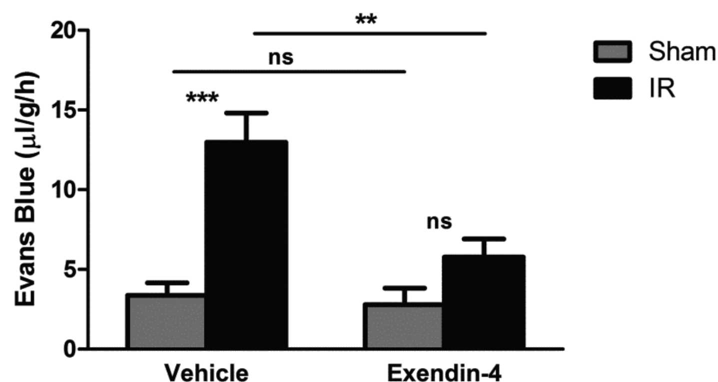

Figure 3.2. Ex-4 protects against the increase in retinal vascular leakage triggered by ischemia-reperfusion. Rats were treated twice-daily with a subcutaneous injection of Ex-4 (10 µg/kg), with two initial administrations prior to ischemia and injections every 12 h for the next 48 h during the reperfusion period. Non-treated animals received saline vehicle injections. One eye of each animal was subjected to retinal ischemia for 45 min, followed by natural reperfusion. The contralateral eyes were subjected to needle puncture and served as sham controls. Evans Blue dye was injected in the tail vein and allowed to circulate, 2 h before flushing and determination of dye accumulation in retinal tissue. Data are expressed as mean ± SEM (n=16 retinas per group). **$P<0.01$, ***$P<0.001$, like-treated Sham and IR retina groups were analyzed by paired Student's *t*-test, with effects of treatments analyzed by unpaired Student's *t*-test.

3.4.2 Exendin-4 treatment alters the inflammatory response following IR injury without affecting the number of CD45-positive cells

Because underlying mechanisms of BRB breakdown include inflammatory changes, we determined by qRT-PCR the expression mRNAs corresponding to classical markers of inflammation at 48 h after IR injury. IR injury induced a significant increase in retinal mRNA expression of several pro-inflammatory cytokines, including IL-1β (3.2-fold, $P<0.05$), IL-6 (4.2-fold, $P<0.05$), TNF (5.6-fold, $P<0.001$) and C-C motif chemokine ligand 2 (CCL2, 116.9-fold, $P<0.05$) (Fig. 3.3 A-D). However, expression of mRNA for cyclooxygenase 2 (COX2) was not altered after 48 h in IR retinas (Fig. 3.3E) and mRNA for ICAM-1 (1.9-fold) trended up (Fig. 3.3F).

Comparison of mRNA expression levels in sham eyes of Ex-4-treated and non-treated rats revealed that the drug alone significantly affected the expression of three of the genes evaluated, including IL-1β (decreased 36 %, $P<0.05$), IL-6 (increased 3.2-fold, $P<0.01$) and COX2 (increased 1.7-fold, $P<0.05$) (Fig. 3.3A, B and E, respectively).

Treatment with Ex-4 significantly inhibited IR-induced up-regulation of cytokines with known effects on vascular permeability, IL-1β (61 % inhibition, $P<0.05$), TNF (30 % inhibition, $P<0.01$) and CCL2 (53 % inhibition, $P<0.05$) mRNAs (Fig. 3.3A, C and D, respectively). No effect on ICAM-1 mRNA expression levels was observed (Fig. 3.3F).

To determine whether Ex-4 treatment decreases leukocyte migration and infiltration into the retinal tissue following IR, retinal whole-mount retinas were stained with CD45, a marker of leukocytes, and with IB4, an isolectin binding to terminal alpha-D-galactose residues of glycoconjugates found on endothelial cells and some macrophages (Fig. 3.3G). The number of CD45 positive cells was significantly higher in IR retinas, relative to sham retinas. Most CD45-positive cells were present in the perivascular space after 48 h reperfusion (Fig. 3.3G, arrows). A few CD45-stained cells were also observed within vessels in IR injured retinas (Fig. 3.3G, arrowhead). Ex-4- treated IR retinas did not demonstrate any decrease in CD45-positive cells when compared to vehicle-treated IR retinas (Fig. 3.3H), suggesting that the anti-inflammatory effects of Ex-4 are not due to a decrease in infiltrating leukocytes and are not sufficient to cause a decrease in leukocyte accumulation.

