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Cover:

Image of the *zonula occludens* (ZO)-1 immunostaining at the cell border of bovine retinal endothelial cells, obtained by confocal microscopy.

Célia Alexandra Ferreira de Oliveira Aveleira

O envolvimento de mediadores inflamatórios na disfunção microvascular da retina: implicações para a ruptura da barreira-hemato retiniana na retinopatia diabética

The role of inflammatory mediators in retinal microvascular dysfunction: implications for the blood-retinal barrier breakdown in diabetic retinopathy

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Dissertação apresentada à Faculdade de Medicina da Universidade de Coimbra para prestação de provas de Doutoramento em Ciências Biomédicas.

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Note: The results presented in this dissertation, included in Chapters 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

ADA	American Diabetes Association
AGEs	Advanced glycation end products
AIF	Apoptosis inducing factor
ANOVA	Analysis of variance
AP-1	Activator protein-1
aPKC	Atypical protein kinase C
AR	Aldose reductase
ARVO	Association for Research in Vision and Ophthalmology
BCA	Bicinchoninic acid
BIM I	Bisindolylmaleimide I
BBB	Blood-brain barrier
BRB	Blood-retinal barrier
BREC	Bovine retinal endothelial cell
BSA	Bovine serum albumin
Calcein AM	Calcein acetoxymethyl ester
cAMP	Cyclic adenosine monophosphate
CBP	cAMP response element binding protein
cDNA	Complementary deoxyribonucleic acid
CHO	Chinese hamster ovary cell line
COX-2	Cyclooxygenase 2
CREB	cAMP response element binding
DAG	Diacylglycerol
DD	Death domain
DCCT	Diabetes Control and Complications Trial
DM	Diabetes mellitus
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
ECF	Enhanced chemifluorescence
ECL	Enhanced chemiluminescence
ETDRS	Early Treatment Diabetic Retinopathy Study
EGF	Epidermal growth factor
ERK1/2	Extracellular signal-regulated kinase 1/2
Etk	Endothelial/epithelial tyrosine kinase
Fab	Fragment antigen binding
FADD	Fas-associated death domain protein
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
Flk-1	Fms-related tyrosine kinase receptor-1
Flt-1	Fetal liver tyrosine kinase receptor-1
GABA	γ-aminobutyric acid
GCL	Ganglion cell layer
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFAP	Glial fibrillary acidic protein
GFAT	Glutamine:fructose-6 phosphate amidotransferase
GFP	Green fluorescent protein

GlcNac	N-acetylglucosamine
GLUT1	Glucose transporter type 1
GR	Glucocorticoid receptor
GREs	Glucocorticoid response elements
GSK-3	Glycogen synthase kinase-3
GuK	Guanylate kinase
HA1c	Hemoglobin A1c
HRP	Horseradish peroxidade
IACUC	Institutional Animal Care and Use Committee
ICAM-1	Intercellular adhesion molecule-1
ICE	Interleukin-1 converting enzyme
IGF	Insulin-like growth factor
IGF-I	Insulin-like growth factor-I
IGF-RI	Insulin-like growth factor receptor I
IκBα	Inhibitor nuclear factor-κB protein α
IKK	Inhibitor nuclear factor-κB protein kinase
ILM	Inner limiting membrane
IL-1	Interleukin-1
IL-1β	Interleukin-1β
IL-1RAcP	Interleukin-1 receptor accessory protein
IL-1RI	Interleukin-1 type I receptor
IL-1RII	Interleukin-1 type II receptor
INL	Inner nuclear layer
iNOS	Inducible nitric oxide synthase isoform
IPL	Inner plexiform layer
IRAK	Interleukin-1 receptor associated kinase
IRMAs	Intraretinal microvascular abnormalities
JAM	Junctional adhesion molecule
JNK	c-Jun N-terminal kinase
KDR	Kinase insert domain-containing receptor
LFA-1	beta 2 integrin lymphocyte function-associated antigen-1
MAGuK	Membrane-associated guanylate kinase
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MDCK	Madin-Darby canine kidney cells
MKP-1	MAPK phosphatase-1
MMPs	Metalloproteinases
mRNA	Messenger ribonucleic acid
NAD^+	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide, reduced form
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
NEMO	Nuclear factor-κB essential modulator
NF-κB	Nuclear factor-κB
NFL	Nerve fiber layer
NGF	Nerve growth factor
nGREs	Negative glucocorticoid response elements
NLS	Nuclear localization sequence

NO	Nitric oxide
NP	Neuropilin
O-GlcNAcase	*O*-GlcNAc β-*N*-acetylglucosaminidase
OLM	Outer limiting membrane
ONL	Outer nuclear layer
OPL	Outer plexiform layer
PAI-1	Plasminogen activator inhibitor-1
PBS	Phosphate buffer saline
PDZ	Postsynaptic protein-95/Drosophila disk large tumor suppressor DlgA/*Zonula occludens*
PEDF	Pigment epithelium-derived factor
PDGF	Platelet-derived growth factor
PI_3K	Phosphoinositide-3 kinase
PIP_3	Phosphoinositide 3,4,5 trisphosphate
PKC	Protein kinase C
PKCζ	Protein kinase C ζ
PKCζI-1	Protein kinase C ζ inhibitor-1
PL	Photoreceptor layer
PLC	Phospholipase C
PMSF	Phenylmethylsulfonyl fluoride
PVDF	Polyvinyllidene difluoride
qPCR	Real-time quantitative polymerase chain reaction
RAGEs	Receptors for advanced glycation end products
RHD	Rel homology domain
RIP	Receptor interacting protein
RIPA	Radioimmunoprecipitation assay
RITC	Rhodamine-isothiocyanate
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPE	Retinal pigment epithelium
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
siRNA	Small interfering ribonucleic acid
SH3	Src homology domain
SOD	Superoxide dismutase
SODD	Silencer of death domain
SRC-1	Steroid receptor coactivator-1
SV-40	Simian vacuolating virus 40
TACE	Tumor necrosis factor converting enzyme
TBARS	Thiobarbituric acid-reacting substances
TBS-T	Tris-buffered saline-Tween 20
TGF	Transforming growth factor
TIR	Toll/Interleukin-1 receptor domain
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-α
TNFR1	Tumor necrosis factor receptor type 1
TNFR2	Tumor necrosis factor receptor type 2

TRADD	TNF receptor associated death domain
TRAF	Tumor necrosis receptor associated factor
TR-iBRB2	Transgenic retinal inner blood-retinal barrier 2 endothelial cell line
UDP	Uridine diphosphate
UDP-GlcNAc	Uridine diphosphate-N-acetylglucosamine
UKPDS	United Kingdom Prospective Diabetes Study
VAMP	Vesicle-associated membrane protein
VAP	VAMP-associated protein
VCAM-1	Vascular cellular adhesion molecule-1
VEGF	Vascular endothelial growth factor
VEGF-R	Vascular endothelial growth factor receptor
WESDR	Wisconsin Epidemiologic Study of Diabetic Retinopathy
ZO	*Zonula occludens*
ZO-1	*Zonula occludens*-1
ZONAB	ZO-1-associated nucleic acid binding protein

Resumo

A retinopatia diabética é uma das complicações mais comuns da diabetes *mellitus*, e uma das principais causas de perda de visão e cegueira em adultos em idade activa nos países desenvolvidos. A ruptura da barreira hemato-retiniana (BHR) é a principal característica desta doença, sendo uma das alterações mais precoces que ocorrem na retina de doentes diabéticos. Evidências recentes sugerem que processos inflamatórios crónicos desempenham um papel importante na patogénese da retinopatia diabética. Os níveis de citocinas como a interleucina-1β (IL-1β) e o factor de necrose tumoral-α (TNF-α) estão aumentados no vítreo de doentes diabéticos e nas retinas de animais diabéticos, e estes aumentos correlacionam-se com um aumento da permeabilidade da BHR. No entanto, os mecanismos moleculares envolvidos na disfunção endotelial e alteração da permeabilidade da vasculatura da retina induzidas por estas citocinas não estão esclarecidos. Assim, este estudo teve como principal objectivo avaliar o efeito da IL-1β e do TNF-α na permeabilidade de células endoteliais dos vasos da retina, prestando particular atenção aos mecanismos moleculares subjacentes ao aumento da permeabilidade celular induzida pelo TNF-α. Além disso, avaliou-se também o efeito da glucose elevada e da IL-1β na regulação do receptor da IL-1 tipo I (IL-1RI).

As citocinas IL-1β e TNF-α induziram um aumento da permeabilidade das células endoteliais da retina de bovino (BREC), dependente da concentração e do tempo de exposição, sendo o TNF-α mais eficaz. O TNF-α induziu uma diminuição, a nível proteico e de RNA mensageiro, da *zonula occludens*-1 (ZO-1) e claudina-5. Contrariamente, a expressão de ocludina aumentou significativamente. Além disso, a localização celular destas proteínas alterou-se após exposição a TNF-α.

Devido à natureza inflamatória da retinopatia diabética, avaliou-se o possível efeito protector do glucocorticóide dexametasona na permeabilidade celular induzida por TNF-α. A dexametasona preveniu completamente as alterações nas junções oclusivas e o aumento da permeabilidade celular induzido por TNF-α. O efeito protector da dexametasona foi dependente da transactivação do receptor dos glucocorticóides, uma vez que o antagonista deste receptor, RU486, reduziu o seu efeito protector. Porém, o efeito do RU486 foi apenas parcial, sugerindo que outros mecanismos estão envolvidos.

O factor de transcrição nuclear κB (NF-κB) constitui um alvo central na via de sinalização do TNF-α, e foi demonstrado que a sua activação pode ser inibida pelo tratamento com

glucocorticóides. A inibição da activação do NF-κB, através da inibição da cinase da proteína inibitória do NF-κB (IKK) e da sobre-expressão da proteína inibitória do NF-κB α (IκBα) mediada por adenovírus, preveniu, parcialmente, o aumento da permeabilidade induzida por TNF-α, sugerindo um novo papel do NF-κB na regulação da permeabilidade vascular. Mais ainda, a inibição da proteína cinase C ζ (PKCζ) reduziu a activação do NF-κB e preveniu completamente o efeito do TNF-α nas proteínas das junções oclusivas, bem como o aumento da permeabilidade em células endoteliais da retina e também dos vasos da retina de rato induzido por TNF-α.

Este conjunto de observações mostra que o TNF-α altera a estrutura das junções oclusivas e aumenta a permeabilidade das células endoteliais de retina. Mais ainda, a inibição da PKCζ previne completamente o aumento de permeabilidade induzida por TNF-α, em parte, pela redução da activação do NF-κB. Estes resultados sugerem que a PKCζ pode ser considerada como um novo alvo terapêutico para a prevenção da permeabilidade vascular induzida por citocinas.

Apesar das células endoteliais da retina constituírem um alvo preferencial da IL-1β e TNF-α, pouco se sabe sobre a regulação dos seus respectivos receptores, como por exemplo o IL-1RI, neste tipo de células. Como a actividade biológica da IL-1β é mediada principalmente pelo IL-1RI, avaliou-se o efeito da glucose elevada e da IL-1β na regulação da expressão do IL-1RI numa linha celular de células endoteliais da retina de rato (TR-iBRB2).

A exposição das células endoteliais a glicose elevada, manitol (controlo osmótico) ou IL-1β, durante períodos relativamente curtos (1-24 h), causou uma diminuição significativa, dependente do tempo de exposição, do receptor IL-1RI. A exposição longa (7 dias) a glicose elevada ou manitol causou também uma diminuição significativa do receptor nas células endoteliais da retina. A diminuição dos níveis proteicos de IL-1RI foi inibida pela presença de anticorpos contra IL-1β e IL-1RI, sugerindo que a activação do IL-1RI pela IL-1β é um evento necessário neste processo. A diminuição do IL-1RI induzida pela glucose elevada ou IL-1β foi prevenida por inibidores do lisossoma, mas não por inibidores do proteossoma. Além disso, observou-se também que o receptor da IL-1β transloca-se para o núcleo, onde se acumula, após a exposição a glucose elevada ou IL-1β. Os resultados obtidos mostram que a glicose elevada, provavelmente devido ao stress osmótico, e a IL-1β regulam o conteúdo do IL-1RI nas células endoteliais da retina. A diminuição de IL-1RI é induzida pela sua activação e depende, em parte, da sua degradação através do lisossoma e da sua translocação e acumulação no

núcleo. Uma vez que a regulação do IL-1RI tem consequências importantes na via de sinalização da IL-1β, o IL-1RI pode ser considerado um possível alvo terapêutico para a tratamento da retinopatia diabética.

Em conclusão, os resultados apresentados neste estudo oferecem uma nova perspectiva sobre o efeito de citocinas na regulação da permeabilidade celular das células endoteliais de retina. Além disso, os receptores de citocinas ou alvos específicos a jusante, tais como a PKCζ, podem ser considerados como potenciais novos alvos terapêuticos para o tratamento da permeabilidade vascular em doenças oculares caracterizadas por níveis elevados de citocinas, tais como a retinopatia diabética.

Abstract

Diabetic retinopathy is one of the most common complications of diabetes *mellitus* and remains a leading cause of vision loss and blindness in working-age adults in developed countries. Retinal microvascular dysfunction is an early feature of diabetic retinopathy, and the blood-retinal barrier (BRB) breakdown is the hallmark of the disease. Growing evidence indicates that low-grade and chronic inflammatory processes play an important role in the pathogenesis of diabetic retinopathy. Elevated levels of the cytokines interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) in the vitreous of diabetic patients and in diabetic rat retinas correlate with increased retinal vascular permeability. However, the mechanisms by which cytokines induce endothelial cell dysfunction and alter retinal vascular permeability remain unclear. Therefore, the main goal of the present study was to investigate the effect of IL-1β and TNF-α on retinal endothelial cell permeability, giving particular attention to the molecular mechanisms underlying the increase in cell permeability induced by the more potent permeabilizing factor, TNF-α. Additionally, the regulation of the interleukin-1 type I receptor (IL-1RI) content in retinal endothelial cells exposed to elevated glucose or IL-1β was also investigated.

Both IL-1β and TNF-α increased bovine retinal endothelial cell (BREC) permeability in a concentration- and time-dependent manner, being TNF-α more effective. TNF-α induced a marked downregulation of *zonula occludens*-1 (ZO-1) and claudin-5 protein content, concomitant with a decrease in the respective transcripts expression. Conversely, TNF-α increased occludin expression. In addition, the junctional localization of these tight junction proteins was altered by TNF-α.

Given the inflammatory nature of diabetic retinopathy, the ability of the glucocorticoid dexamethasone to prevent TNF-α-induced cell permeability was evaluated. Dexamethasone completely prevented the disruption of the tight junction complex and the increase in cell permeability induced by TNF-α. Moreover, the protective effect of dexamethasone was dependent on transcriptional activation of the glucocorticoid receptor, since RU486, a glucocorticoid receptor antagonist, reduced the protective effect of dexamethasone on TNF-α-induced cell permeability. However, RU486 effect was only partial, suggesting that additional mechanisms are involved.

The transcription factor nuclear factor-κB (NF-κB) is a critical target of the TNF-α signaling pathway, and its activation can be inhibited by glucocorticoid treatment. Preventing NF-κB

activation with a chemical inhibitor of the inhibitor NF-κB protein kinase (IKK) or with adenovirus-mediated overexpression of the inhibitor NF-κB protein α (IκBα) partially prevented the increase in cell permeability induced by TNF-α, implying a new role for NF-κB in cell permeability regulation. Furthermore, protein kinase C ζ (PKCζ) inhibition reduced NF-κB activation and completely prevented the alterations in the tight junction complex and cell permeability induced by TNF-α, both in cell cultures and in the retinal vasculature of rats.

Taken together, these results demonstrate that TNF-α alters the tight junction complex and increases retinal endothelial cell permeability. Further, inhibition of PKCζ completely prevents TNF-α-induced cell permeability, in part, by reducing NF-κB activation. These results suggest that PKCζ may be considered as a specific therapeutic target for the prevention of cytokine-induced vascular permeability.

Although retinal endothelial cells are primarily affected by IL-1β and TNF-α, little attention has been given to the regulation of their cognate receptors, such as IL-1RI, in these cells. As IL-1β activity is regulated primarily by IL-1RI, the effect of high glucose and IL-1β on IL-1RI regulation was also investigated in a rat retinal capillary endothelial cell line (TR-iBRB2).