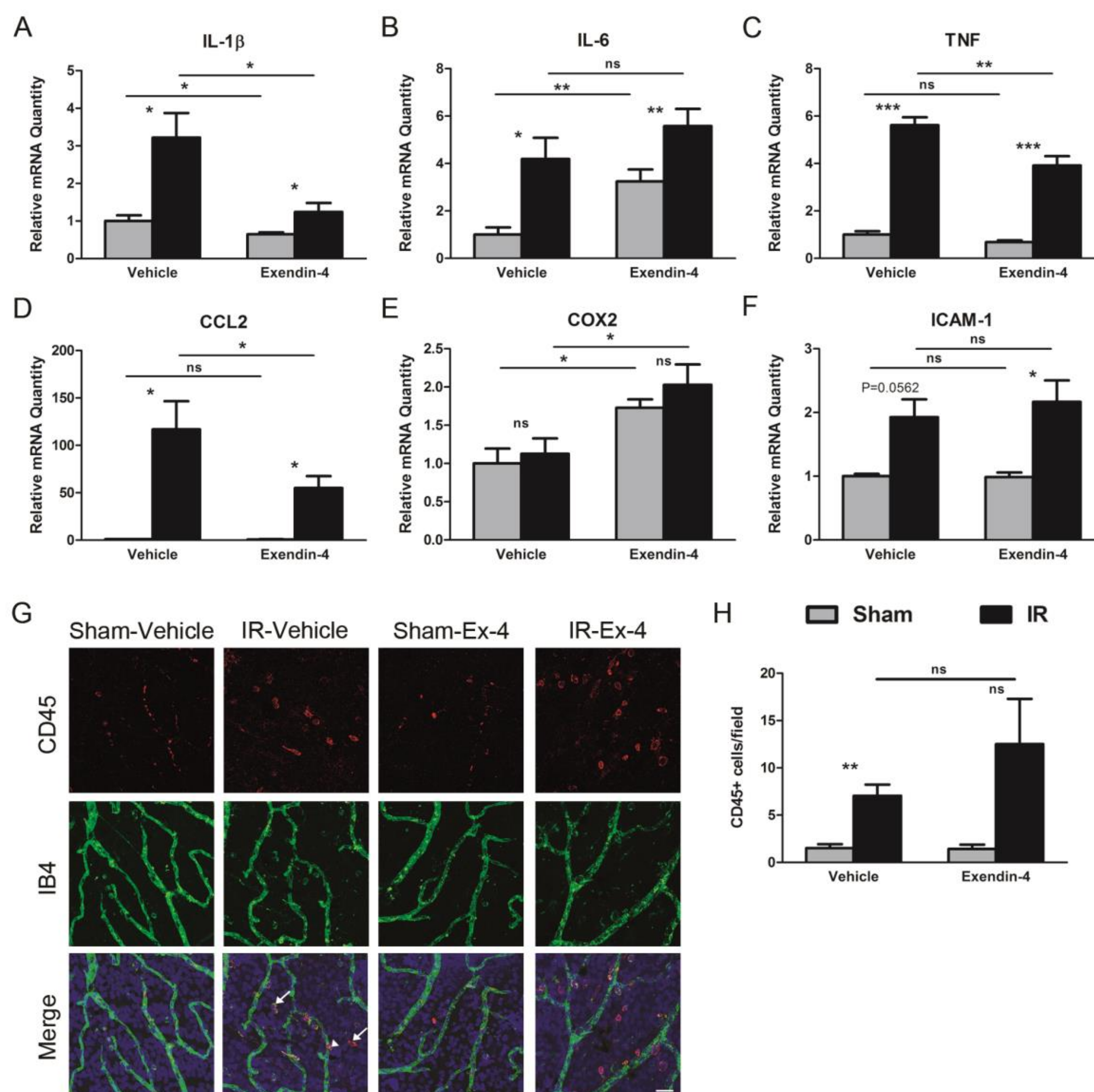

Figure 3.3. Ex-4 inhibits the induction of the expression of IR-responsive genes associated with inflammation without affecting the number of CD45-positive cells. Rats were treated with Ex-4 (10 µg/kg) twice-daily with two initial administrations prior to ischemia, and injections every 12 h for the next 48 h during the reperfusion period. Non-treated animals received saline vehicle injections. One eye of each animal was subjected to retinal ischemia for 45 min, followed by natural reperfusion. The contralateral eyes were subjected to needle puncture and served as sham controls. Total RNA was isolated from the retinas and the relative levels of mRNA of **(A)** IL-1β, **(B)** IL-6, **(C)** TNF, **(D)** CCL2, **(E)** COX2 and **(F)** ICAM-1 were determined by duplex qRT-PCR with β-actin serving as control. Data are expressed as mean ± SEM (n=8 retinas per group). **(G)** Retinas were isolated and stained with antibodies to CD45 (red), isolectin B4 (IB4, green) and Hoechst nuclear stain (blue) and then flat mounted. The majority of CD45-positive cells are present in the perivascular region (arrows) with few cells positively staining inside the vessels (arrowhead). Scale bar: 25 µm **(H)** CD45-positive cells were quantified in each retina from each

group. Data are presented as CD45 positive cells per field and represent the mean ± SEM (n=8 retinas per group). *P<0.05, **P<0.01, ***P<0.001, like-treated Sham and IR retina groups were analyzed by paired Student's *t*-test, with effects of treatments analyzed by unpaired Student's *t*-test.

3.4.3 3.4.3 Exendin-4 has no effect on TNF and/or VEGF-induced endothelial cell permeability

Given that Ex-4 was able to prevent the IR induced BRB permeability *in vivo*, we hypothesized that it could have direct beneficial effects on endothelial barrier integrity. We therefore evaluated the effect of Ex-4 pretreatment on *in vitro* endothelial cell monolayer permeability to RITC-dextran following treatment with TNF and/or VEGF. As shown in Figure 3.4, treatment with TNF and/or VEGF resulted in a significant increase in 70 kDa RITC-dextran permeability across BREC monolayer. Pre-treatment of BREC with Ex-4 did not prevent or reduce the increase in permeability induced by TNF, VEGF or both combined (Fig. 3.4).

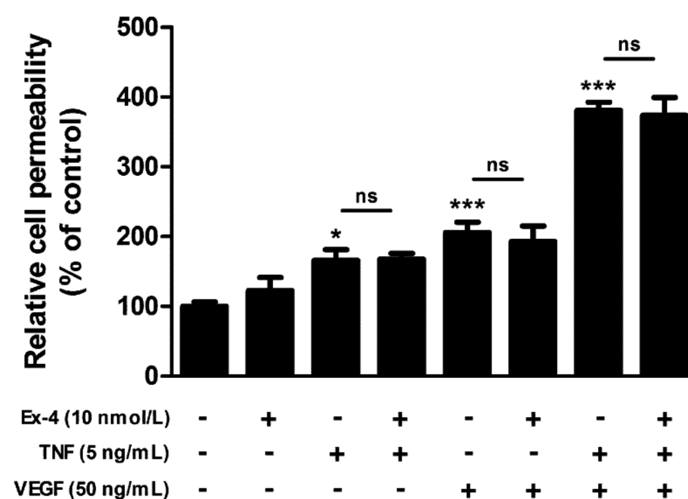

Figure 3.4. Effect of Ex-4 on TNF and/ or VEGF-induced retinal endothelial cells permeability. BREC were grown to confluence on transwell filters and the monolayer permeability to RITC-70 kDa dextran was measured over the following 4 h after the several treatments. Ex-4 does not block TNF and/or VEGF-induced permeability in cultured BREC. BREC were treated with 10 nmol/L Ex-4 1 h before TNF (5 ng/mL, 1.5 h) and/or VEGF (50 ng/mL, 30 min) treatment. Average rate of diffusive flux (P_o) for the control was 5.5 x 10^{-7} cm/s. The results represent the mean ± SEM (n≥3). *P<0.05, ***P<0.001 vs. Control, ns: non-significant; One-way ANOVA followed by Bonferroni's *post hoc* test.