A time-dependent downregulation of IL-1RI occurred in cells exposed to high glucose, mannitol (osmotic control) or IL-1β (1-24 h). Long-term exposure (7 days) of retinal endothelial cells to high glucose or mannitol also decreased IL-1RI protein content. The downregulation of IL-1RI was completely prevented by anti-IL-1RI or anti-IL-1β antibodies, indicating that IL-1RI activation by IL-1β is a necessary event in this process. IL-1RI downregulation was prevented by lysosome inhibitors but not by proteasome inhibitors. It was also found that IL-1RI translocates to the nucleus after high glucose, mannitol or IL-1β treatment. These results indicate that high glucose, probably due to osmotic stress, and IL-1β downregulate IL-1RI in retinal endothelial cells. The downregulation of IL-1RI is triggered by its activation and is due, at least partially, to lysosomal degradation and translocation and accumulation in the nucleus. Given the potential important role of IL-1β on the pathogenesis of diabetic retinopathy, the inhibition of IL-1β action by targeting IL-1RI may be considered another potential therapeutic strategy for the treatment of diabetic retinopathy.

In summary, the results presented herein provide new insight into the role of pro-inflammatory cytokines on retinal endothelial cell barrier function. Moreover, cytokine receptors or specific downstream targets, such as PKCζ, may be considered potential novel therapeutic targets for

the treatment of vascular permeability in ocular diseases characterized by elevated levels of cytokines, such as diabetic retinopathy.

CHAPTER 1

Introduction

CHAPTER 1

Introduction

1.1 Anatomy of the eye

The eye is a highly specialized organ which gives us the sense of sight. The outermost layer of the eye is a tough, white outer layer of connective tissue called the sclera (Figure 1.1). A delicate layer of epithelial cells forms a mucous membrane, the conjunctiva that covers the outer surface of the sclera and helps to keep the eye moist. At the front of the eye, the sclera becomes the transparent cornea, which lets light pass into the eye and acts as a fixed lens. The iris, a colored circular muscle, controls the amount of light that enters the eye owing to its ability to change the diameter of the pupil, the iris aperture.

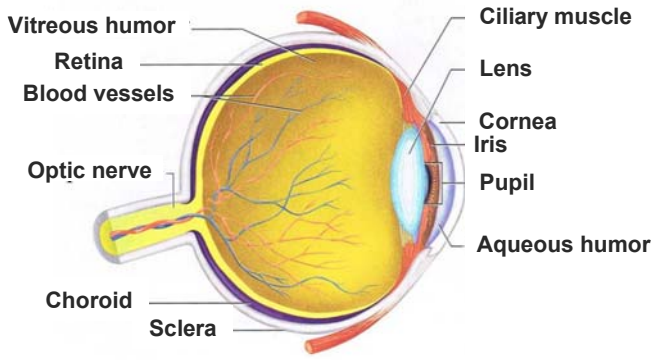

Figure 1.1 Schematic diagram of the human eye anatomy (adapted from Widmaier *et al.*, 2004).

Underneath the iris is the crystalline lens, a transparent biconvex structure that, together with the cornea, regulates the refraction of the light entering the eye to be focused on the retina. The lens can change its shape to focus on objects at various distances. The lens divides the eye into two cavities: the anterior cavity, between the lens and the cornea, and the posterior cavity, behind the lens within the eyeball itself. The ciliary body produces the clear, watery aqueous humor that fills the anterior cavity of the eye and provides nourishment to the avascular lens and cornea. The posterior cavity is filled with a thick, jellylike fluid known as the vitreous humor that constitutes most of the volume of the eye and helps to maintain the shape of the eyeball.

Light from the lens passes through the vitreous body and enters the retina, the innermost layer of the eye. At the posterior part of the eye, inner to the sclera, is the choroid that supports the retina. The retina is a thin transparent layer of neural tissue that is responsible for converting the light stimuli from the environment into neuronal impulses that are relayed to the optic nerve and then to the brain, where the information is integrated and processed as a visual image (Figure 1.1).

1.2 The retina

1.2.1 Anatomy of the retina

The retina is constituted by several cell types of which neurons predominate; other cell types include glial cells (Müller cells, astrocytes and microglial cells), endothelial cells and pericytes, and epithelial cells. Retinal cells are arranged in a highly organized manner, consisting of alternate layers of neuron cell bodies and synaptic processes (Figure 1.2).

The outermost layer of the retina is the retinal pigment epithelium (RPE), a continuous monolayer of cuboidal epithelial cells enriched in melanin pigment that lies between the photoreceptors and the Bruch's membrane, adjacent to choroid. Photoreceptors extend through the next four layers: their outer and inner segments lie in the photoreceptor layer (PL); the outer nuclear layer (ONL), constituted by the nuclei of the photoreceptors; the outer limiting membrane (OLM), along the border between the inner segments and the outer nuclear layer, consisting of a series of adherent junctions between photoreceptors and Müller cells, which are the main type of glial cells in the retina; and the outer plexiform layer (OPL), constituted by the photoreceptors axons and terminal endings that synapse with the dendrites of bipolar and horizontal cells. The inner nuclear layer (INL) contains the nuclei of bipolar, horizontal, amacrine and Müller cells. The bipolar cells have their axon terminals in the inner plexiform layer (IPL), where they synapse with the ganglion cell dendrites and the processes of amacrine cells. The ganglion cell layer (GCL) contains the nuclei of ganglion cells and some displaced amacrine cells. The axons of ganglion cells converge to form the nerve fiber layer (NFL) that leaves the eye as the optic nerve. The innermost retinal layer, the inner limiting membrane (ILM), separates the retina from the vitreous humor and is formed by the end-feet processes of Müller cells, which extend from the inner surface of the retina out to the photoreceptor layer (Figure 1.2). The area of the retina is approximately 1250 mm^2 and varies in thickness from 100 μm in

the periphery to 230 µm near the optic nerve (Forrester *et al.*, 2002). The neural layers of the retina are actually a forward extension of the central nervous system. In the retina, the light must pass through several neural layers before striking the photoreceptors. The photoreceptors then synapse with bipolar cells, which in turn synapse with ganglion cells, so that the nerve impulses are conducted outward the retina.

1.2.2 Retinal cell types

The unique anatomic and physiologic specialization required for vision demands an intact cell-cell communication between the several cell types within the retina. The retina includes several types of cells that perform sensory, regulatory, nutritional and immunomodulatory functions: neurons (photoreceptors, bipolar cells, horizontal cells, amacrine cells and ganglion cells), glial cells (Müller cells, astrocytes and microglial cells), retinal pigment epithelial cells, and the cells that constitute the retina blood vessels (endothelial cells and pericytes).

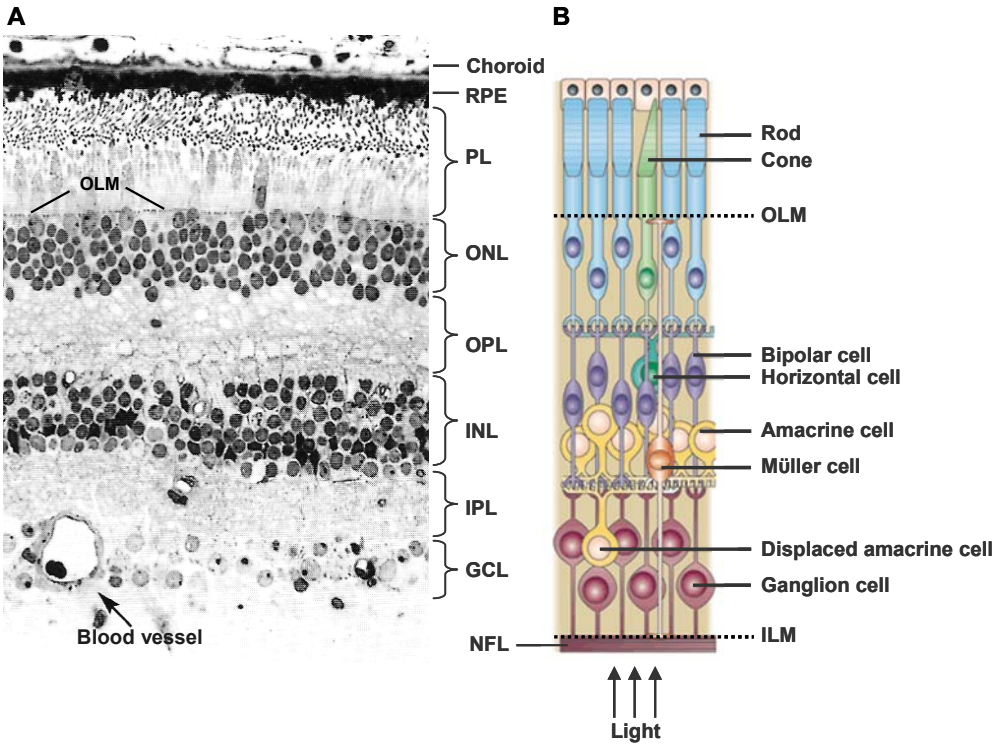

Figure 1.2. The anatomy of the retina. A. Histological cross section of a human retina showing the retinal layers. Original magnification of 150x (adapted from Forrester *et al.*, 2002). **B.** Schematic representation of a retinal cross section showing the different cell types of the retina (adapted from Dyer and Cepko, 2001).

Legend: RPE, retinal pigment epithelium; PL, photoreceptors layer; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer, NFL, nerve fiber layer; OLM, external limiting membrane; ILM, inner limiting membrane.

1.2.2.1 The neuronal retina

The neurons in the retina are divided into five major types: photoreceptors, bipolar cells, horizontal cells, amacrine cells and ganglion cells. The neurons perform sensory functions (phototransduction) and define color perception, spatial resolution and contrast discrimination (Masland, 2001). Phototransduction takes place in photoreceptors, cells that are sensitive to light, and is the process through which light photons are converted into nerve impulses. Signals form the photoreceptors may follow either vertical or lateral pathways (Figure 1.3). In the vertical or direct pathway, nerve impulses from the photoreceptors are transmitted to bipolar cells and then to ganglion cells that convey the information along the optic nerve to the brain. The horizontal and amacrine cells provide the lateral integration of visual signals.

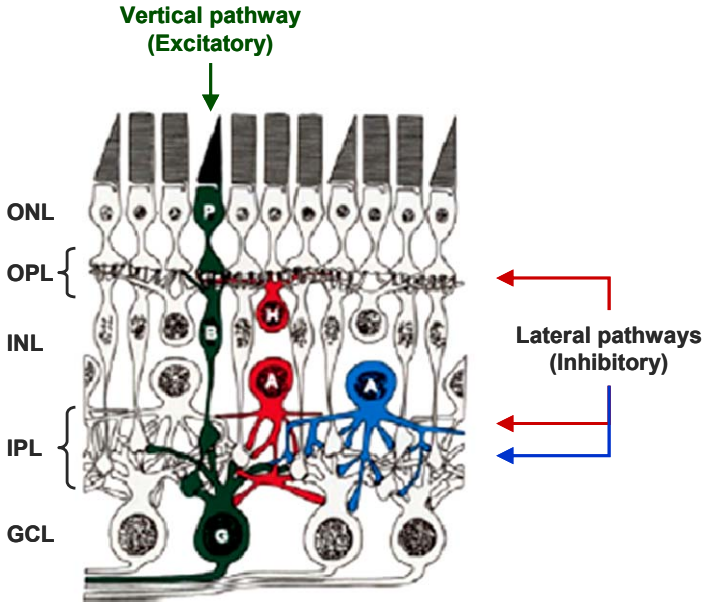

Figure 1.3. Schematic representation of the retina showing the five classes of retinal neurons and the main neurotransmitters released by neurons. Photoreceptors (P), bipolar cells (B) and ganglion cells (G) constitute the vertical pathway, and release the neurotransmitter glutamate (green); the horizontal cells (H) and amacrine cells (A) release the neurotransmitter γ-aminobutyric acid (GABA; red) or glycine (blue) and mediate the lateral integration of the visual signals (adapted from Brandstatter and Hack, 2001).

The horizontal cells receive inputs from photoreceptors and make connections between neighboring photoreceptors in the OPL. The signals going to the bipolar cells in the vertical pathway are a result of a direct photoreceptor input, as well as of an input from surrounding photoreceptors, being mediated by the horizontal cells. Amacrine cells contribute to the modulation of the vertical pathways by mediating lateral interactions between bipolar cell terminals and dendrites of ganglion cells. Amacrine cells may receive input from bipolar cells and other amacrine cells; their output may be bipolar cells, other amacrine cells, and ganglion cells. A simplified schematic diagram of the retina circuitry is shown in Figure 1.3.

Photoreceptors

In the vertebrate eye there are two main types of photoreceptors: rods and cones, localized in the outer part of the retina. The photoreceptor outer and inner segments are localized at the PL (Figure 1.2). The outer segments consist of stacked membrane disks that contain high concentrations of photopigments responsible for light absorption and initiation of the phototransduction cascade. The rod outer segments have a cylindrical shape while cone outer segments are generally conical. The inner segments are packed with mitochondria and contain several organelles involved in protein synthesis, essential for the regeneration of the membrane discs. The outer and inner segments are joined by a connecting cilium. The nucleus is localized at the ONL and the synaptic terminals are localized at the OPL where they communicate with bipolar cells and horizontal cells. Rod and cone terminals are enriched in neurotransmitter-filled vesicles and differ in structure. Rod terminals are relatively small and roughly spherical and are called spherules. Cone terminals are called pedicles because their footlike appearance (Figure 1.4). Besides the structural differences, rods and cones also contain different photopigments. In rods there is only one type of photopigment, the rhodopsin, which is most sensitive to wavelengths around 505 nm, while in cones it is possible to distinguish three types of pigments, one for each type of cone photoreceptor, based on wavelength sensitivity. As shown in figure 1.4B, long wavelength (red) sensitive cones, middle wavelength (green) sensitive cones and short wavelenght (blue) sensitive cones contain opsins more sensitive to wavelengths around 559 nm, 531 nm and 419 nm, respectively (Widmaier *et al.*, 2004). Rods are very sensitive to light so they are used essentially under dim light and are responsible for sensing contrast, brightness, motion and dark vision. Cones are less sensitive to light than rods being used under daylight, and provide color vision, fine detail perception and spatial resolution (Kolb *et al.*, 2001).

There are approximately 115 million rods and 6.5 million cones in the human retina. The density of rods and cones varies within different regions of the retina. The periphery of the retina is rod dominated while the cone density increases near the macula. The fovea, the central region of the macula, responsible for the highest resolution and color vision, is absent of rods. In the optic disc of the retina, where the optic nerve leaves towards the brains, there are no photoreceptors and, therefore there is no visual perception is this area. As a result, it is known as the "blind spot" (Forrester *et al.*, 2002).

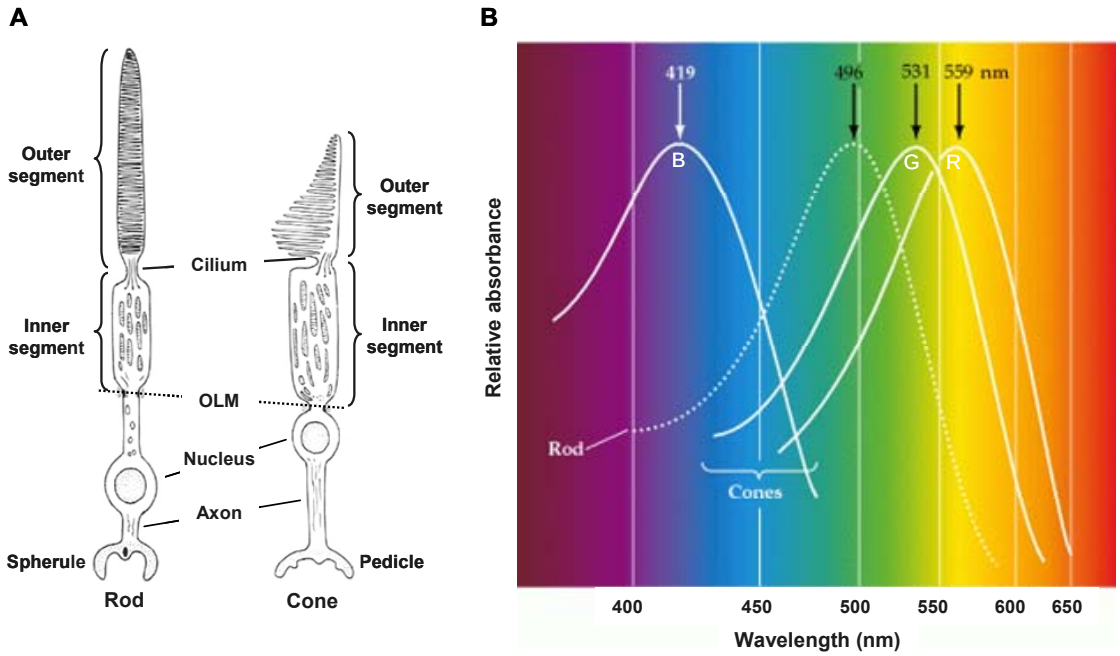

Figure 1.4. Photoreceptors physiology. A. Structural differences between rods and cones. Although generally similar in structure, rods and cones differ in their size and shape, as well as in the arrangement of the membranous disks in their outer segments (adapted from Forrester *et al.*, 2002). **B.** The light absorption spectra of rod and cone photoreceptors in the human retina. Relative light absorption is plotted against wavelength, in nanometers (nm); the wavelength of maximal absorption of rods and blue (B), green (G) and red (R) cones are indicated above the curves (adapted from Purves *et al.*, 2004). Legend: OLM, external limiting membrane.

Bipolar cells

The retina contains approximately 35 million bipolar cells, which are responsible for transmitting the signals from the photoreceptors and horizontal cells to the ganglion cells (Kolb *et al.*, 2001). The bipolar cell nuclei lie in the INL and are parallel-oriented to the photoreceptors. Bipolar cells

accept synapses from either rods or cones, but not both, and they are designated rod bipolar or cone bipolar cells, respectively. In the fovea, the central region of the retina, a bipolar cell may receive input from only one cone while in the periphery, a single bipolar cell can receive input from up to-50-100 rods (Forrester *et al.*, 2002).

Ganglion cells

The retinal ganglion cells are the final output neurons of the vertebrate retina. They receive input from photoreceptors via bipolar cells and amacrine cells. The cell bodies of the ganglion cells are located in the innermost nucleated layer of the retina, the GCL. Retinal ganglion cells vary significantly in terms of their size, dendritic tree spread, connections, and responses to light stimulation. The ganglion cell axons form the optic nerve and establish synapses in brain visual centers, principally the lateral geniculate nucleus and the superior colliculus.

There are about 1.2 to 1.5 million retinal ganglion cells in the human retina. On average each retinal ganglion cell receives inputs from about 100 rods and four to six cones. However, these numbers vary greatly among individuals and as a function of retinal location. In the fovea, the ratio of ganglion cells to photoreceptors is close to 1, where almost every photoreceptor has one ganglion cell receiving input from it. In the peripheral retina, the number of photoreceptors connected to a single ganglion cell increases markedly (Forrester *et al.*, 2002).

Horizontal cells

Horizontal cells are characterized by the extensive horizontal extensions of their processes and their cell bodies are located in the outer part of the INL. Horizontal cells help to regulate and integrate the input from multiple photoreceptor cells into bipolar cells at the OPL. The selective inhibition of signals from neighboring photoreceptors improves the sensitivity to contrast and differences in light intensity (Forrester *et al.*, 2002).

Amacrine cells

Amacrine cells are interneurons that synapse with bipolar cells and ganglion cells in the IPL of the retina. They are characterized by their large size and oval shape and their cell bodies locate in the inner part of the INL. Amacrine cells display a wide morphological diversity. In the human retina, around 30 types of amacrine cells can be distinguished, depending on their dendritic tree size, stratification of their dendrites in the IPL and the neurotransmitter type they release. Most

of the amacrine cells are inhibitory neurons containing the inhibitory neurotransmitter γ-aminobutyric acid (GABA) or glycine. Like horizontal cells, amacrine cells modulate the output of bipolar cells, enhancing motion perception and sensitivity to differences in light intensity (Forrester *et al.*, 2002).

1.2.2.2 Retinal neuroglia

The glial cells of the retina constitute the interface between neurons and the retinal vasculature and provide nutritional and regulatory support for neurons. There are two main types of glial cells in the retina: macroglia (Müller cells and astrocytes) and microglia.

Müller cells

Müller cells are the principal supporting glial cell of the retina. They have a radial orientation and span the entire thickness of the retina, from the ELM, where they have adherens junctions with photoreceptor inner segments and other Müller cells, to the ILM, a basement membrane formed by the Müller cell end-feet that interfaces with the vitreous humor (Figure 1.2). Müller cell bodies are located in the INL and these cells project processes, irregularly, either in direction to the OLM or ILM. Müller cells contact and ensheath neurons cell bodies and processes. Also, Müller cells end-feet envelop retinal blood vessels at the inner surface of the retina, regulating blood flow and permeability of retinal endothelial cells (Hollander *et al.*, 1991).

Müller cells have several functions essential to the survival of retinal neurons and maintenance of the retinal homeostasis. They regulate the extracellular ionic and osmotic environment, remove neural waste products and protect neurons from exposure to high concentrations of neurotransmitters, such as glutamate, using well developed uptake mechanisms to recycle this transmitter (Kolb *et al.*, 2001; Reichenbach *et al.*, 2007). In fact, being the only cells of the retina expressing the enzyme glutamine synthetase (Newman and Reichenbach, 1996), Müller cells transform glutamate taken up via high-affinity carriers into glutamine, which is then returned to the neural cells for glutamate resynthesis (Poitry-Yamate *et al.*, 1995). Müller cells are the primary site of glucose uptake and phosphorylation in the retina (Poitry-Yamate *et al.*, 1995). They are endowed with glucose transporter 1 (GLUT1) (Kumagai *et al.*, 1994) and metabolize glucose intensely through glycolysis to produce lactate that fuels neuronal metabolism. In addition, Müller cells store glycogen for conversion to lactate, synthesize retinoic acid from

retinol and phagocyte neuronal debris. Müller cells can also secrete growth factors, cytokines and neurotransmitters (Bringmann and Reichenbach, 2001; Walker and Steinle, 2007).

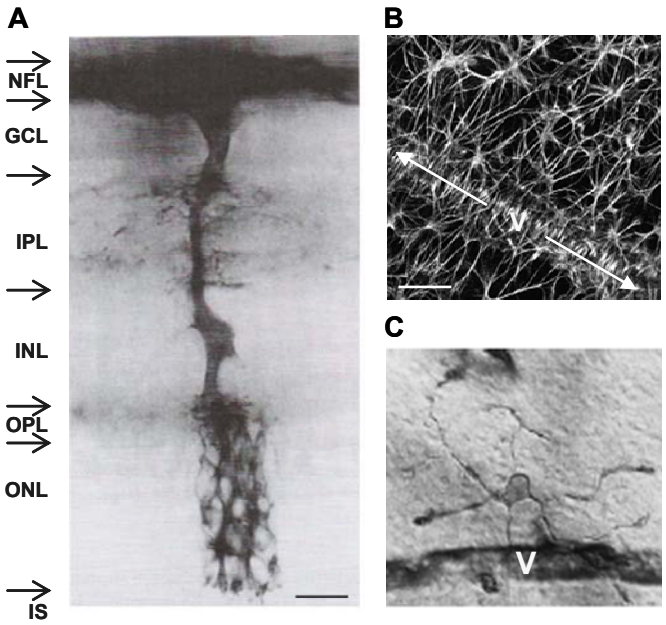

Figure 1.5. Retinal glial cells. A. Micrograph of a horseradish peroxidase (HRP) filled Müller cell of a rabbit retina. The dark band at the top is composed of Müller cell end-feet and the labeled axons of ganglion cells in the nerve fiber layer. Scale bar: 10 µm (adapted from Forrester *et al.*, 2002). **B.** Astrocytes immunoreactivity to glial fibrillary acid protein (GFAP) was detected by immunofluorescence histochemistry in flat-mounted rat retinas. V, astrocytic ensheatment of a blood vessel. Scale bar: 50 µm (adapted from Barber *et al.*, 2000). **C.** A ramified retinal microglia in close contact with a blood vessel (V). Interference contrast microscopy image of a rat retina flat mount preparation stained with isolectin B4-peroxidase (adapted from Rungger-Brandle *et al.*, 2000). Legend: NFL, nerve fiber layer; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; IS, inner segments.

Astrocytes

Astrocytes are predominantly located in the GCL and NFL but they can also be found in the inner plexiform and nuclear layers. They have a characteristic morphology of a flattened cell body and a fibrous series of radiating processes. Their processes are filled with intermediate filaments consisting of glial fibrillary acid protein (GFAP). Their morphology changes from the optic nerve head to the periphery: from extremely elongated near the optic nerve to a symmetrical stellate form in the far peripheral retina (Schnitzer, 1988).

Astrocytes migrate into the retina along the optic nerve during development (Watanabe and Raff, 1988; Ling *et al.*, 1989). Although astrocytes have a close relationship with retinal neurons, they are mainly associated with retinal blood vessels. Indeed, the presence and distribution of astrocytes correlates with the presence and distribution of retinal blood vessels: avascular retinas contain no astrocytes whereas retinas that are vascularized contain astrocytes only in the vascularized region (Watanabe and Raff, 1988; Abbott *et al.*, 1992; Newman and Reichenbach, 1996). Astrocytes, like Müller cells, envelop retinal blood vessels (Figure 1.5B), regulating the blood-retinal barrier (BRB) properties (Tout *et al.*, 1993). Also, astrocytes contain glycogen, which they degrade to provide glucose to neurons, remove neural waste products and regulate ionic homeostasis by maintaining the extracellular levels of potassium.

Microglial cells

Microglial cells are widely regarded as the resident mononuclear phagocyte of the central nervous system and they are responsible for the immunomodulatory functions in the retina. Microglial cells are ubiquitously distributed through the retina and they serve as sensors of the local environment by interacting with neurons, glia and endothelium. Microglial cells enter the retina coincident with the mesenchymal precursors of retinal blood vessels during development (Chan-Ling, 1994). Under normal conditions, microglial cells are characterized by a highly ramified morphology and are termed the resting microglia (Figure 1.5C). Upon any disturbance of the environment, these cells rapidly transform into an activated state characterized by an ameboid morphology. Activated microglial cells can migrate to the site of injury, proliferate, and release a variety of factors such as cytokines, nitric oxide and reactive oxygen species (ROS). Also, these cells transform themselves into phagocytic cells capable of presenting antigen to circulating immune cells, participating in the immune responses. Activated microglia participates in the resolution of local injury (Fetler and Amigorena, 2005; Nimmerjahn *et al.*, 2005), but chronic activation may lead to exaggerated microglia responses, leading to retinal damage and neuronal apoptosis.

1.2.2.3 Retinal pigmented epithelium

The RPE is the outermost layer of the retina which is composed by a monolayer of hexagonal polarized epithelial cells. These cells are characterized by the presence of apical microvilli and basal membrane infoldings, being also densely packed with pigment granules, especially

melanin. The apical membrane of RPE is intimately associated with the light-sensitive outer segments of photoreceptors while the basolateral membrane faces the Bruch's membrane, separating the neurosensorial retina from the choroid (Figure 1.6).

Although RPE is not directly involved in the vision process, it is critical for the normal function and support of the outer retina, specially the photoreceptors (Young, 1967). As a layer of pigmented cells, RPE absorbs the scattered light focused by the lens on the retina. To maintain photoreceptors excitability, RPE stabilizes the ion composition of the sub-retinal space, phagocytes the shed outer segments of photoreceptors and recycles the light-sensitive pigments, essential for the visual cycle.

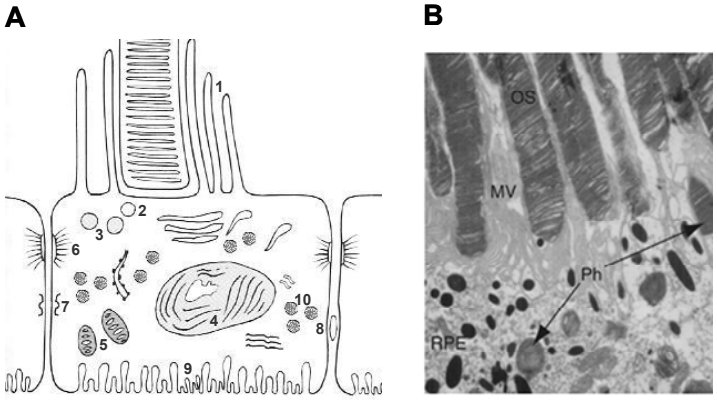

Figure 1.6. Retinal pigment epithelium. A. Diagram summarizing the main ultrastructural features of retinal pigment epithelium (RPE). Legend: 1, apical microvilli; 2, endosomes; 3, lysosomes; 4, nucleus; 5, mitochondria; 6, tight junction; 7, adherens junctions; 8, gap junctions; 9, basal infoldings; 10, melanin granules (adapted from Forrester *et al.*, 2002). **B**. Transmission electron micrograph of rat retina RPE (3000x magnification). Legend: OS, outer segment; MV, microvilli; Ph, phagosome (adapted from Tschernutter *et al.*, 2005).

In addition, the RPE serves as a selective permeable barrier between the retina and the choroid blood vessels due to the existence of tight junctions between epithelial cells, know as the outer BRB. The RPE is also involved in the transport of nutrients, such as glucose, amino acids, ascorbic acid and fatty acids from the blood to nourish photoreceptors, as well as in the transport of ions, water and metabolic end products from the sub-retinal space to the blood. Further, RPE secretes growth and trophic factors, such as pigment epithelium-derived factor (PEDF), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), insulin-like growth factor-I (IGF-I), among others, which are essential to maintain the survival and structural

integrity of photoreceptors and choroid blood vessels endothelium (Thumann and Hinton, 2000; Strauss, 2005).

1.2.2.4 Endothelial cells and pericytes

Endothelial cells and pericytes of the retinal vasculature form the primary barrier between the blood and retinal parenchyma. They provide nutritional support and waste product removal for the inner retina. The retina blood vessels constitute less than 5% of the retinal mass and are the only structures visible by clinical examination.

1.2.3 Retinal blood-supply

The retina is a highly metabolic tissue with the highest oxygen consumption per weight of any human tissue. The dominant oxygen-consuming layers in the adult rat retina are the inner segments of the photoreceptors, the OPL, and the deeper region of the IPL (Yu and Cringle, 2001). The retina requires a highly specialized circulation to meet its demanding metabolic requirements without compromising the extracellular environment. The retina has a dual blood-supply: 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 1.7). While the retinal circulation has a low flow rate, high oxygen exchange and non-fenestrated capillaries, the choroidal circulation has a high flow rate, low oxygen exchange and a fenestrated capillary bed.

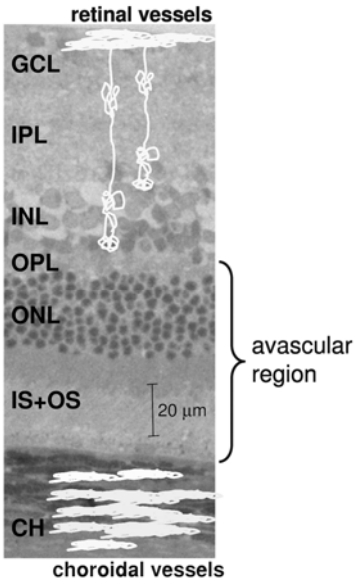

Figure 1.7. Retinal blood supply. Histological section of a normal adult rat retina stained with toluidine blue, depicting the embedded retinal vascular layer and a distinct choroidal vascular layer (white drawings). GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; IS+OS, inner and outer photoreceptor segment; CH, choroidal vascular layer (adapted from Cheng *et al.*, 2006).

1.2.3.1 Retinal vasculature

The retinal circulation that supplies the inner retina, can be observed ophthalmoscopically as a geometric network of blood vessels, and their tree-dimensional complexity reflects the intricate cellular distribution of the neural retina. The retinal vascular endothelium constitutes the inner BRB, which serves primarily to regulate the optimal extracellular neural environment necessary to proper neuronal signaling and glial interaction. This inner BRB exists at the level of the vascular endothelium in the retina. Retinal capillaries are characterized by a continuous endothelium monolayer surrounded by pericytes embedded and supported by the basement membrane (Figure 1.8). Both endothelial cells and pericytes directly communicate via gap junctions and exchange paracrine signals through their shared basement membrane (Hirschi and D'Amore, 1996). Retinal pericytes are modified smooth muscle cells that envelop endothelial cells and occur in a 1:1 ratio with endothelial cells, in the human retina, which is a unique feature of this microvasculature and translates to an even greater pericyte coverage than in brain capillaries (Frank *et al.*, 1990). These cells have been shown to possess

contractile properties (Chakravarthy and Gardiner, 1999) and are involved in the control of blood flow through the capillaries (Rucker *et al.*, 2000; Bandopadhyay *et al.*, 2001).