3.4.4 Ex-4 modulates inflammatory response in BV2 microglial cells

Having established that Ex-4 reduced the production of pro-inflammatory cytokines without inhibition of induction of expression of ICAM-1 or accumulation of leukocytes occurring in IR retinas, we proceeded by assessing the impact of Ex-4 in microglia, the resident innate immune cells of the retina and other neural tissues. Production of nitric oxide and pro-inflammatory cytokines by microglia may contribute to neuroinflammation in the IR injury model (Madeira *et al.*, 2015). In order to test the ability of Ex-4 to inhibit microglial activation, we used BV2, a microglia cell line, stimulated with LPS, which is a potent inducer of microglial activation and inflammatory gene expression.

Ex-4 is known to activate GLP-1R, a G-protein coupled receptor, leading to activation of adenylyl cyclase, which results in generation of cyclic AMP (cAMP) (Campbell and Drucker, 2013). We observed by Western blotting that Ex-4 and LPS each induced a significant increase in GLP-1R protein levels ($P<0.01$) that was not additive ($P<0.001$) (Fig. 3.5A).

Treatment with 100 nmol/L of Ex-4 promoted a significant increase of intracellular cAMP levels by 15 min ($P<0.001$) that was maintained for at least 1 h ($P<0.01$) confirming GLP-1R activation (Fig. 3.5B).

Activation of GLP-1R was further confirmed by measuring the PKA-mediated phosphorylation of the transcription factor cAMP-response element binding protein (CREB). Treatment with Ex-4 promoted a peak in CREB phosphorylation after 30 min ($P<0.01$) (Fig. 3.5C). Further, Ex-4-induced CREB activation was maintained after incubation with LPS (Fig. 3.5D).

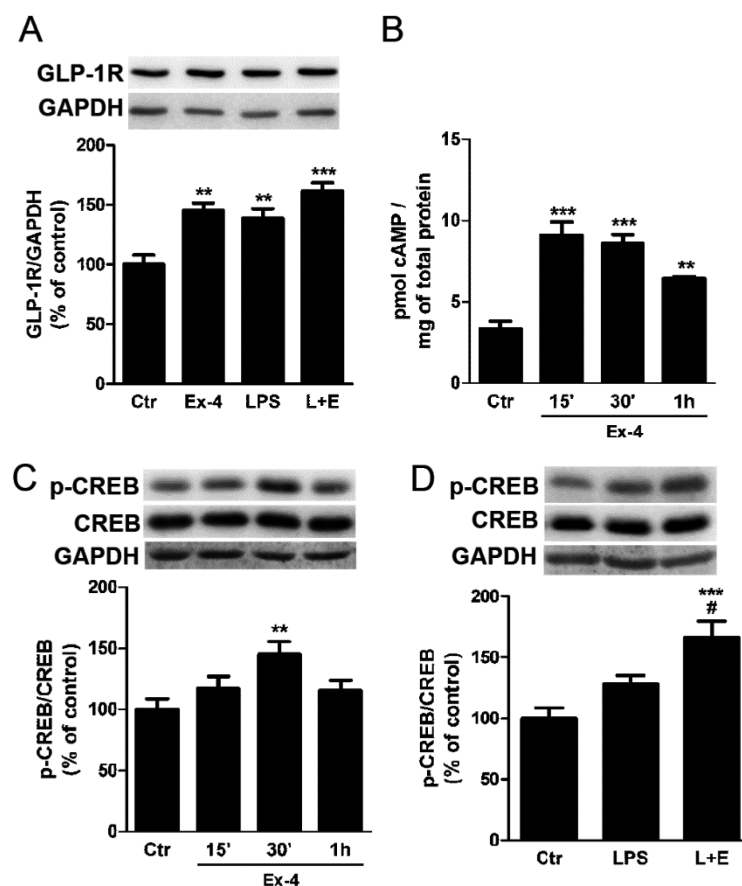

Figure 3.5. Ex-4 induces activation of GLP-1R and CREB in LPS-treated microglial BV2 cells. (A) BV2 cells were treated with Ex-4 (100 nmol/L) 1 h prior to the LPS stimulus (100 ng/mL for 4 h), and protein levels of GLP-1R were evaluated by Western blotting. GAPDH was used as a loading control. Quantification of GLP-1R protein levels was performed by densitometric analysis. Data represent the mean ± SEM (n=5) **(B)** Intracellular cAMP levels were determined by ELISA after stimulating BV2 cells with Ex-4 (100 nmol/L) for 15 min, 30 min and 1 h. Data are presented as pmol of cAMP per mg of total protein and represent the mean ± SEM (n=3). **(C)** BV2 cells were incubated with Ex-4 as described in (B) and protein levels of p-CREB and CREB were determined by Western blotting. **(D)** The effects of LPS on CREB phosphorylation were also assessed. Data represent the mean ± SEM (n=5). ***P*<0.01, ****P*<0.001 vs. Control; #*P*<0.05 vs. LPS, one-way ANOVA followed by Bonferroni's *post hoc* test.

To investigate the anti-inflammatory effects of Ex-4, BV2 microglial cells were pre-incubated with Ex-4 for 1 h and then stimulated with LPS for 4 h or 24 h. Treatment with Ex-4 was able to inhibit LPS-induced increase in mRNA expression of IL-1β (43 % inhibition, *P*<0.05) after 4 h, and IL-6 (64 % inhibition, *P*<0.05), CCL2 (58 % inhibition, *P*<0.001), nitric oxide synthase 2 (NOS2) (76 % inhibition, *P*<0.01) after 24 h (Fig 3.6A-E).

A non significant trend toward a decrease in TNF mRNA levels was observed after Ex-4 treatment at both 4 h (40 % inhibition) and 24 h (63 % inhibition) (Fig 5A-E).