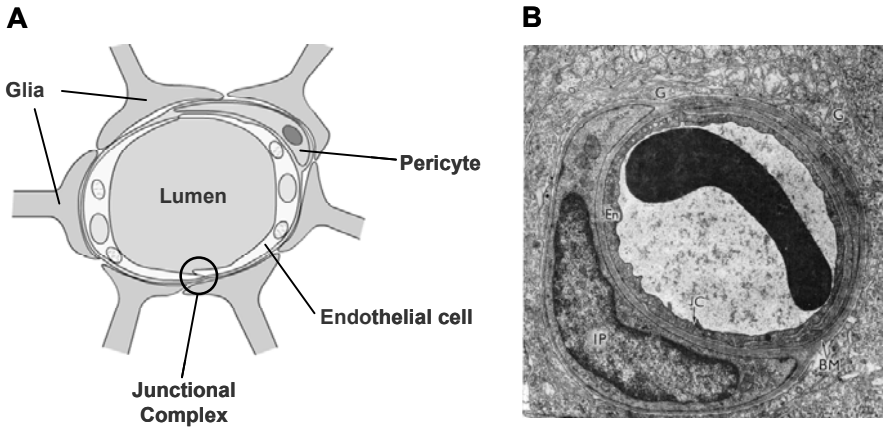

Figure 1.8. Retinal capillary. A. Illustration of a retinal capillary structure. **B.** Transmission electron micrograph of a typical retinal capillary (Magnification 20,000x). Legend: En, endothelial cells; BM, endothelium basement membrane; P, pericyte; IP, intramural pericyte; G, glial cell; JC, junctional complexes (adapted from Cunha-Vaz *et al.*, 1966).

1.2.3.2 Choroidal circulation

The choroid lies between the sclera and the RPE. The choroid is a thin, pigmented, highly vascularized loose connective tissue, whose principal function is to nourish the outer part of the retina. Also, choroidal pigments absorb light to prevent its reflection back to the retina. The choriocapillaris consists of a lobular network of capillaries that form the inner vascular layer of the choroid, responsible for the nutritional support of the outer retina, especially photoreceptors and the RPE (Forrester *et al.*, 2002). Unlike the retinal capillaries, choroid capillaries are highly fenestrated and leaky (Figure 1.9).

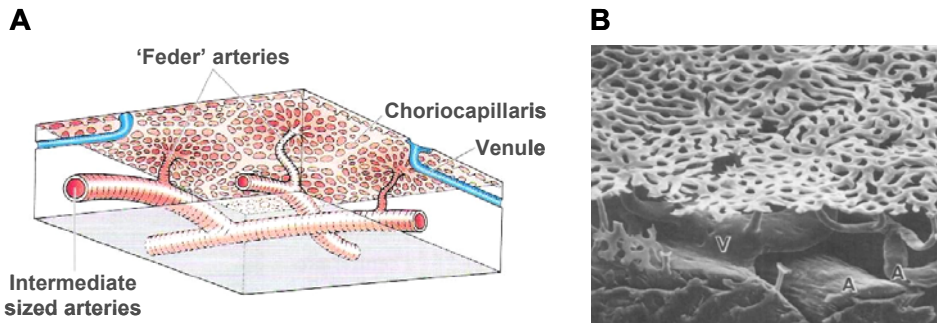

Figure 1.9. Retinal choriocapillaris. A. Schematic representation of the hexagonal units in the choriocapillaris fed by small arteries (adapted from Forrester *et al.*, 2002). **B.** Scanning electron micrograph of the choriocapillaris network. Choroidal arteries (A) and veins (V) can be seen beneath the choriocapillaris (adapted from Risco and Nopanitaya, 1980).

1.3 Blood-retinal barrier

Homeostasis is essential for the normal function of the retina and it depends on the existence of the BRB. The concept of BRB was developed four decades ago based on the similarities with the blood-brain barrier (Ashton and Cunha-Vaz, 1965; Cunha-Vaz *et al.*, 1966). The BRB consists of an inner and an outer component. The inner BRB is formed by tight junctions between juxtaposed endothelial cells of the retinal vasculature in the inner retina. Although the vascular endothelium in the retina and brain are similar, the paucity of endocytic vesicles suggest that retinal vessels are more permeable than brain capillaries (Vinores, 1995).

Specifically, the inner BRB controls the flow of ions, water, nutrients, and potential toxins into and out of the retinal parenchyma in order to maintain the specialized environment proper to neuronal function. It also restricts the access of immunoglobulins and intravascular immune cells to the retina (Gardner *et al.*, 1999). Furthermore, retinal capillaries possess an array of abluminal anionic pumps contained within plasmalemmal caveolae that assist the removal of excess fluid and waste products from the extracellular space into the retinal circulation (Minshall *et al.*, 2003). The inner BRB development and maintenance is regulated in part by the surrounding neuroglia (Müller cells and astrocytes) (Janzer and Raff, 1987; Tout *et al.*, 1993) and by pericytes, that together promote tight junction integrity and the non-fenestrated phenotype of the retinal vascular endothelium (Antonetti *et al.*, 1999b). When these cell relationships are disrupted by a pathological condition, as in diabetic retinopathy, the barrier properties of the retinal vessels may be compromised or lost. In such situations the endothelial

cells can lose tight-junction integrity and may even assume a fenestrated phenotype. The outer BRB is established by the tight junctions between RPE cells in the outer retina and regulates the selective transport of ions and nutrients from the fenestrated choriocapillaris to the outer retina. The ability of RPE to regulate transepithelial transport depends on apical tight junctions that regulate diffusion through the paracellular spaces of the monolayer, and an asymmetric distribution of proteins to regulate vectorial transport across the monolayer.

During development, these properties form gradually. Initially, the tight junctions are leaky, and the RPE exhibits only partial polarity. As the neural retina and choriocapillaris develop, there are progressive changes in the composition of the apical junctional complexes, the expression of cell adhesion proteins, and the distribution of membrane and cytoskeletal proteins. These mechanisms are regulated by the interactions that are established between the RPE and its neighboring tissues. Unlike the inner BRB, the RPE barrier properties do not depend on the presence of other cell types (Ban *et al.*, 2000; Steuer *et al.*, 2005).

1.3.1 Tight junctions

The movement of ions, water and solutes across endothelial and epithelial barriers occurs both through cells (transcellular flux) and between cells (paracellular flux). Transcellular transport is membrane-associated, and is mediated through membrane receptors, transporters, pumps or channels. Paracellular flux is mostly regulated by the tight junctions, the most apical component of the intercellular junctional complex, which also includes adherens junctions, desmosomes and gap junctions (Figure 1.10). Tight junctions provide the paracellular diffusion barrier that is critical for the normal functioning of the retina. Nevertheless, this barrier is not absolute but rather semi-permeable, since it allows the selective passage of certain solutes but not others (Anderson *et al.*, 2004). Tight junctions also maintain cell polarity by preventing lipids and proteins from diffusing between the apical and basolateral plasma membranes (fence function) (Farquhar and Palade, 1963; van Meer and Simons, 1986). More recently, another role for tight junctions has begun to be unraveled. It involves the control of cell proliferation and gene expression and requires the shuttling of tight junction associated components to the nucleus (Gonzalez-Mariscal *et al.*, 2007).

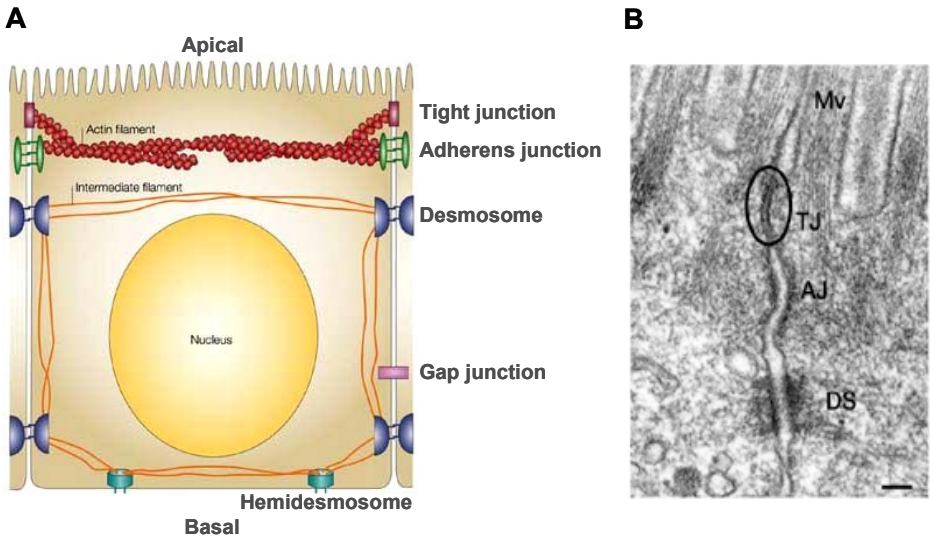

Figure 1.10. Intercellular junctions. A. Schematic representation of the several intercellular junctional structures (adapted from Matter and Balda, 2003). **B.** Scanning electron micrograph showing the main types of intercellular junctions in epithelial and endothelial cells (adapted from Kobielak and Fuchs, 2004). Legend: Mv, microvilli, TJ, tight junction, AJ, adherens junction, DS, desmosome.

Tight junctions fibrils are visualized by electron microscopy and appear as sites where the intercellular space between adjacent cells is obliterated and the adjoining membranes appear to fuse (Farquhar and Palade, 1963). These fibrils are composed of transmembrane proteins that interact with proteins on adjacent cells (Figure 1.11). Tight junctions are composed of an intricate combination of transmembrane proteins including occludin, claudins and junctional adhesion molecule (JAM), and several cytosolic accessory proteins such as *zonula occludens* (ZO)-1, ZO-2, ZO-3, 7H6 antigen, cingulin and symplekin (Figure 1.12). The transmembrane proteins, claudins and occludin, form the seal between adjacent cells and the paracellular diffusion barrier. The accessory proteins are multidomain proteins necessary for the structural support of tight junctions, and are also involved in signal transduction (Fanning *et al.*, 1999; Matter and Balda, 2003; Aijaz *et al.*, 2006).

Occludin

Occludin, a 65 kDa protein, was the first tight junction integral membrane to be identified (Furuse *et al.*, 1993) and was originally though to be the main sealing protein. Occludin is a

tetraspan transmembrane protein with two extracellular loops and intracellular amino- and carboxy-termini.

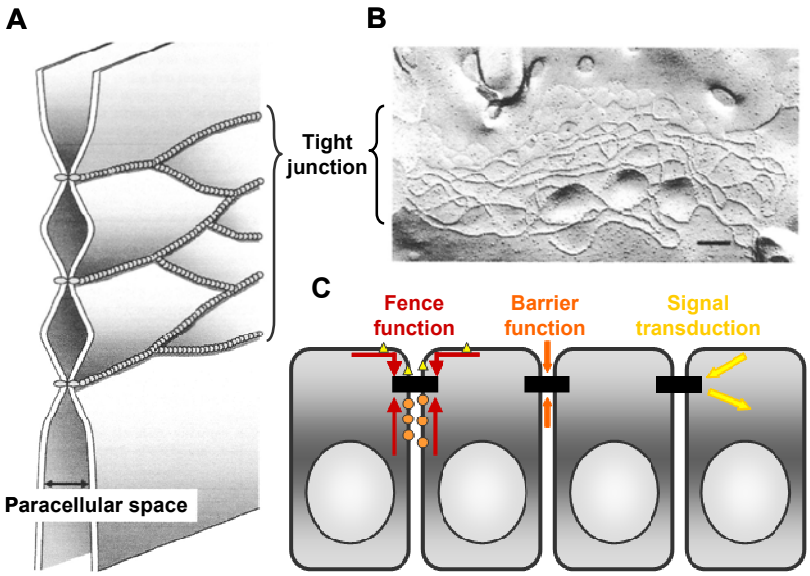

Figure 1.11. Morphology and functions of tight junctions. A. Schematic diagram of tight junction strands. **B.** Freeze-fracture replica electron micrograph of tight junction fibrils. Tight junctions appear as a set of continuous, anastomosing intramembranous particle strands or fibrils. Bar: 50 nm. **C.** Functions of tight junctions. Tight junctions provide a selective permeable barrier to paracellular flux (barrier function), maintain cell polarity by preventing lipids and proteins from diffusing between the apical and basolateral plasma membranes (fence function), and coordinate a variety of signaling and trafficking molecules regulating cell differentiation, proliferation and polarity, thereby serving as a multifunctional complex (adapted from Sawada *et al.*, 2003).

Occludin is expressed predominantly in both endothelial and epithelial cells (Furuse *et al.*, 1993). Occludin, through its carboxy-terminal cytoplasmic domain interacts with several structural proteins such as occludin itself (Nusrat *et al.*, 2000), ZO-1 (Furuse *et al.*, 1994), ZO-2 (Jesaitis and Goodenough, 1994), ZO-3 (Haskins *et al.*, 1998), and connexin-32 (Nusrat *et al.*, 2000), a gap junction protein. The ZO-1 interaction with occludin establishes a link between occludin and the actin cytoskeleton (Fanning *et al.*, 1998). Occludin may also interact with Itch, an E3-ubiquitin-protein ligase, via its amino-terminal cytoplasmic domain, suggesting that occludin may be regulated by ubiquitination (Traweger *et al.*, 2002). The expression of occludin correlates with barrier properties. For example, occludin expression is higher in the endothelium of neuronal tissues in the brain and retina than in non-neuronal tissues that have lower barrier

properties (Hirase *et al.*, 1997; Kevil *et al.*, 1998; Mitic and Anderson, 1998). Further, overexpression of occludin increases the electrical resistance in Madin-Darby canine kidney (MDCK) cells (Balda *et al.*, 1996; McCarthy *et al.*, 1996), whereas suppression of occludin expression by occludin antisense oligonucleotides or small interfering RNA (siRNA) decreases occludin content and increase solute flux (Kevil *et al.*, 1998; Yu *et al.*, 2005; Phillips *et al.*, 2008). Occludin also confers calcium-independent adhesiveness when transfected into occludin-null fibroblasts suggesting that occludin play an important role in the formation of paracellular barriers. However, occludin is not required for the formation of intact tight junctions since structurally intact tight junctions with normal barrier properties were formed in the absence of occludin (Saitou *et al.*, 1998). Furthermore, occludin-deficient mice are viable, able to form intact tight junctions and maintain an intestinal epithelium barrier (Saitou *et al.*, 2000). However, a host of abnormalities are observed, including growth retardation, brain calcification, hyperplasia of the gastric epithelium and the males are unable to produce offspring. Together, these studies demonstrate that occludin is not required for the formation of intact tight junctions but regulates barrier properties.

Claudins

Claudins are the major structural constituents of tight junctions and are required to restrict the paracellular flux to ions and small molecules (Turksen and Troy, 2004). Claudins are small (20-27 kDa) tetraspan transmembrane proteins with two extracellular loops, and cytoplasmic amino- and carboxy-termini. Claudins interact with ZO proteins through their carboxy-terminus (Itoh *et al.*, 1999a). At least 24 isoforms have been identified and their extracellular loops display high variability in the distribution and number of charged residues, a critical feature that determines the paracellular ionic selectivity of the tight junction. In fact, several reports have shown that claudin family members form charge-selective paracellular channels (Amasheh *et al.*, 2002; Colegio *et al.*, 2002; Hou *et al.*, 2007). Unlike conventional ion channels, which mediate ion transport across lipid bilayers, the tight junction channels must orient parallel to the plane of the plasma membranes to support paracellular ion movements. This new class of paracellular-tight junction channels facilitates the transport of ions between separate extracellular compartments (Angelow *et al.*, 2008; Balkovetz, 2009).

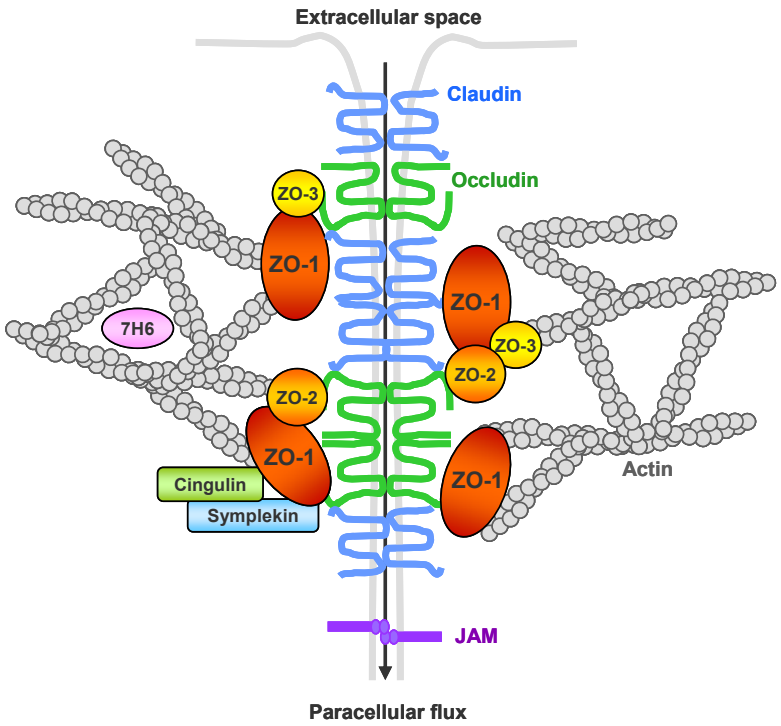

Figure 1.12. Schematic representation of the basic structural components of tight junctions. Tight junctions are composed of transmembrane proteins (occludin, claudins, and JAMs) and several cytosolic accessory proteins such as ZO proteins, cingulin, symplekin and 7H6. Transmembrane proteins are linked to the actin cytoskeleton *via* cytoplasmic ZO proteins. ZO proteins are important for clustering claudins and occludin, resulting in the formation of tight junctional strands.

Claudins show an intrinsic ability to polymerize into linear fibrils, as shown by the extensive networks resulting from the transfection of claudin-1 or claudin-2 into claudin-null fibroblasts (Furuse *et al.*, 1998). Similarly, expression of claudin-5 in fibroblasts reconstituted tight junction strands that resemble those of endothelial cells *in vivo* (Morita *et al.*, 1999). In contrast, expression of occludin in fibroblasts resulted in a punctuate pattern and the formation of short fragments of strands (Furuse *et al.*, 1998). When occludin is transfected into claudin-expressing fibroblasts, it is recruited into the claudin fibrils. These observations suggest that claudins are necessary and sufficient for the formation of tight junctions by their homophilic and heterophlic binding to adjacent cells (Furuse *et al.*, 1998; Furuse *et al.*, 1999). Also, expression of claudins in fibroblasts confers cell adhesion that is calcium independent (Kubota *et al.*, 1999).

Claudins display a varied tissue expression pattern and claudin composition determines the tissue barrier properties (Kollmar *et al.*, 2001; Rahner *et al.*, 2001; Kiuchi-Saishin *et al.*, 2002).

Claudin-5 is predominantly expressed in brain and retinal endothelial cells (Antonetti *et al.*, 1998; Morita *et al.*, 1999). Claudin-5 deficient mice studies demonstrated that claudin-5 has a critical role in the regulation of blood-brain barrier (BBB). These mice developed normally but died few hours after birth. Despite, in the absence of claudin-5, brain endothelial cells presented well-developed tight junctions. However, the BBB against small molecules (800 Da), but not larger molecules, was selectively affected (Nitta *et al.*, 2003).

Junctional adhesion molecule family

The JAM protein family consists of three members called JAM-A, JAM-B, and JAM-C, which belong to the immunoglobulin superfamily, characterized by two immunoglobulin domains, a single transmembrane domain and a short cytoplasmic tail (Martin-Padura *et al.*, 1998; Ebnet *et al.*, 2004). The prototypical member of the family, JAM-A, has been initially described as a tight junction molecule expressed by endothelial and epithelial cells and involved in monocyte migration *in vivo* (Martin-Padura *et al.*, 1998). JAM-A is also a β2-integrin lymphocyte function-associated antigen 1 (LFA-1) involved in transendothelial migration of leukocytes by regulating the integrity and permeability of cell junctions (Ostermann *et al.*, 2002). The two other members of the family, JAM-B and JAM-C, are expressed by endothelial and lymphatic cells (Palmeri *et al.*, 2000; Aurrand-Lions *et al.*, 2001), and have also been shown to be involved in leukocyte transendothelial migration (Johnson-Leger *et al.*, 2002; Zen *et al.*, 2004). JAM-A and JAM-B expression in chinese hamster ovary (CHO) cells reduced the paracellular permeability of the cell monolayer, suggesting that these molecules promotes cell–cell adhesion (Martin-Padura *et al.*, 1998; Aurrand-Lions *et al.*, 2001). Also, the increase in monocyte transmigration and BBB permeability in response to interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) is inhibited by an anti-JAM antibody (Del Maschio *et al.*, 1999). Furthermore, Liu *et al.* demonstrated that monoclonal antibodies generated to the extracellular domain of JAM inhibit the recovery of transepithelial resistance of epithelial monolayers after disruption of tight junctions (Liu *et al.*, 2000). The inability to recover barrier function correlated with the lack of redistribution of JAM and occludin, but not ZO-1, to the region of the tight junction. JAM interacts with several tight junction proteins such as ZO-1, occludin, cingulin and AF-6/afadin (Bazzoni *et al.*, 2000; Ebnet *et al.*, 2000). Although JAM lacks the ability to form tight junction strands (Tsukita and Furuse, 2000), it is involved in the regulation of tight junction assembly and cell permeability.

The *zonula occludens* family

ZO-1 was first identified as a ~220 kDa antigen for a monoclonal antibody raised against a junction-enriched fraction from the liver (Stevenson *et al.*, 1986). Subsequent studies identified ZO-2 (160 kDa) (Gumbiner *et al.*, 1991) and ZO-3 (130 kDa) (Balda and Anderson, 1993) as binding partners that co-immunoprecipitate with ZO-1 from cell lysates. ZO proteins are peripheral membrane proteins associated with the cytoplasmic surface of transmembrane tight junction proteins and are present ubiquitously within tight junctions of both epithelial and endothelial cells. In cells that do not form tight junctions, such as cardiac miocytes and fibroblasts, ZO-1 and ZO-2 are localized to adherens junctions (Itoh *et al.*, 1999b).

ZO proteins belong to the membrane-associated guanylate kinase (MAGuK) homologue family, containing three postsynaptic protein-95/Drosophila disc large tumor suppressor DlgA/zonula occludens-1 (PSD-95/DlgA/ZO-1; PDZ) domains, a src homology (SH3) domain, and a non-catalytic guanylate kinase (GuK) homology domain (Woods and Bryant, 1993; Jesaitis and Goodenough, 1994; Haskins *et al.*, 1998). ZO proteins interact with several proteins through multiple protein-binding domains. ZO-1 has been reported to interact with both ZO-2 and ZO-3 via PDZ domains (Fanning *et al.*, 1998; Haskins *et al.*, 1998; Wittchen *et al.*, 1999). In addition, ZO-1 binds claudins and occludin via its PDZ-1 and GuK domains, respectively (Furuse *et al.*, 1994; Itoh *et al.*, 1999a). The carboxy-terminus domain of ZO proteins interacts with perijunctional filamentous actin (Wittchen *et al.*, 1999; Fanning *et al.*, 2002), thereby anchoring the transmembrane proteins occludin and claudin to the cytoskeleton. Also, ZO-1 binds to adherens junctions-associated peripheral membrane proteins, such as α-catenin and AF-6/afadin, via its amino-terminus, being this way linked to adhesion molecules of adherens junctions like cadherin and nectin (Itoh *et al.*, 1993; Itoh *et al.*, 1997; Yamamoto *et al.*, 1997; Fanning *et al.*, 1998).

Recent studies demonstrated that the formation and organization of tight junction complex is dependent on ZO proteins. In ZO-1 knockout/ZO-2 knockdown Eph4 mouse mammary gland epithelial cells, the formation of tight junctions was completely disrupted and claudins failed to polymerize at tight junctions (Umeda *et al.*, 2006). Moreover, ZO-1-deficient mice died at the embryonic stage, with embryonic and extraembryonic defects, including massive apoptosis of the notochord and neural tube area and impaired vascular development in the yolk sac (Katsuno *et al.*, 2008).

ZO proteins are scaffolds that establish numerous protein-protein interactions that cluster at the tight junction diverse kinases, phosphatases, small G proteins and nuclear transcription factors (Lopez-Bayghen *et al.*, 2006). In addition, ZO-1 and ZO-2 have been reported to localize to the nucleus in proliferating epithelial cells (Gottardi *et al.*, 1996; Islas *et al.*, 2002) and during remodeling of cell-cell contacts, but not in differentiated cells. ZO-1 and ZO-2 associate with proteins involved in the regulation of gene transcription and cell proliferation such as the transcription factors ZO-1–associated nucleic acid binding protein (ZONAB) (Balda *et al.*, 2003), Jun/Fos (Betanzos *et al.*, 2004), the cell cycle regulator cyclin D1 (Huerta *et al.*, 2007) and the heat shock protein Apg-2 (Tsapara *et al.*, 2006). Therefore, ZO proteins may be involved in cell growth and proliferation, in addition to the central role in the organization and assembly of tight junction complexes.

Other tight junction associated proteins

Several other peripheral membrane associated proteins have been localized to tight junctions including cingulin (Cordenonsi *et al.*, 1999), 7H6 (Satoh *et al.*, 1996), symplekin (Keon *et al.*, 1996), protein kinase C (PKC) ζ isoform (Dodane and Kachar, 1996) and AF-6/afadin (Yamamoto *et al.*, 1997). The small G proteins, rab 3B (Weber *et al.*, 1994), rab 13 (Zahraoui *et al.*, 1994), the Gα family member $G\alpha_{12}$ (Dodane and Kachar, 1996) and vesicle-associated membrane protein (VAMP)-associated protein (VAP)-33, which binds occludin (Lapierre *et al.*, 1999), are involved in vesicle transport processes and have been also found to localize at the tight junction complex. Many of these proteins interact with each other and this complex network of adaptors, signaling and regulatory proteins appear to be involved in the maintenance and/or regulation of tight junction assembly. However, additional studies are needed to fully understand the role of these proteins.

1.4 Diabetes mellitus

Diabetes mellitus (DM) is a group of metabolic diseases characterized by chronic hyperglycemia caused by defects in either insulin secretion or insulin action, or both. The impaired insulin action leads to an impairment of carbohydrates, lipids and proteins metabolism. DM is the most common endocrine disease in all age groups worldwide. DM incidence is increasing dramatically, and the total number of people with diabetes is estimated to be 366 million in 2030 (Wild *et al.*, 2004).

The chronic elevation of glucose levels in diabetes is associated with long-term damage, dysfunction, and failure of various organs, especially the eye, kidney and heart, but the vasculature and nervous system might also be seriously affected.

The vast majority of cases of diabetes fall into two broad etiological categories. Type 1 DM, previously designated as insulin-dependent DM or juvenile-onset diabetes, is characterized by absolute lack of insulin due to autoimmune destruction of the pancreatic β cells, which are responsible for insulin production. This form of diabetes generally occurs in children and young people, usually under 30 years of age, and represents 10% of diabetic patients. Type 2 DM, previously known as non insulin-dependent DM, is characterized by a diminished insulin secretion from β cells and/or insulin resistance, which results from impaired ability of insulin to regulate the glucose levels (ADA, 2008). Type 2 DM accounts for 90% of all cases of diabetes and occurs mostly in adult individuals. Its incidence increases with age, especially after 40 years of age and is mostly associated with obesity and a sedentary lifestyle (Zimmet *et al.*, 2001).

1.4.1 Diabetic retinopathy

Diabetic retinopathy is one of the most common complications of diabetes and a leading cause of vision impairment and blindness worldwide (Aiello *et al.*, 1998; Cunha-Vaz, 2000). For both type 1 and type 2 DM, the prevalence of diabetic retinopathy is strongly related with the duration of diabetes. During the first two decades of the disease, nearly all patients with type 1 DM and more than 60% of patients with type 2 DM have some degree of retinopathy. In the Wisconsin Epidemiologic Study of Diabetic Retinopathy (WESDR), the prevalence of any retinopathy among patients with type 1 DM was 8% at 3 years, 25% at 5 years, 60% at 10 years, and 80% at 15 years. Further, 3.6% of patients with type 1 DM and 1.6% of patients with type 2 DM were legally blind. In the group of patients with type 1 DM, 86% of blindness was attributable to diabetic retinopathy. In the group of type 2 DM patients, in which other eye diseases were common, one-third of the cases of legal blindness were due to diabetic retinopathy (Klein *et al.*, 1984; Fong *et al.*, 2003). Overall, diabetic retinopathy is the most frequent cause of new cases of blindness among adults aged 20-74 years (Aiello *et al.*, 1998).

1.4.1.1 Classification of diabetic retinopathy

Diabetic retinopathy is a progressive disease and several intents were made to classify the retinal lesions observed during the course of the pathology (Cunha-Vaz and Bernardes, 2005). In the Early Treatment Diabetic Retinopathy Study (ETDRS) classification, considered as the reference classification, the development of diabetic retinopathy is graded into 13 stages, based on the presence of visible ophthalmologic alterations and retinal neovascularization (ETDRS Research Group, 1991a; ETDRS Research Group, 1991b). This classification was developed on the basis that diabetic retinopathy, given time, uniformly progresses to proliferative retinopathy. However, this is a matter of controversy since some evidence supports the notion that proliferative retinopathy occurs in diabetes as a result of the extensive capillary closure and ischemia and is, therefore, relatively independent of the diabetic general metabolic status (Cunha-Vaz and Bernardes, 2005). Since the use of this classification in everyday clinical practice has not proven to be practical, another classification has been proposed in an effort to simplify the ETDRS grading scale (Wilkinson *et al.*, 2003). The five-stage disease severity classification for diabetic retinopathy, with reference to the ETDRS grading system, includes: no apparent retinopathy, mild nonproliferative diabetic retinopathy, moderate nonproliferative retinopathy, severe nonproliferative diabetic retinopathy and proliferative diabetic retinopathy. Diabetic macular edema is classified as apparently present or apparently absent and it can be present at any levels of non-proliferative or proliferative diabetic retinopathy. More recently, Cunha-Vaz has proposed another classification, that will be described herein and includes the following stages: preclinical retinopathy, nonproliferative retinopathy, diabetic macular edema, preproliferative retinopathy, and proliferative retinopathy (Cunha-Vaz, 2006).

Pre-clinic diabetic retinopathy

Pre-clinic diabetic retinopathy is characterized by the absence of detectable lesions in the retina by the eye fundus observation (Figure 1.13A). However, more sensitive diagnostic techniques, such as fluorescein angiography, and histological studies have revealed that some alterations occur in this early stage. The initial histological alterations include microaneurysms, due to the endothelial cell proliferation in capillaries and venules, arterioles and capillaries occlusion and pericyte and endothelial cell degeneration and loss (Cunha-Vaz, 1972).

The fluorescein angiography evaluates the abnormal leak of fluorescein from retinal vessels, and therefore it can detect very early an increase in the permeability of capillary vessels in the

retina without clinical evident lesions. Indeed, this technique allowed, for the first time, the demonstration that alteration in the BRB permeability is one of the earliest changes in diabetic patients (Cunha-Vaz and Maurice, 1967). Also, the capillary occlusions can be early detectable by the fluorescein angiography. These capillary occlusions are important markers for the progression of ischemia in the retina.

In addition to the early retinal vasculature dysfunction, other functional alterations occur in the retina. Loss of color vision and contrast sensitivity (Sokol *et al.*, 1985; Roy *et al.*, 1986; Daley *et al.*, 1987) and changes in electroretinograms (Sakai *et al.*, 1995) are early signs of neural retinal dysfunction in diabetic patients. Also, a decline in night vision and in the visual sensitivity recovery after bright light exposure occurs in some diabetic patients.