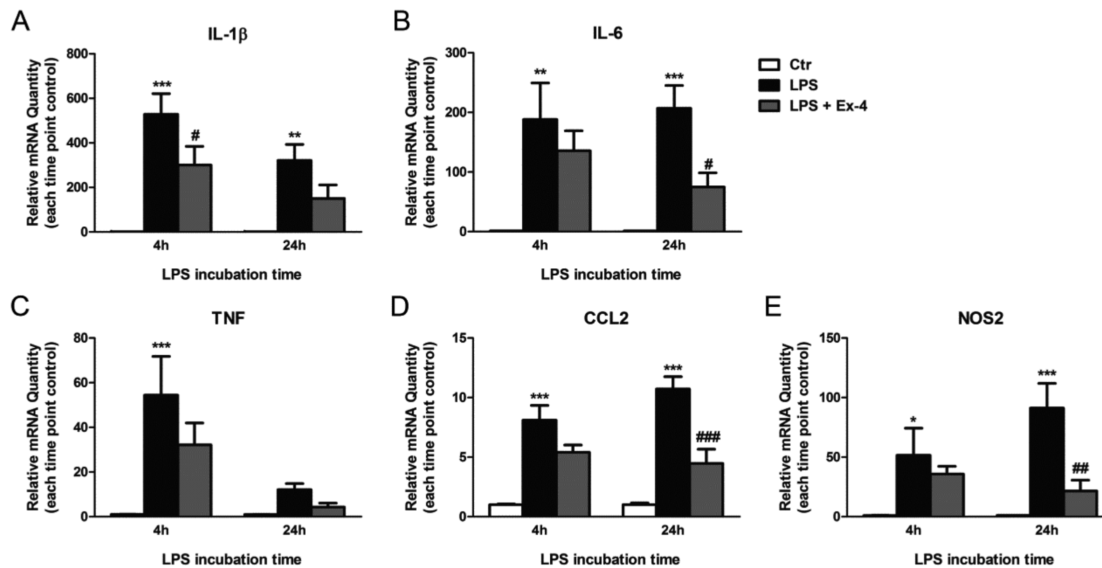

Figure 3.6. Ex-4 attenuates the inflammatory response of BV2 microglial cells to LPS. BV2 cells were treated with Ex-4 (100 nmol/L) 1 h prior to the LPS stimulus (100 ng/mL for 4 h or 24 h). Total RNA was isolated from the cells and the relative levels of mRNA of **(A)** IL-1β, **(B)** IL-6, **(C)** TNF, **(D)** CCL2 and **(E)** NOS2 were determined by duplex qRT-PCR with β-actin serving as control. Data are presented as the relative mRNA quantity and represent the mean ± SEM (n=4). **P*<0.05, ***P*<0.01, ****P*<0.001 vs. Control; #*P*<0.05, ##*P*<0.01, ###*P*<0.001 vs. LPS. Two-way ANOVA followed by Bonferroni's *post hoc* test.

We next examined the effects of Ex-4 on NF-κB, which is an important transcription factor modulating cytokine gene expression in microglia (Sen and Smale, 2010). Immunocytochemical analysis shows that stimulation of BV2 cells with LPS for 30 min resulted in a strong nuclear expression of NF-κB p65 subunit, which was significantly inhibited by pre-treatment with Ex-4 (Fig. 3.7A-B). Additionally, these results were confirmed by Western blotting using nuclear extracts from BV2 cells (Fig. 3.7C). These data suggest that modulation of NF-κB activation may be involved in Ex-4 ability to inhibit the expression of inflammatory mediators in microglial cells.

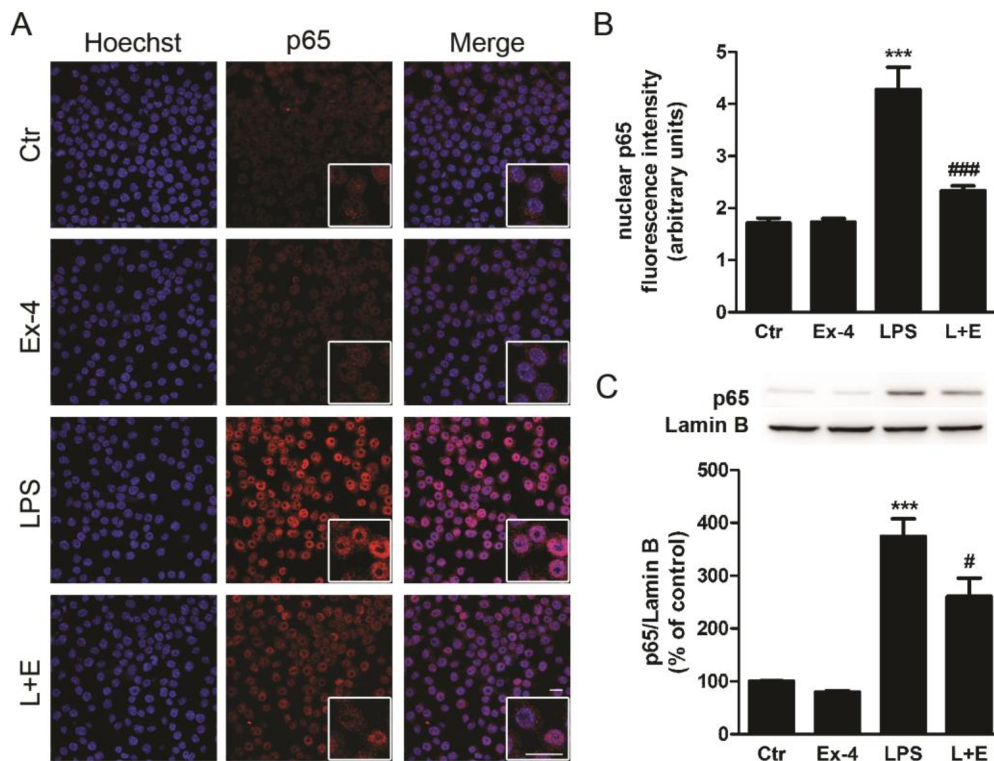

Figure 3.7. Ex-4 inhibits the nuclear accumulation of the NF-κB p65 subunit in LPS-stimulated BV2 microglial cells. **(A)** BV2 cells were treated with Ex-4 (100 nmol/L) 1 h prior to the LPS stimulus (100 ng/mL for 30 min). Subcellular localization of p65 subunit (red) was evaluated by immunocytochemistry. Hoechst staining (blue) was used to visualize nuclei. Scale bar: 20 µm **(B)** Quantification of nuclear fluorescence intensity for p65 immunoreactivity in BV2 cells. Data are presented as arbitrary fluorescence units and represent the mean ± SEM (n=3). **(C)** BV2 cells were stimulated as described in (A). Subcellular fractionation was performed and nuclear extracts were separated by SDS-PAGE and immunoblotted with anti-p65 antibody. Lamin B was used as a loading control. Quantification of p65 protein levels was performed by densitometric analysis. Data are presented as mean ± SEM (n=5). ****P*<0.001 vs. Control; #*P*<0.05, ###*P*<0.001 vs. LPS, one-way ANOVA followed by Bonferroni's *post hoc* test.