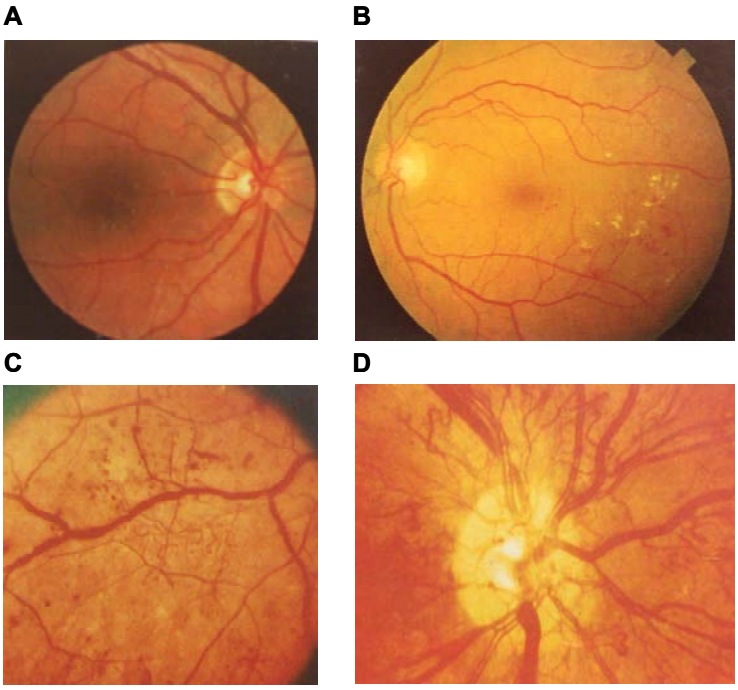

Figure 1.13. Eye fundus photographs of diabetic patients. A. Eye fundus of a diabetic patient without alterations. **B.** Non-proliferative diabetic retinopathy. Several red dots, hemorrhages and hard exudates are observed. **C.** Pre-proliferative diabetic retinopathy. **D.** Proliferative diabetic retinopathy: neovascularization with origin in the optic disk (adapted from Cunha-Vaz, 1972).

Nonproliferative diabetic retinopathy

Nonproliferative diabetic retinopathy occurs when lesions in the eye fundus can be detected by the ophthalmoscopic exam. This stage is characterized by the presence of microaneurysms,

intraretinal hemorrhages, lipid exudates, commonly known as hard exudates (Figure 1.13B), and a certain level of retinal edema (Cunha-Vaz and Bernardes, 2005).

Microaneurysms, which appear as small red spots, are the first visible alterations seen under ophthalmoscopic examination and fundus photography. Microaneurysm formation is associated with localized proliferation of endothelial cells, loss of pericytes and alterations of the capillary basement membrane (Ashton, 1963; Cunha-Vaz, 1978). The counting of red dots has been suggested as an appropriate marker of retinopathy progression (Klein *et al.*, 1995a; Klein *et al.*, 1995b). The intraretinal hemorrhages are the consequence of the breakdown of microaneurysms, capillaries and venules and are mainly localized in the IPL and in the INL. The abnormal leakage of retinal vessels leads to retinal edema and the formation of hard yellow exudates (precipitates of lipids and lipoproteins), often localized around the macula. Retinal edema results from the increase in the fluid content in the retinal tissue, with the consequent increase in the thickness of the retina.

Diabetic macular edema

Diabetic macular edema is the main cause of vision loss in diabetic patients (Aiello *et al.*, 1998). Macular edema is characterized by an increase in the macula thickness as a result of an abnormal fluid accumulation in the central area of the retina. Either extracellular or intracellular fluid accumulation may result in edema, and in fact both are likely to occur in many retinal disorders. The intracellular edema, also called cytotoxic edema, is caused by alterations in the cellular ionic exchanges, resulting in the accumulation of sodium ions inside the cell. In this case, the water movement from its normal extracellular location to the intracellular space causes cell damage. The extracellular edema, also called vasogenic edema, results of the extracellular accumulation of fluid directly associated with BRB breakdown. In this later situation, the unbalance of hydrostatic, oncotic and osmotic pressure gradients across the BRB contributes to further water movements and edema formation (Cunha-Vaz and Travassos, 1984).

Preproliferative diabetic retinopathy

Preproliferative diabetic retinopathy is characterized by an increase in retinal ischemia signs, such as cotton-wool spots, intraretinal microvascular abnormalities (IRMAs), the appearance of several regions without capillary perfusion due to an extensive capillary occlusion and

significant venous beading (increase in the venous caliber) (Figure 1.13C). Cotton-wool spots, also termed soft exudates, are very common in diabetic retinopathy, especially if the patient is also hypertensive. They appear as puffy white patches in the NFL and result from the occlusion of retinal pre-capillary arterioles supplying the NFL with concomitant swelling of local nerve fiber axons. A high number of cotton-wool spots (higher than 5) is a sign of an advanced stage of diabetic retinopathy with the risk of new vessel formation (Kohner and Oakley, 1975). IRMAs are abnormal, dilated retinal capillaries that grow within the retinal tissue towards areas of capillary occlusion. IRMAs represent intra-retinal neovascularization in response to the retinal ischemia (Frank, 1995). IRMAs can be distinguished from normal retinal blood vessels by their haphazard branching, with unusual large angles between branches and an irregular caliber. With the increase of widespread IRMAs, and thus capillary non-perfusion, the risk of developing proliferative retinopathy within a year increases four-fold (ETDRS Research Group, 1991a).

Proliferative diabetic retinopathy

Proliferative diabetic retinopathy refers to a severe stage of the disease characterized by the proliferative growth of abnormal new blood vessels (neovascularization) on the surface of the retina as a result of a widespread retinal ischemia (Figure 1.13D). Retinal ischemia due to extensive capillary occlusion results in the production of vasoproliferative factors, such as VEGF, that promote neovascularization (Aiello *et al.*, 1994; Miller *et al.*, 1997). Neovascularization can involve the retina, optic disc or the iris (Frank, 1995). The new blood vessels are leaky. They have weak walls with a profound loss of the barrier function, as demonstrated by the substantial leakage of intravenously-injected fluorescein dye through their endothelial walls. Bleeding from the fragile new blood vessels involving the retina or optic disc can result in vitreous or retinal hemorrhage, which results in retinal damage. The alterations in the vitreous associated to the contraction of associated fibrous tissue and to cellular proliferation can result in distortion of the retina and tractional retinal detachment.
In conclusion, visual impairment is usually associated with the later stages of diabetic retinopathy, and is mainly due to neovascularization and macular edema.

1.4.1.2 Hyperglycemia and biochemical alterations in diabetic retinopathy

Hyperglycemia is considered the major factor for the initiation and progression of diabetic retinopathy. Prolonged hyperglycemia results in microvascular dysfunction, including basement

membrane thickening of retinal vessels, loss of pericytes and endothelial cells, and BRB breakdown (DCCT Research Group, 1993). It has been demonstrated that intensive glycemic control can prevent or delay the progression of diabetic retinopathy. The Diabetes Control and Complications Trial (DCCT) showed that intensive insulin therapy reduces or prevents the development of retinopathy by 76% in patients with type 1 DM (DCCT Research Group, 1993). The protective effect of glycemic control has also been confirmed for patients with type 2 DM. The U.K. Prospective Diabetes Study (UKPDS) demonstrated that improved blood glucose control reduces the risk of developing retinopathy and nephropathy and possibly neuropathy. The overall rate of microvascular complications was decreased by 25% in patients receiving intensive insulin therapy versus conventional therapy (UK Prospective Diabetes Study (UKPDS) Group, 1998).

Multiple biochemical pathways have been proposed to link hyperglycemia to retinal microvasculature injury. These metabolic pathways are activated after the onset of hyperglycemia, including increased polyol flux, increased intracellular formation of advanced glycation end products (AGEs), oxidative stress and PKC activation.

Polyol pathway

The polyol pathway is a metabolic pathway in which glucose is reduced to sorbitol by aldose reductase (AR), using nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor. Sorbitol is then oxidized to fructose and nicotinamide adenine dinucleotide (NAD^+) by sorbitol dehydrogenase (Greene *et al.*, 1987). Under normal conditions, glucose is channeled preferentially into the glycolytic pathway catalyzed by the enzyme hexokinase, which has higher affinity for glucose than AR. Secondary to hyperglycemia, the levels of intracellular glucose increase and a considerable portion of glucose is shuttled to the polyol pathway. In tissues such as kidney, retina, lens and nervous tissues, which do not require insulin for the intracellular transport of glucose, AR activity has been shown to be increased in a hyperglycemic environment (Nishimura *et al.*, 1994), concomitant with intracellular sorbitol accumulation. Sorbitol is an alcohol, strongly hydrophilic, and therefore does not diffuse readily through cell membranes, accumulating intracellularly and causing osmotic damage to vascular cells (Gabbay, 1975). The increased flux through the polyol pathway increases sorbitol and fructose as well as the cytosolic NADH/NAD+ ratio causing a hypoxia-like redox imbalance, a state termed “pseudohypoxia” (Greene *et al.*, 1987; Williamson *et al.*, 1993). This increase in the

NADH/NAD+ ratio may contribute to increased ROS production, inhibited fatty acid oxidation, increased diacylglycerol (DAG) and prostaglandins synthesis, decreased nitric oxide (NO) production, as well as to abnormal growth factor expression and vascular permeability (Pugliese *et al.*, 1991; Wolf *et al.*, 1991; Williamson *et al.*, 1993; Van den Enden *et al.*, 1995). Also, increased AR activity decreases the availability of NADPH, which is also a critical cofactor in the conversion of oxidized glutathione to reduced glutathione by the glutathione reductase. By reducing the amount of reduced glutathione, a major antioxidant, the polyol pathway increases the susceptibility of intracellular oxidative stress (Williamson *et al.*, 1993). All these metabolic changes contribute to tissue damage and alterations within the retinal vasculature. In experimental diabetes, the accumulation of sorbitol in the retinas is associated with basement membrane thickening, pericyte loss and microaneurysms formation (Frank *et al.*, 1983; Engerman and Kern, 1984), and these abnormalities are prevented by administration of AR inhibitors (Vinores *et al.*, 1993; Neuenschwander *et al.*, 1997; Robison *et al.*, 1998; Kato *et al.*, 2003; Sun *et al.*, 2006). Further, in AR knockout mice, AR deficiency prevented diabetes-induced oxidative stress, retinal ischemia and increased vascular permeability (Cheung *et al.*, 2005; Cheung *et al.*, 2007), suggesting that AR contributes to vascular dysfunction in diabetes. However, clinical trials of AR inhibitors have failed to demonstrate the efficacy of this drug to prevent diabetic vascular complications in diabetic patients (Sorbinil Retinopathy Trial Research Group, 1990; van Gerven *et al.*, 1994), suggesting that other mechanisms can have a significant role in the pathogenesis of diabetic retinopathy.

Advanced glycation end products

AGEs are a heterogeneous group of molecules formed from the nonenzymatic reaction of reducing sugars with free amino groups of proteins, lipids, and nucleic acids. The initial product of this reaction is called a Schiff base, which spontaneously rearranges itself into an Amadori product, as is the case of the well-known hemoglobin A1c (HA1c). These initial reactions are reversible depending on the concentration of the reactants. However, with time, these intermediates are transformed into irreversibly bound and chemically reactive adducts, the AGEs (Bucala and Cerami, 1992). AGEs form at slow but constant rate in the normal body, starting in early embryonic development, and accumulate with time. However, their formation is markedly accelerated in diabetes because of the increased availability of glucose (Schleicher *et al.*, 1997). A key characteristic of certain reactive or precursor AGEs is their ability for covalent

crosslink formation between proteins, which alters their structure and function, as in cellular matrix, basement membranes, and vessel-wall components (Brownlee *et al.*, 1988; Sell *et al.*, 1992).

AGEs exert their vascular effects by both receptor-independent and receptor-dependent pathways. The receptor-dependent effects of AGEs are mediated by interaction with specific receptors for AGEs (RAGEs) and also with binding proteins including AGE receptors 1, 2 and 3 (AGE-R1, AGE-R2 and AGE-R3) (Thornalley, 1998), the formers acting mainly as AGE scavengers, limiting AGE-mediated cell and tissue injury. The interaction of AGEs with their receptors leads to oxidative stress and nuclear factor-κB (NF-κB) activation, with the consequent increase in inflammatory genes expression (Mohamed *et al.*, 1999).

The accumulation of AGEs in the vitreous of diabetic patients (Sebag, 1993; Stitt, 2003) or in the retina of diabetic animals has been correlated with retinal vascular dysfunction (Vlassara *et al.*, 1994; Chibber *et al.*, 1997; Karachalias *et al.*, 2003; Nakamura *et al.*, 2003). AGEs were also reported to induce apoptosis in retinal pericytes in culture (Chibber *et al.*, 1997). Further, AGE accumulation has been reported to upregulate the expression of VEGF and increase leukocyte adhesion in retinal microvascular endothelial cells (Lu *et al.*, 1998; Moore *et al.*, 2003).

Aminoguanidine, a potent inhibitor of AGE crosslinks, has been shown to have a protective effect in the development of retinal microvasculature dysfunction in experimental diabetes. In diabetic animals, treatment with aminoguanidine reduces pericyte loss, microaneurysms formation, basement membrane thickening and BRB permeability (Cho *et al.*, 1991; Hammes *et al.*, 1991; Kern and Engerman, 2001; Gardiner *et al.*, 2003). Similar beneficial effects in the retinal vasculature of diabetic animals have been observed with other inhibitors of AGE formation, including pyridoxamine and benfotiamine (Stitt *et al.*, 2002; Hammes *et al.*, 2003; Canning *et al.*, 2007). In addition, RAGE inhibition was shown to reduce retinal capillary lesions induced by diabetes (Barile *et al.*, 2005).

Protein kinase C activation

The PKC family is a large group of structurally related serine/threonine kinases that includes at least eleven isoforms that are classified into three subgroups depending on the organization of their catalytic and regulatory domains and second messenger requirement (Jaken, 1996; Newton, 1997): conventional PKCs (cPKCs: α, βI, βII, γ), are activated by phosphatidylserine,

DAG, phorbolesters and Ca^{2+}; novel PKCs (nPKCs: δ, ε, η, θ, μ) are Ca^{2+}-independent, but phosphatidylserine and DAG responsive; atypical PKCs (aPKCs: ζ, ι/λ) are insensitive to DAG and Ca^{2+} but can be activated by phosphatidylserine (Jaken, 1996; Newton, 1997). PKCs represent the major downstream targets for lipid second messengers or phorbol esters, being primarily activated by DAG (Nishizuka, 1992; Shiba *et al.*, 1993; Nishizuka, 1995).The source of DAG that activates PKC can be derived from the hydrolysis of phosphatidylinositides or from the metabolism of phosphatidylcholine by phospholipase C (PLC) or phospholipase D. Although recent evidence has demonstrated that each isoform could be regulated by more than one lipid second messenger (Sharma and Ziyadeh, 1995), such as the activation of aPKC by phosphoinositide 3,4,5 trisphosphate (PIP_3) (Nakanishi *et al.*, 1993; Nishizuka, 1995).

Increased PKC activation and DAG levels have been shown to increase in several tissues associated with diabetic vascular complications, including the retina (Shiba *et al.*, 1993), heart (Inoguchi *et al.*, 1992) and renal glomeruli (Craven *et al.*, 1995). Hyperglycemia leads to *de novo* synthesis of DAG which directly activates PKC in vascular endothelial cells. The activation of DAG-PKC in the vasculature induced by hyperglycemia is maintained chronically (Xia *et al.*, 1994), suggesting that PKC activation may have a profound effect on retinal damage. Hyperglycemia may also activate PKC isoforms indirectly through AGE receptors (Portilla *et al.*, 2000) and increased flux of the polyol pathway (Keogh *et al.*, 1997) probably by increasing ROS. In experimental diabetes, it has been shown that PKC activation is correlated with the development of microvascular abnormalities, including basement membrane thickening, increased extracellular matrix components synthesis, changes in blood flow, via alterations in NO and endothelin-1, increased leukocyte adhesion and vascular permeability, and angiogenesis (Koya and King, 1998; Das Evcimen and King, 2007). PKC has also been shown to regulate several growth factors responsible for cell survival and growth such as VEGF, platelet derived growth factor (PDGF), IGF, epidermal growth factor (EGF) and FGF. These growth factors are of further importance in mediating endothelial cell proliferation and neovascularization, hallmark features of the latter stages of diabetic retinopathy (Blakesley, 1996; Newton, 1997).

Several PKC isoforms (α, βI, βII, and ε) are preferentially elevated in vascular tissues in the diabetic state. However PKCβ has been suggested to have a predominant role in the pathogenesis of diabetic retinopathy (Inoguchi *et al.*, 1992; Shiba *et al.*, 1993; Ishii *et al.*, 1996; Idris *et al.*, 2001; Aiello, 2002). In fact, oral administration of ruboxistaurin (LY333531), a

specific PKCβ inhibitor, to diabetic animals for a 2-week period, reduced the increase in PKC activity and the retinal mean circulation time (Ishii *et al.*, 1996). PKCβ has also been implicated as a mediator of VEGF-induced retinal vascular permeability (Xia *et al.*, 1996). Indeed, oral or intravitreal administration of ruboxistaurin abrogated the increase in retinal vessels permeability induced by VEGF (Aiello *et al.*, 1997). More recently, it has been reported that VEGF-induced retinal endothelial cell permeability and occludin phosphorylation are mediated, at least in part, by PKCβ activation (Harhaj *et al.*, 2006). In addition, general PKC inhibitors can reverse VEGF-induced BRB breakdown (Aiello *et al.*, 1997; Xu *et al.*, 2004), suggesting that other PKC isoforms might be involved. The PKCβ inhibitor ruboxistaurin has also been demonstrated to prevent leukocyte adhesion to the retinal endothelium in diabetic retinas (Nonaka *et al.*, 2000; Abiko *et al.*, 2003). The potential beneficial effects of ruboxistaurin in diabetic microvascular disease have also been evaluated in clinical trials, in patients with diabetic retinopathy. Although ruboxistaurin was not able to prevent the progression of nonproliferative to proliferative diabetic retinopathy, it was found to increase visual acuity, reduce vision loss and the need for laser treatment (Aiello *et al.*, 2006a; Aiello *et al.*, 2006b). In fact, the clinical trials results further support an important role for PKCβ in the development of retinal microvascular pathology in diabetes.

Hexosamine pathway activation

In normal conditions, glucose enters the glycolysis pathway to be converted to glucose-6-phosphate, and then to fructose-6-phosphate. Only a small part of the intracellular glucose is channeled to the hexosamine pathway. In this pathway, fructose-6-phosphate is converted to N-acetylglucosamine-6-phosphate by glutamine:fructose-6-phosphate amidotransferase (GFAT), the rate-limiting enzyme of this pathway. N-acetylglucosamine-6-phosphate is rapidly metabolized to uridine diphosphate (UDP)-N-acetylglucosamine (UDP-GlcNAc), which along with other hexosamines, are essential substrates for the *O*-linked glycosylation involved in the synthesis of glycoproteins, proteoglycans and glycolipids. During hyperglycemia, increased flux of glucose through the hexosamine pathway may contribute to insulin resistance (Marshall *et al.*, 1991; Schleicher *et al.*, 1997; Nerlich *et al.*, 1998). Also, inhibition of GFAT prevents hyperglycemia-induced increase in the transcription of transforming growth factor (TGF)-α, TGF-β1 (Kolm-Litty *et al.*, 1998) and plasminogen activator inhibitor-1 (PAI-1) (Du *et al.*, 2000). The mechanism by which increased flux through the hexosamine pathway mediates

hyperglycemia-induced gene transcription is not known, but covalent modification of transcription factors by *O*-GlcNAc may explain the link between hexosamine pathway activation and hyperglycemia-induced changes in gene transcription (Chen *et al.*, 1998). Indeed, it was demonstrated that hyperglycemia-induced activation of the hexosamine pathway increases *O*-GlcNAcylation of the transcription factor Sp1, increases Sp1 transactivation and Sp1-dependent expression of both TGFβ1 and PAI-1 (Du *et al.*, 2000). It was also reported that excessive glucose flux through the hexosamine pathway may direct retinal neurons to undergo apoptosis in a bimodal fashion, i.e., via perturbation of the neuroprotective effect of insulin mediated by Akt and via induction of apoptosis possibly by altered glycosylation of proteins (Nakamura *et al.*, 2001), emphasizing that hexosamine pathway may be involved in retinal degeneration in diabetes.

Oxidative stress

The retina has the highest oxygen consumption in the body and an elevated rate of glucose oxidation, thus making the retina extremely susceptible to increase oxidative stress (Anderson *et al.*, 1984). Oxidative stress, defined by an increase of ROS formation, is indeed elevated in the retina in diabetes, and contributes to the development of diabetic retinopathy (Baynes, 1991; Kollmar *et al.*, 2001). Hyperglycemia may induce ROS production through glucose auto-oxidation, increased flux through the polyol pathway, and increased protein glycation (Giugliano *et al.*, 1996). In turn, ROS may increase AGEs and DAG production and activate AR, PKC and NF-κB (Nishikawa *et al.*, 2000a). Indeed, an increase in ROS is considered a causal link between elevated glucose and other metabolic abnormalities important for the development of diabetic retinopathy (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). 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. Elevated levels of superoxide anions were found in diabetic rat retinas and in endothelial cells exposed to high glucose, probably due to mitochondrial activation (Nishikawa *et al.*, 2000b; Du *et al.*, 2003; Kowluru and Abbas, 2003). Increased levels of thiobarbituric acid-reacting substances (TBARS), which are markers of oxidative stress, concomitant with reduced levels of glutathione were reported in diabetic rat

retinas (Kowluru *et al.*, 2000). Also, increased TBARS levels and decreased levels of superoxide dismutase and glutathione peroxidase were found in serum samples obtained from diabetic patients, however without significant association with the severity of retinopathy (Hartnett *et al.*, 2000). The levels of reduced glutathione and the activity of glutathione reductase, glutathione peroxidase, superoxide dismutase (SOD) and catalase are markedly diminished in the retinas of diabetic animals (Tagami *et al.*, 1992; Kern *et al.*, 1994; Kowluru *et al.*, 1997; Kowluru *et al.*, 2001b). The treatment with antioxidants, such as N-acetyl cysteine, inhibited the formation of TBARS in diabetic rat retinas and endothelial cells exposed to high glucose (Kowluru, 2001; Kowluru *et al.*, 2001a). In addition, administration of the antioxidants vitamin C and E prevented the decrease in the activity of several antioxidant enzymes in the retinas of diabetic animals (Kowluru *et al.*, 1997; Bursell *et al.*, 1999; Bursell and King, 1999). Recently, it was found that overexpression of mitochondrial SOD in mice protects the retina from diabetes-induced oxidative stress (Kowluru *et al.*, 2006). In diabetic rats, the treatment with antioxidants has been shown to ameliorate the capillary cell loss by apoptosis and reduce the number of acellular capillaries in the retina (Ansari *et al.*, 1998; Kowluru and Odenbach, 2004a), suggesting that oxidative stress is involved in microvascular cell loss in the course of diabetic retinopathy.

Antioxidant therapy has also provided encouraging results in clinical trials with diabetic patients. Administration of vitamin E has been shown to reverse some alterations in the retinal vessels and normalize retinal blood flow (Bursell *et al.*, 1999; Bursell and King, 1999). In addition, chronic administration of vitamin E has been reported to improve metabolic control and reduce protein glycation in diabetic patients (Ceriello *et al.*, 1991; Paolisso *et al.*, 1993). Calcium dobesilate (2,5-dihydroxybenzenesulfonate), a compound with potent antioxidant capacity against hydroxyl radical (Brunet *et al.*, 1998), and a registered compound for the treatment of diabetic retinopathy, was shown to reduce blood viscosity, BRB permeability, retinal hemorrhages and delay the progression of diabetic retinopathy (Garay *et al.*, 2005; Ribeiro *et al.*, 2006). Calcium dobesilate has also been reported to reduced the formation of vascular abnormalities and BRB permeability in the retinas of diabetic animals (Rota *et al.*, 2004; Padilla *et al.*, 2005). Pycnogenol, a compound with both free radical scavenging and anti-inflammatory properties, was also reported to have beneficial effects on the progression of retinopathy in diabetic patients (Spadea and Balestrazzi, 2001). In contrast, other studies have shown no beneficial effects of supplementation with vitamins C and E (Millen *et al.*, 2003; Millen *et al.*,

2004). However, further clinical studies are needed to determine the appropriate regimen, and also whether these therapies could have long-term effects that may help diabetic patients to slow the progression of this sight-threatening complication of diabetes.

Growth factors

Retinal vascular dysfunction has also been associated with alterations in the expression of several growth factors, such as VEGF, IGF-I and PEDF, in diabetic retinopathy.

Vascular endothelial growth factor

VEGF is a member of a large family of angiogenic growth factors. There are six known members of the VEGF family: VEGF-A (commonly referred as VEGF), placental growth factor, VEGF-B, VEGF-C, VEGF-D and VEGF-E (Robinson and Stringer, 2001). VEGF-A is the predominant form and a key molecular regulator of angiogenesis and hyperpermeability (Bacic *et al.*, 1995). VEGF isoforms have different affinities for the VEGF receptors, VEGF-R1 (fms-like tyrosine kinase-1 or Flt-1), VEGF-R2 (kinase insert domain-containing receptor/fetal liver kanse-1 – KDR/Flk-1) and VEGF-R3 (Flt-4), which are tyrosine kinase receptors, as well as for VEGF co-receptors, neuropilin-1 and neuropilin-2 (NRP1, NRP2), which have also been identified in the retina (Witmer *et al.*, 2003). Interaction between VEGF and its receptors has been shown to activate several downstream key kinases including PLCγ (Takahashi *et al.*, 2001) and phosphatidylinositol 3-kinase (PI_3K) (Qi and Claesson-Welsh, 2001), in endothelial cells. Activation of PLCγ may have an important role in retinal endothelial cells since it is involved in DAG synthesis, causing activation of PKC that engages subsequent changes in endothelial cells (Guo *et al.*, 1995; Enaida *et al.*, 1999).

VEGF is increased in the retinas of diabetic patients and diabetic rats (Aiello *et al.*, 1994; Mathews *et al.*, 1997; Hammes *et al.*, 1998; Qaum *et al.*, 2001), which has been correlated with increased BRB permeability. Diabetes increases VEGF expression in the retina (Tilton *et al.*, 1997), particularly in endothelial cells, pericytes, RPE cells, astrocytes, Müller cells and ganglion cells (Pe'er *et al.*, 1995; Dorey *et al.*, 1996; Amin *et al.*, 1997; Hammes *et al.*, 1998; Lu and Adamis, 2002). VEGF expression is likely to be hypoxia-induced since elevated levels of VEGF are present in areas of retinal occlusion and neovascularization in diabetic patients and in an animal model of ischemia-induced neovascularization (Malecaze *et al.*, 1994; Simpson *et al.*, 1999). Flt-1 and Flk-1, the major high-affinity VEGF receptors, are similarly upregulated in the

diabetic retina (Hammes *et al.*, 1998) and localize to the retinal vasculature (Thieme *et al.*, 1995).

VEGF is considered to be a major factor in the breakdown of BRB (Caldwell *et al.*, 2003). Intravitreal injection of VEGF triggers BRB breakdown and induces angiogenesis in primate retinas (Tolentino *et al.*, 1996; Aiello *et al.*, 1997). The inhibition of VEGF with specific VEGF-neutralizing soluble constructs can prevent the increase in BRB permeability induced by diabetes (Qaum *et al.*, 2001; Saishin *et al.*, 2003). Moreover, VEGF is involved in the formation of new vessels in diabetic retinas, and intravitreal injection of anti-VEGF antibodies prevents retinal neovascularization in animal models (Aiello *et al.*, 1994). Several lines of evidence show that VEGF increases retinal vascular permeability by altering tight junction complexes. VEGF treatment reduces occludin content and induces a rapid phosphorylation of occludin and ZO-1 in rat retinas and cultured bovine retinal endothelial cells concomitant with increased permeability (Antonetti *et al.*, 1998; Antonetti *et al.*, 1999b; Harhaj *et al.*, 2006).

The strong supportive evidence from animal studies defined VEGF as an optimal therapeutic target for treatment of ocular diseases in which retinal edema and neovascularization leads to blindness. Pegaptanib (Macugen), ranibizumab (Lucentis) and bevacizumab (Avastin), anti-VEGF agents, are currently used as treatments for proliferative diabetic retinopathy and diabetic macular edema (Simo and Hernandez, 2008). Pegaptanib is a neutralizing RNA aptamer, which binds with high specificity and affinity to human VEGFA (Yasukawa *et al.*, 2004). Ranibizumab is a fragment antigen-binding (Fab) domain derived of a full-length monoclonal antibody against human VEGF (Kim *et al.*, 1992) whereas bevacizumab is a full-lenght antibody (Kim *et al.*, 1992). In contrast to pegaptanib, ranibizumab and bevacizumab bind and inhibit the biological activity of all isoforms of human VEGF. Results from a phase 2 clinical trial demonstrate that patients with diabetic macular edema, who are treated with pegaptanib, had better visual acuity, reduction in macular edema and reduced need for laser treatment (Cunningham *et al.*, 2005). In addition, neovascularization progression is reduced by pegaptanib treatment in diabetic patients (Adamis *et al.*, 2006). Studies using ranibizumab and bevacizumab have also found a regression of retinal neovascularization, an improvement in visual acuity and a decrease in retinal edema (Avery *et al.*, 2006; Nguyen *et al.*, 2006; Spaide and Fisher, 2006). Nevertheless, larger studies are needed to investigate the effectiveness of these anti-VEGF drugs in the diabetic population.

Insulin-like growth factor-I

IGF is a polypeptide hormone that shares a high degree of homology with insulin. Growth hormone stimulates the production of IGF mainly in the liver and muscle in a paracrine/autocrine manner. IGF-1 exerts its biological effects via coupling with the tyrosine kinase IGF-1 receptor (IGF-1R). Three key signaling pathways are activated by IGF-1: PI_3K (Shoba *et al.*, 2001), PLCγ (Chattopadhyay and Carpenter, 2002) and Ras/raf/mitogen-activated protein kinase (MAPK) (Boney *et al.*, 2001), which are involved in cell survival and proliferation (Arsenijevic *et al.*, 2001; Yamada *et al.*, 2001).

IGF-1 was one of the first growth factors to be directly linked with diabetic retinopathy (Hyer *et al.*, 1989). Indeed, pituitary ablation acutely improved visual acuity and suppressed retinal neovascularization in some patients with diabetic retinopathy, raising the hypothesis that growth hormone and IGF-I have a role in the pathogenesis of diabetic retinopathy (Poulsen, 1953; Luft *et al.*, 1955). Initial reports demonstrated that an acute increase in serum levels of IGF-1 preceded the onset of neovascularization in diabetic animals (Hyer *et al.*, 1988; Grant *et al.*, 1993) whereas reduction of serum IGF-I levels inhibits retinal neovascularization in an ischemic murine model (Smith *et al.*, 1997). Increased IGF-1 levels were also found in the serum and vitreous of patients with proliferative diabetic retinopathy (Meyer-Schwickerath *et al.*, 1993; Lee *et al.*, 1994), indicating that IGF-1 plays a role in retinal neovascularization. In fact, several reports demonstrate that IGF-1 induces neovascularization either in cultured endothelial cells (King *et al.*, 1985; Nakao-Hayashi *et al.*, 1992; Nicosia *et al.*, 1994) or in animal retinas (Grant *et al.*, 1993; Danis and Bingaman, 1997). In addition, a recent report demonstrated that IGF-1 induces upregulation of VEGF and intercellular adhesion molecule-1 (ICAM-1) and increased BRB permeability by altering the tight junction complex (Haurigot *et al.*, 2009). Further, systemic inhibition of IGF-IR with a neutralizing antibody suppressed VEGF and ICAM-1 expression, leukocyte adhesion and BRB permeability in diabetic rat retinas (Poulaki *et al.*, 2004).

Pigment epithelium-derived factor

PEDF is a glycoprotein originally identified as a neuronal differentiation factor produced by cultured human RPE cells (Tombran-Tink *et al.*, 1991). PEDF has been recognized as a potent anti-angiogenic factor in the eye (Dawson *et al.*, 1999; Hutchings *et al.*, 2002) by inhibiting endothelial cell proliferation. PEDF levels decrease in the vitreous or aqueous humor of diabetic patients with proliferative diabetic retinopathy (Spranger *et al.*, 2001; Ogata *et al.*, 2002; Boehm

et al., 2003), suggesting that PEDF is a negative regulator of neovascularization. Indeed, PEDF was shown to inhibit retinal and choroidal neovascularization in ischemia-induced retinopathy animal models (Mori *et al.*, 2001; Stellmach *et al.*, 2001). Inhibition of vessel formation appears to be mediated by PEDF-induced up-regulation of the Fas ligand on the surface of endothelial cells, with subsequent induction of apoptosis in actively dividing cells (Volpert *et al.*, 2002). In addition, a recent report has demonstrated that PEDF inhibits AGE-induced BRB breakdown by reducing VEGF levels (Yamagishi *et al.*, 2006b) Moreover, PEDF also inhibits diabetes- and AGEs-induced rat retinal leukostasis by suppressing ICAM-1 expression (Yamagishi *et al.*, 2006a). These observations suggest that the loss of PEDF activity in the eye may contribute to the pathogenesis of proliferative diabetic retinopathy. Furthermore, the balance between the angiogenic and anti-angiogenic factors rather than angiogenic factors themselves appears to be crucial in determining the progression of proliferative diabetic retinopathy.