3.4.5 Role of microglia inflammatory response on endothelial cells permeability

Inflammatory mediators have been known to contribute to an increase in retinal endothelium permeability. In fact, TNF and IL-1β are known to promote an increase in permeability both *in vivo* and *in vitro*.(Carmo *et al.*, 2000; Aveleira *et al.*, 2010) Therefore, to clarify the role of microglia modulation by Ex-4 we evaluated the effect of microglia conditioned medium (MCM) on endothelial cells permeability. BREC monolayer permeability significantly increased by 1.8-fold (*P*<0.05) after exposure to

MCM from BV2 cells treated with LPS alone for 24h (Fig. 3.8A). MCM from BV2 pre-treated with Ex-4 for 1h and then stimulated with LPS for 24 h did not induce any significant alteration in BREC permeability, cell permeability values (113.5 ± 13.7 % of BV2 medium) are similar to the ones observed for BREC exposed to MCM from control BV2 cells (112.0 ± 17.6 % of BV2 medium) (Fig. 3.8A). Importantly, when BREC were directly treated with LPS and Ex-4, there was no reduction on the increase in permeability induced by LPS alone (Fig. 3.8B). These results suggest that the effects observed with MCM were not due to remaining LPS/Ex-4 present in the medium and point to the important role of microglia activation on barrier function.

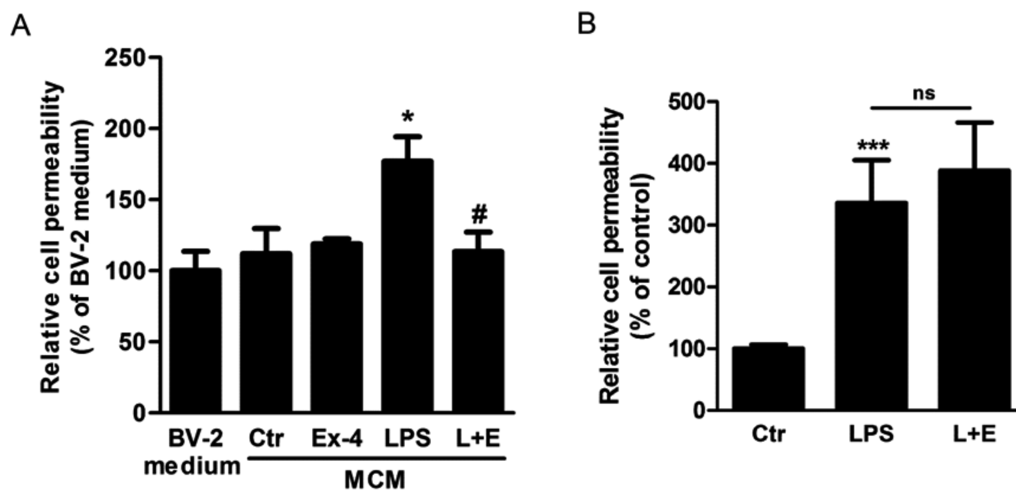

Figure 3.8. Modulation of microglia activation by Ex-4 contributes to vascular barrier properties. BREC were grown to confluence on transwell filters and the monolayer permeability to RITC-70 kDa dextran was measured over the following 4 h after the several treatments. **(A)** BREC monolayer permeablity was assessed after exposure to 1 h of microglia (BV2) medium, microglia conditioned medium (MCM) from BV2 cells non-treated, treated with Ex-4 (100 nmol/L), LPS (100 ng/mL, 24 h) and treated with Ex-4 1 h prior to the LPS stimulus. Average rate of diffusive flux (Po) for the BV2 medium condition was 4.6 x 10^{-6} cm/s. **(B)** Ex-4 does not prevent LPS induced increase in monolayer permeability. BREC were incubated with LPS (100 ng/mL) and Ex-4 (100 nmol/L) for 1 h prior to the permeability assay. Average rate of diffusive flux (Po) for the control was 6.2 x 10^{-7} cm/s. The results represent the mean ± SEM (n ≥3). *P<0.05, ***P<0.001 vs. MCM Ctr or Ctr, #P<0.05 vs. MCM LPS; One-way ANOVA followed by Bonferroni's post hoc test.

3.5 Discussion

In the present study, we provide evidence that Ex-4, a GLP-1 analog, prevents the increase in BRB permeability and reduces the expression of several classical inflammatory markers induced by IR injury in rat retinas. Moreover, using a microglia cell line for *in vitro* studies we demonstrate that Ex-4 also inhibits the inflammatory response to LPS activation, while reducing the amount of NF-κB p65 in the nucleus. Collectively, these results demonstrate, for the first time, that Ex-4 is able to modulate inflammation and prevent loss of the BRB in a retinal ischemia model.

Intraocular pressure–induced retinal IR injury provides a useful model of VEGF-driven vascular permeability followed by inflammatory response that maintains BRB loss, such as may be observed in a number of retinal eye diseases, including diabetic retinopathy and retinal vein occlusions (Abcouwer *et al.*, 2010). Evidence of retinal neurodegeneration, inflammation, including microglial activation, and BRB loss associated with TJ alterations all may be observed after IR injury. Furthermore, this model does not include any systemic metabolic alterations, providing an opportunity to verify the direct effects of Ex-4 previously observed on diabetic retinas. Given its high lipophilicity, Ex-4 was shown to readily cross the BBB, where it binds to the ubiquitously expressed GLP-1 receptors (Kastin and Akerstrom, 2003). Thus, in contrast to previous studies (Zhang *et al.*, 2011; Fan *et al.*, 2014), where Ex-4 was delivered by intravitreal injection, we decided to administer Ex-4 by subcutaneous injection, which is the route of administration indicated for GLP-1R agonists in the clinical practice. Furthermore, repeated intravitreal injections present a high risk for developing infectious endophthalmitis which would mask any anti-inflammatory effects of Ex-4 in case this route of administration was used.

Ex-4 has been reported to have multiple cellular protective effects, including the protection of endothelial cells and barrier function. A recent study showed that intravitreal injection of Ex-4 reduces retinal vascular leakage in type 2 diabetic Goto-Kakizaki rats (Fan *et al.*, 2014). Moreover, Ex-4 was found to suppress LPS-mediated release of nuclear DNA-binding protein high-mobility group box 1 (HMGB1) and inhibits HMGB1-mediated hyperpermeability and leukocyte migration in septic mice (Lee *et al.*,

2014). An earlier study showed that GLP-1 attenuates LPS-induced mesenteric endothelium permeability via GLP-1R/cAMP/PKA signaling pathway (Dozier *et al.*, 2009). Moreover, in human umbilical vein endothelial cells (HUVEC), Ex-4 significantly prevents the thrombin-induced permeability of endothelial monolayers via both GLP-1R/cAMP/PKA and GLP-1R/cAMP/Epac1 pathways (Li *et al.*, 2015). In the present study, systemic administration of Ex-4 was able to prevent increased retinal vascular permeability induced by IR injury at 48 h reperfusion. However, in primary cultures of retinal endothelial cells, Ex-4 could not prevent the increase in permeability induced by permeabilizing agents that are known to be increased in IR retinas, such as VEGF and TNF. These results point to indirect mechanisms accounting for the *in vivo* protective effects of Ex-4 in BRB, other than directly targeting the endothelial cells.

Inflammation triggered by reperfusion is a key mediator in retinal damage after ischemic injury and anti-inflammatory effects of Ex-4 have been proposed by recent studies (Iwai *et al.*, 2006; Arakawa *et al.*, 2010; Kodera *et al.*, 2011; Darsalia *et al.*, 2014). However, the current study is the first demonstration of the anti-inflammatory effects of Ex-4 on retinal IR injury. Ex-4 inhibited the increase in the expression of classical inflammatory genes (IL-1β, TNF and CCL2) that are responsive to IR injury. We also observed that the expression of another IR-responsive gene, IL-6, was further exacerbated by Ex-4. IL-6 appears to have a dual role in inflammatory processes (Suzuki *et al.*, 2009) as increased IL-6 in microglia medium protects retinal ganglion cells (RGC) from pressure-induced death (Sappington *et al.*, 2006) and intravitreal injection of IL-6 protects RGC layer neurons from IR injury (Sanchez *et al.*, 2003). Thus, besides having anti-inflammatory effects, Ex-4 may also play an important role in neuroprotection, a question that was not addressed in the present study.

In response to neural tissue injury, such as in IR, microglial cells become activated, altering their morphology and gaining increased phagocytic ability. They migrate to the site of injury, proliferate and release a variety of factors, such as cytokines, NO, reactive oxygen species and MMPs, which can contribute to BRB dysfunction and breakdown (Madeira *et al.*, 2015). Weakening of the barrier and increased expression of adhesion molecules by endothelial cells promotes the infiltration of circulating leukocytes that may further exacerbate inflammation and retinal damage (Sprague and Khalil, 2009; Madeira *et al.*, 2015).

In the present study, Ex-4 was neither able to prevent the increase in the number of CD45-positive cells nor inhibit the induction of expression of ICAM-1 in IR retinas, suggesting that the anti-inflammatory effects of Ex-4 are not due to inhibition of the accumulation of inflammatory cells, nor was it sufficient to block the accumulation of leukocytes in the injured retina. Further studies are warranted to discern between the populations of CD45-positive cells and their activation state present in the ischemic retinas. Our analysis of CD45-positive cells in retinal whole-mounts cannot definitively differentiate activated resident microglia from invading leukocytes. Nevertheless, we observed a decrease in pro-inflammatory factors induced by Ex-4 in IR retinas and we clearly demonstrated that Ex-4 can modulate a microglia cell line's response to activation by inhibiting nuclear accumulation of NF-κB and decreasing the expression of classical inflammatory markers (IL-1β, IL-6, CCL2 and NOS2). Furthermore, Ex-4 is also known to suppress macrophage activation. Treatment of isolated mouse macrophages with Ex-4 suppresses LPS-induced gene expression of TNF and CCL2 by a mechanism dependent on adenylate cyclase and PKA activation, and prevents nuclear translocation of p65 (Arakawa *et al.*, 2010). Additionally, Ex-4 also attenuates high glucose-induced TNF and IL-1β expression and secretion by macrophages derived from the THP-1 human monocytic cell line in a GLP-1R dependent-mannert (Kodera *et al.*, 2011). Thus, it is probable that both microglia and circulating monocytes were affected by systemic treatment with Ex-4. In addition, two studies identified microglia as a mediator of GLP-1R-induced anti-inflammatory effects *in vivo*. Ex-4 attenuated microglia activation in an experimental model of transient cerebral ischemia and in diabetic rats subjected to experimental stroke by reducing the number of Iba-1 positive microglia (Lee *et al.*, 2011; Darsalia *et al.*, 2012). It was also suggested that the neuroprotective effects of Ex-4 in transient focal cerebral ischemia were due to enhanced polarization of microglia towards a reparative M2-like phenotype (Darsalia *et al.*, 2014). Furthermore, in a model of Parkinson's disease, peripheral administration of Ex-4 inhibits microglial activation and the release of microglia-derived pro-inflammatory mediators (Kim *et al.*, 2009).

It is well known that GLP-1R activates the cAMP/PKA signaling pathway and a previous report (Ollivier *et al.*, 1996) has revealed that an increase in activity of this pathway suppresses NF-κB activity in monocytic THP-1 cells and in HUVEC. These findings support our observation that Ex-4 inhibited LPS-induced NF-κB activation, decreasing p65

nuclear accumulation, possibly via enhancement of cAMP following GLP-1R activation. Therefore, Ex-4 anti-inflammatory effects in microglia cells may be due to NF-κB inhibition and consequent decrease in pro-inflammatory cytokine gene transcription. Additionally, cAMP stimulates the expression of numerous genes via the protein kinase A-mediated phosphorylation of CREB (Gonzalez *et al.*, 1989). The present results are consistent with the previous reports demonstrating that CREB was significantly activated by Ex-4 (Pugazhenthi *et al.*, 2010; Teramoto *et al.*, 2011). CREB is a transcription factor that regulates a variety of genes involved in proliferation, survival, differentiation (Wen *et al.*, 2010). Activation of CREB can also contribute to a decrease in inflammation in microglia as it promotes the expression of IL-10 (Koscso *et al.*, 2012), an anti-inflammatory cytokine that is responsible for decreasing pro-inflammatory cytokine expression via activation of transcription factor STAT3 (Murray, 2005). Furthermore, p-CREB and NF-κB p65 subunit compete for the same co-activator CBP/p300 to optimize their transcriptional activity (Parry and Mackman, 1997). Activation of one pathway or the other differentially regulates the association with the co-activator and subsequent gene transcription, favoring an anti or pro-inflammatory response (Shenkar *et al.*, 2001; Martin *et al.*, 2005). In the present study, pre-treatment with Ex-4 stimulated the phosphorylation of CREB. Thus, the Ex-4 associated inhibition of NF-κB could result, in part, from the increased p-CREB competing with the LPS-induced p65 for the limiting amount of the co-activator and consequently limiting the pro-inflammatory response.
Conditioned medium experiments revealed that modulation of the inflammatory response of microglia cells with Ex-4 could prevent the increase in endothelial cell permeability induced by media conditioned by microglia exposed to LPS. These results suggest that microglia-derived soluble factors may, in fact, contribute to an increase in endothelial cell permeability, and that modulating microglia activation with Ex-4 may decrease the inflammatory response and protect barrier properties.

In conclusion, we report herein that Ex-4 confers significant protective effects in the ischemic retina, inhibiting inflammation and preventing BRB breakdown. Our *in vitro* studies suggest that the effects of Ex-4 on BRB are not mediated by direct effects on endothelial cells but by an inhibition of microglia activation, that leads to a decrease in the inflammatory response and protects against barrier breakdown. These data identify

Ex-4 as a potential therapeutic option for the treatment of retinal diseases characterized by increased vascular permeability and neuroinflammation, such as diabetic retinopathy.

3

Chapter 4

General discussion

Diabetic retinopathy is a leading cause of vision impairment and blindness in working-age adults worldwide (Fong *et al.*, 2004). In the last two decades, several important findings have improved our knowledge about the pathogenesis of diabetic retinopathy. Chronic hyperglycemia is considered to be a driving force in endothelial cell dysfunction, resulting in leukocyte adhesion to retinal vessels and increased BRB permeability, among other effects. Increased levels of growth factors and pro-inflammatory molecules have been associated with increased vascular permeability, implying a pro-inflammatory component in this pathology. However, the disease is still not curable, and there are limited therapeutic options to preserve vision in patients with diabetic retinopathy at risk of vision loss.

Two of the most recently approved classes of therapeutic agents for the treatment of type 2 diabetes, GLP-1 receptor agonists and DPP-IV inhibitors, act on the incretin system. Both exert their actions through the potentiation of incretin receptor signaling, mainly via GLP-1. GLP-1 has an important physiologic role in controlling glucose homeostasis by enhancing glucose-stimulated insulin secretion and inhibition of glucagon secretion (Baggio and Drucker, 2007). However, recent studies have also demonstrated extrapancreatic effects of incretin-based therapies in the vasculature (Shah *et al.*, 2011; Shiraki *et al.*, 2012), kidney (Mega *et al.*, 2011), heart (Bose *et al.*, 2005) and brain (Gaspari *et al.*, 2011).

We previously demonstrated that the DPP-IV inhibitor sitagliptin preserves BRB integrity, and prevents nitrosative stress, inflammation and apoptosis, in the retina of type 2 diabetic animals, induced by chronic hyperglycemia, while improving glycemic control. In fact, it is reasonable expecting that any glucose lowering drug may be, theoretically, beneficial in preventing or arresting diabetic microvascular complications, including diabetic retinopathy. However, information regarding beneficial direct effects of anti-diabetic agents on the diabetic retina, by an insulin-independent mechanism and glycemic control improvement, is lacking. Thus, a major goal was to evaluate the direct beneficial effects of sitagliptin in the diabetic retina (Chapter 2) in an animal model of type 1 diabetes (STZ-induced diabetes), taking into consideration that sitagliptin did not improve glucose, glycated hemoglobin or insulin levels in this animal model.

Retinal microvasculature dysfunction is one of the earliest detectable events occurring in the development of diabetic retinopathy. Indeed, the breakdown of BRB is the hallmark

of this disease (Cunha-Vaz *et al.*, 1975). The increase in BRB permeability may lead to retinal edema, which along with the formation of new blood vessels in the later stages of the disease, is associated with visual loss (Klein *et al.*, 1995). In the present study, sitagliptin effectively inhibited the increase in permeability of retinal vessels induced by diabetes. Previous studies have shown that BRB breakdown induced by diabetes is correlated with changes in the levels and distribution of TJ proteins within the retinal vascular endothelium (Antonetti *et al.*, 1999; Barber *et al.*, 2000; Leal *et al.*, 2007; Leal *et al.*, 2010). In our study we found that sitagliptin prevented the downregulation or subcellular redistribution of the TJ proteins claudin-5, occludin, and ZO-1, as well as the increase in BRB permeability induced by diabetes, thus protecting barrier function. It is well known that various factors, including inflammation, affect BRB permeability. In fact, diabetic retinopathy is considered a low-grade chronic inflammatory disease, characterized by the upregulation of pro-inflammatory mediators in the retina, that has been associated with the increase in leukocyte adhesion to retinal vessels, vascular permeability, and retinal cell death (Miyamoto *et al.*, 1999; Krady *et al.*, 2005). Our results are consistent with these findings, since diabetes upregulated IL-1β and ICAM-1 in the rat retinas, along with increased BRB permeability. Sitagliptin appears to have anti-inflammatory effect, as it prevented the increase of both inflammatory mediators in the retinas of diabetic animals, and this anti-inflammatory capacity might contribute for the prevention of the BRB breakdown, as both IL-1β and ICAM-1 have been correlated with increased retinal vascular permeability (Miyamoto *et al.*, 1999; Carmo *et al.*, 2000; Leal *et al.*, 2010).

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 (Barber *et al.*, 2011). 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. These effects may be mediated by increased GLP-1 levels in the retina, as intravitreal injection of Ex-4, in the same animal model, was shown to prevent the reduction in retinal thickness and cell loss (Zhang *et al.*, 2011). Nevertheless, as DPP-IV has other substrates, such as chemokines,

adipokines and neuropeptides, the inhibition of this enzyme could have multiple pleiotropic effects.

It remains to be established whether the effects of sitagliptin in the retina are dependent or independent of GLP-1. To address this question, the direct effects of GLP-1R activation on BRB breakdown were assessed (Chapter 3). We used an IR injury model, as this model recapitulates several features found in diabetic retinopathy, such as neurodegeneration, inflammation associated with microglia activation and BRB disruption associated with alterations in TJ. The GLP-1R agonist, Ex-4, was able to prevent the increase in retinal vascular permeability induced by IR injury at 48 h reperfusion. However, in primary cultures of retinal endothelial cells, Ex-4 was not capable of preventing the increase in endothelial cell monolayer permeability induced by permeabilizing agents, such as VEGF and TNF, which are known to be increased in IR and diabetic retinas. These results indicate that the protective effects of Ex-4 against BRB breakdown were not due to direct actions of Ex-4 on retinal endothelial cells, and are likely to be due to direct actions on other retinal cell types.

In addition, we demonstrated that Ex-4 exerts anti-inflammatory effects in the retina challenged with IR injury. Ex-4 inhibited the upregulation of classical pro-inflammatory genes (IL-1β, TNF and CCL2) that are responsive to IR injury. However, Ex-4 neither prevented the increase in the number of positive cells for the general leukocyte marker CD45 nor inhibited the upregulation of ICAM-1 in the retinas exposed to IR injury. These observations suggest that the anti-inflammatory effects of Ex-4 are not due to inhibition of accumulation of inflammatory cells in the retina, nor due to the inhibition of ICAM-1-mediated leukostasis in the injured retina.

In recent years, there has been increasing evidence implicating microglial cells in IR injury-induced inflammation. In fact, these cells migrate to the site of injury, proliferate and release a variety of factors, such as pro-inflammatory cytokines, NO, reactive oxygen species and MMP, which can contribute to BRB breakdown (Madeira *et al.*, 2015). In this study, we demonstrated that Ex-4 can modulate microglia activation by inhibiting the accumulation of NF-κB in the nucleus and decreasing the expression of classical inflammatory markers (IL-1β, IL-6, CCL2 and NOS2), suggesting that the anti-inflammatory effects detected in the animal model may be due to modulation of microglial cells. Since Ex-4 is also known to suppress macrophage activation (Arakawa *et*

al., 2010; Kodera *et al.*, 2011), it seems likely that both microglia and circulating monocytes are affected by systemic treatment with Ex-4. In accordance with our findings, other studies have pointed microglia as a key target of GLP-1R-induced anti-inflammatory effects in animal models of neurodegenerative diseases (Kim *et al.*, 2009; Lee *et al.*, 2011; Darsalia *et al.*, 2012; Darsalia *et al.*, 2014).

The anti-inflammatory effects of Ex-4 in microglia are likely due to NF-κB inhibition and consequently to the decrease in pro-inflammatory cytokine gene transcription. This decrease could be explained, at least in part, by the activation of CREB triggered by Ex-4, as p-CREB and p65 subunit of NF-κB compete for the same co-activator for optimal transcription, thus limiting the pro-inflammatory response (Parry and Mackman, 1997). Moreover, CREB activation can also contribute to a decrease in inflammation, as it promotes the expression of the anti-inflammatory cytokine IL-10 (Koscso *et al.*, 2012).

As previously suggested, inflammatory mediators such as TNF and IL-1β contribute to the increase in retinal endothelial permeability (Carmo *et al.*, 2000; Aveleira *et al.*, 2010). We here demonstrated that the modulation of the inflammatory response of microglial cells by Ex-4 was able to prevent the increase in endothelial cell permeability triggered by exposure of endothelial cells to conditioned media of microglial cell cultures previously exposed to LPS. These results suggest that microglia-derived soluble factors may, in fact, contribute to an increase in endothelial cell permeability and that modulating microglia activation with Ex-4 decreases the inflammatory response and protects barrier properties.

Hereby, by validating the effects of GLP-1R activation in a model that recapitulates a number of features that are common to several ocular diseases, such as diabetic retinopathy, we could envisage an even broader usage of incretin-based therapies in other retinopathies, characterized by neuroinflammation and vascular hyperpermeability.

Early effective treatments of diabetic retinopathy will be a key step for reducing the disease progression to more advanced stages. Given that there are no specific treatments for background retinopathy or non-proliferative diabetic retinopathy, any new therapeutic strategies that efficiently target BRB dysfunction will be of great importance.

In summary, the results presented in this dissertation provide a better understanding of the effects of incretin-based therapies on BRB breakdown. The underlying basis for the protective effects is believed to derive from the anti-inflammatory properties of these drugs. Clarifying the mechanisms of action of sitagliptin and Ex-4 might contribute to envisage new potential therapeutic options for the treatment of diabetic retinopathy, even for patients diagnosed with type 1 diabetes, which account for the highest risk of developing diabetic retinopathy.

Chapter 5

Main conclusions

The results presented in this dissertation allowed drawing the following main conclusions:

- Sitagliptin treatment prevents BRB breakdown and TJ disassembly induced by diabetes.

- Sitagliptin treatment decreases the levels of the pro-inflammatory cytokine IL-1β and the adhesion molecule ICAM-1 in the retina of diabetic animals.

- Sitagliptin prevents retinal neuronal cell death induced by diabetes.

- The protective effects of sitagliptin in the diabetic retina were not due to its insulinotropic actions, as treated diabetic animals remained hyperglycemic and hypoinsulinemic.

- Ex-4 prevents the increase in BRB permeability triggered by IR injury; however it does not inhibit the increase in cultured retinal endothelial cells monolayer permeability triggered by permeabilizing agents.

- Ex-4 inhibits the expression of IR-responsive genes associated with inflammation without affecting the number of CD45-positive cells.

- *In vitro,* Ex-4 reduces the reactivity of microglial cells triggered by LPS, decreasing the expression of inflammatory markers, possibly due to NF-κB inhibition and CREB activation.

In summary, these results suggest that sitagliptin and Ex-4 have protective effects against BRB breakdown and that their anti-inflammatory properties seem to greatly contribute to this protection. These mechanistic insights may contribute to envisage incretin-based therapies as a therapeutic option for the treatment of diabetic retinopathy.

Chapter 6

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