1.4.1.3 Blood-retinal barrier breakdown

Retinal microvasculature dysfunction is one of the earliest event that occurs in the development of diabetic retinopathy and the breakdown of BRB is considered the hallmark of diabetic retinopathy (Cunha-Vaz *et al.*, 1975). The increase in BRB permeability progresses to retinal edema which is directly associated with vision loss (Klein *et al.*, 1995a; Moss *et al.*, 1998). Although both inner BRB and outer BRB are affected by diabetes, the retinal vasculature seems to be a prime target either in diabetic patients or diabetic animal models (Vinores *et al.*, 1989; Carmo, 1998). Given the importance of tight junctions in regulating BRB permeability, the BRB breakdown is associated with changes in the tight junction complex. However, microvascular cell death, leukostasis and glial activation also contribute to BRB breakdown.

Alterations in tight junctions

Tight junctions form a physical barrier to control water and solute flux from blood-circulation and the retinal parenchyma, crucial for the homeostasis and normal function of the retina. The increase in retinal vascular permeability is associated with alterations in the content and distribution of tight junction proteins in endothelial cells. The most known diabetes-induced changes in the tight junction complex involve the transmembrane protein occludin. Occludin is of particular interest since there is a direct correlation between occludin content and cell permeability (Furuse *et al.*, 1993; Hirase *et al.*, 1997; Mitic and Anderson, 1998). Experimental

diabetes decreases the content of occludin and occludin immunoreactivity at the endothelial cell borders in rat retinal capillaries and arterioles concomitant with increased retinal vascular permeability (Antonetti *et al.*, 1998; Barber *et al.*, 2000; Barber and Antonetti, 2003; Leal *et al.*, 2007). Occludin cellular localization may be regulated by phosphorylation, which may also promote tight junction permeability. Indeed, intravitreal injection of VEGF into rat retinas or VEGF stimulation of bovine retinal endothelial cells reduces occludin content and enhances occludin phosphorylation, prior to increased tissue and monolayer permeability (Antonetti *et al.*, 1999a). Inhibition of PKC, specially the β isoform, completely prevents VEGF-induced increase in occludin phosphorylation but only partially prevents the VEGF-induced cell monolayer permeability, suggesting that in addition to PKCβ, other PKC isoforms or protein kinases may be involved (Antonetti *et al.*, 1999a; Harhaj *et al.*, 2006). Recently, multiple VEGF-induced occludin phosphorylation sites have been identified and, one of these sites, Ser490, is VEGF responsive. Also, phosphorylation at this site reduces occludin interaction with ZO-1, which may lead to tight junction disruption (Sundstrom *et al.*, 2009).

Changes in other tight junction proteins have also been associated with increased retinal vascular permeability. In fact, the decrease of claudin-5 and ZO-1 content correlates with increased BRB permeability in diabetic rat retinas (Jung *et al.*, 2001; Leal *et al.*, 2007; Ambrosio *et al.*, 2009; Klaassen *et al.*, 2009). Also, VEGF treatment has also been shown to reduce claudin-5 (Klaassen *et al.*, 2009) and ZO-1 (Ghassemifar *et al.*, 2006; Peters *et al.*, 2007) mRNA levels in retinal endothelial cells. Further, VEGF induces a rapid phosphorylation of ZO-1 in rat retinas concomitant with increased cell permeability (Antonetti *et al.*, 1999a). These observations demonstrate that alterations in the content, localization and phosphorylation state of tight junction proteins, specially occludin and ZO-1, affects the tight junction complex integrity and therefore contribute to increased vascular permeability.

Retinal capillary cell death

BRB breakdown is related to the microvascular pathology that affects retinal capillaries, which includes capillary cells death, basement membrane thickening, the formation of microaneurysms and capillary occlusion. The earliest histopathological abnormalities in retinal capillaries are the loss of pericytes (Cogan *et al.*, 1961; Cogan and Kuwabara, 1963; Engerman and Kern, 1995), together with capillary basement membrane thickening and microaneurysm formation. Pericyte loss by apoptosis occurs both in human diabetic patients and diabetic

animal models (Mizutani *et al.*, 1996). The exact cause of pericyte demise is unknown but is probably the result of cumulative biochemical insults coupled with the limited ability of these cells to repair and proliferate. Indeed, intracellular accumulation of sorbitol and AGEs (Hohman *et al.*, 1989; Stitt *et al.*, 1997) have toxic effects on pericyte growth and function (Chibber *et al.*, 1997). Retinal endothelial cell loss by apoptosis also occurs in human and experimental diabetes resulting in acellular capillaries (Mizutani *et al.*, 1996), a major feature of the ischemic retina.

Leukocyte adhesion to retinal vessels

The increased adhesion of leukocytes to the retinal microvasculature is one of the earliest changes detected in the diabetic retina, occurring before clinically detectable microvascular pathology (Miyamoto *et al.*, 1999). Leukocyte adhesion coincides with the development of BRB breakdown, capillary nonperfusion, and endothelial cell injury and death (Schroder *et al.*, 1991; Miyamoto *et al.*, 1999; Joussen *et al.*, 2001b).

Leukocyte adhesion to the diabetic retinal vasculature is mediated by ICAM-1 (McLeod *et al.*, 1995), which is expressed in endothelial cells, and the β2 integrin CD18, expressed in leukocytes (Barouch *et al.*, 2000). The increase in retinal leukostasis correlates with an increase in the expression of ICAM-1 (Miyamoto *et al.*, 1999; Joussen *et al.*, 2001a; Leal *et al.*, 2007) and CD18 (Barouch *et al.*, 2000) in diabetic retinas. Moreover, ICAM-1 or CD18 inhibition by monoclonal antibodies prevented diabetes-induced leukostasis and retinal vascular leakage (Miyamoto *et al.*, 1999; Barouch *et al.*, 2000; Joussen *et al.*, 2001a). A similar result was obtained in ICAM-1- and CD18-deficient mice (Joussen *et al.*, 2004). Recently, it was found that the inducible nitric oxide synthase isoform (iNOS) is involved in retinal leukostasis during diabetes, since diabetes-induced ICAM-1 expression, leukocyte adhesion and BRB permeability were reduced in diabetic iNOS-deficient mice (Leal *et al.*, 2007). Leukocyte adhesion has also been shown to be mediated by PKC activation. Oral administration of LY333531, a specific inhibitor of PKCβ, which as been shown to decrease vascular permeability (Aiello *et al.*, 1997; Antonetti *et al.*, 1999a; Harhaj *et al.*, 2006), also decreases leukostasis in diabetic retinas (Nonaka *et al.*, 2000; Abiko *et al.*, 2003). VEGF has also been considered to have a role in leukostasis. Intravitreal injection of VEGF upregulates the expression of ICAM-1 and leukocyte adhesion in retinal endothelial cells (Lu *et al.*, 1999; Joussen *et al.*, 2002b). The inhibition of VEGF with a highly specific VEGF neutralizing antibody prevented the increase in ICAM-1

expression and leukocyte adhesion in the retinas of diabetic animals (Joussen *et al.*, 2002a). Moreover, leukocyte adhesion has also been shown to induce disorganization of adherens junctions (Del Maschio *et al.*, 1999) and tight junctions (Bolton *et al.*, 1998; Krady *et al.*, 2005) between endothelial cells, thereby increasing vascular permeability.

Leukocyte adhesion might also play a role in capillary cell death, since it was demonstrated that leukostasis is temporally and spatially associated with retinal endothelial cell death. Further, both ICAM-1 and CD18 neutralizing antibodies prevented retinal endothelial cell death (Joussen *et al.*, 2001a) and thus vascular permeability.

All together, these results clearly demonstrate that leukocyte adhesion to the retinal vessels in the early stages of diabetic retinopathy plays a critical role in microvasculature pathology and BRB breakdown.

Retinal glial cell dysfunction

Retinal macroglial cells (astrocytes and Müller cells) are closely associated with retinal vessels and have a critical role in the formation and maintenance of the BRB (Schnitzer, 1987; Schnitzer, 1988; Tout *et al.*, 1993).

Astrocytes are known to induce barrier properties of the retinal vascular endothelium by enhancing the expression of the tight junction protein ZO-1 (Gardner *et al.*, 1997). This observation is supported by *in vivo* studies demonstrating a primary role for astrocytic contact in the expression of occludin, claudin-5 and ZO-1 in the mature brain vasculature (Willis *et al.*, 2004). Müller cells are known to support the neuronal activity in the retina and maintain the proper functioning of the inner BRB under normal conditions (Reichenbach *et al.*, 2007). They are involved in the homeostasis of potassium and other ions and in the regulation of extracellular pH (Bringmann *et al.*, 2000). Müller cell dysfunction contributes to BRB breakdown (Tretiach *et al.*, 2005) under hypoxic-ischemic conditions. Under normal conditions, Müller cells secrete PEDF (Eichler *et al.*, 2004), which is known to antagonize VEGF action and hence reduce vascular permeability (Duh *et al.*, 2002). Expression of PEDF by Müller cells decreases in hypoxic conditions resulting in an increased VEGF/PEDF ratio, which facilitates neovascularization (Duh *et al.*, 2002; Lange *et al.*, 2008). Barrier function is also impaired by metalloproteinases (MMPs) from Müller cells (Behzadian *et al.*, 2001), as these MMPs lead to proteolytic degradation of the tight junction occludin (Giebel *et al.*, 2005). Müller cells also undergo apoptosis in diabetes (Hammes *et al.*, 1995), and the increase in Müller cells apoptosis

is concomitant with an increase in pericyte loss and capillary occlusion. Treatment of diabetic rats with the nerve growth factor (NGF) prevented both Müller cells apoptosis and the formation of acellular capillaries, suggesting a link between Müller cell dysfunction and retinal vasculature damage (Hammes *et al.*, 1995).

Glial reactivity, characterized by increased GFAP expression, is a general response to injury and inflammation in the adult brain (O'Callaghan, 1991). Early in the course of the disease, there is an increase in GFAP expression in the retinas of diabetic animals (Lieth *et al.*, 1998; Barber *et al.*, 2000; Rungger-Brandle *et al.*, 2000; Zheng *et al.*, 2004). Müller cells and astrocytes react differently in diabetes (Barber *et al.*, 2000). Diabetes reduces GFAP content in astrocytes, which normally exhibit high levels of GFAP in the retina, while markedly upregulates GFAP expression in Müller cells, which express low levels of GFAP under normal conditions (Mizutani *et al.*, 1998; Barber *et al.*, 2000). Further, alterations in the redistribution of GFAP in astrocytes and Müller cells were concomitant with a reduction and redistribution of occludin in retinal endothelial cells in diabetic rat retinas (Barber *et al.*, 2000).

Diabetes also induces microglia reactivity. It was reported that the number of microglial cells is increased in the retinas of rats four weeks after the onset of diabetes (Rungger-Brandle *et al.*, 2000; Zeng *et al.*, 2000). Also, alterations in the morphology of these cells were accompanied by an increased expression of OX-42, a marker for microglial reactivity. Activated microglia exhibit strongly enhanced proliferation, migration, phagocytosis, and production of many different bioactive molecules. In fact, diabetes increases the expression of mRNAs for IL-1β and TNF-α, two proinflammatory cytokines that can be produced by microglia as a consequence of its activation (Krady *et al.*, 2005). Minocycline is a second-generation tetracycline with anti-inflammatory properties, and is known to inhibit the proliferation and activation of microglia (Tikka *et al.*, 2001). Minocycline administration to diabetic rats repressed the increase in the proinflammatory cytokines as well as caspase-3 activity, thus supporting a role of microglial cells in diabetic retinopathy (Krady *et al.*, 2005). More recently, it was reported that microglia activation occurs concomitantly with alterations in the electroretinogram in diabetic mice treated with insulin (Gaucher *et al.*, 2007), in the absence of neuronal cell death or upregulation of GFAP expression, suggesting that microglia activation may precede neuronal degeneration and macroglial activation. Furthermore, a recent report showed that microglia cells are hypertrophic and their number is increased in the retinas of patients with proliferative diabetic retinopathy (Zeng *et al.*, 2008). These cells clustered around the retinal vasculature, especially near

microaneurysms, intraretinal hemorrhages, and retinal neovascularization, suggesting that microglia might have a role in neovascularization as well.
In conclusion, the evidences presented above demonstrate that glial cells are critical for the BRB integrity maintenance and that glial dysfunction induced by diabetes contributes to increased retinal vascular permeability.

1.4.1.4 Retina neuronal dysfunction in diabetes

Diabetic retinopathy is generally characterized as a retinal microvascular dysfunction, with pericyte and endothelial cell loss, increase in vessel permeability, retinal capillary occlusion and nonperfusion, leukostasis, and ultimately, neovascularization. The fact that microvasculature lesions are detectable ophthalmoscopically has led to the general assumption that diabetic retinopathy affects primarily the retinal microvasculture. However, recent findings indicate that diabetes also affects the neuronal and glial cells of the retina (Barber *et al.*, 1998; Abu-El-Asrar *et al.*, 2004; Santiago *et al.*, 2006b; Santiago *et al.*, 2007) and alterations occurring in each cell type will certainly affect other cells. In fact, histopathological studies performed four decades ago have already shown the loss of neurons, especially in the inner retina, during the course of diabetic retinopathy (Wolter, 1961; Bloodworth, 1962). Moreover, an increase in apoptosis of retinal neurons, mainly ganglion cells, was observed in diabetic animals (Hammes *et al.*, 1995; Barber *et al.*, 1998; Abu-El-Asrar *et al.*, 2004) and patients (Bek, 1994; Kerrigan *et al.*, 1997; Barber *et al.*, 1998; Lieth *et al.*, 2000). Studies using electroretinography, dark adaptation, contrast sensitivity, and color vision tests have demonstrated that neuroretinal function is compromised as early as two weeks after the onset of diabetes in diabetic animals and prior to the development of detectable vascular lesions in humans (Greenstein *et al.*, 1992; Lieth *et al.*, 2000; Parisi and Uccioli, 2001; Li *et al.*, 2002; Bearse *et al.*, 2004). Furthermore, loss of oscillatory potentials on electroretinograms predict the onset of proliferative diabetic retinopathy better than vascular lesions evaluation by fundus photography or fluorescein angiograms (Bresnick and Palta, 1987). Several evidences suggest that glutamate, the main neurotransmitter in the retina may be involved in retinal neurodegeneration in diabetic retinopathy. A recent study has shown that high glucose and diabetes increase the evoked release of D-aspartate in retinal neural cells and diabetic rat retinas, respectively, suggesting that the extracellular levels of glutamate might be increased and therefore exert an excitotoxic effect on retinal neural cells (Santiago *et al.*, 2006a). It was also reported that high glucose

alters the content of glutamate receptor subunits, which may contribute to impaired glutamatergic neurotransmission, and affects calcium homeostasis in retinal neural cells, which may cause cell death (Santiago *et al.*, 2006b). Indeed, elevated glucose induces apoptosis in cultured retinal neural cells. The increase in apoptosis is independent of caspase activation, but it is correlated with the translocation of the apoptosis inducing factor (AIF) from the mitochondria to the nucleus, where it may cause DNA fragmentation (Santiago *et al.*, 2007).

Overall, all retinal cell types are affected by diabetes. Regardless of whether the initial alterations occur first in vascular or neural cells, it is clear that vascular dysfunction and neuronal degeneration are interdependent processes. It is therefore crucial to understand and unravel the mechanisms underlying cell dysfunction in diabetes for the design of specific therapeutic strategies to prevent the progression of the disease and its complications.

1.4.2 Inflammation in diabetic retinopathy

A growing body of evidence indicates that diabetic retinopathy has several features of a chronic and low-grade inflammatory disease, which includes increased expression of cytokines and adhesion molecules, increased leukocyte adhesion, increased vascular permeability and microglia activation (Mohr, 2004; Antonetti *et al.*, 2006; Kern, 2007). In addition, it has been shown that these inflammatory events occur early in the course of the disease (Carmo *et al.*, 1999; Miyamoto *et al.*, 1999; Rungger-Brandle *et al.*, 2000; Joussen *et al.*, 2001a).

It has been demonstrated that several genes involved in inflammatory processes are upregulated early in the diabetic rat retinas (Joussen *et al.*, 2001a). Increased levels of cytokines, such as IL-1β and TNF-α, have been shown in vitreous fluid of diabetic patients (Abu el Asrar *et al.*, 1992; Demircan *et al.*, 2006; Patel *et al.*, 2008) and in the retinas of diabetic rats (Carmo *et al.*, 1999; Joussen *et al.*, 2002a; Kowluru and Odenbach, 2004b; Gerhardinger *et al.*, 2005; Krady *et al.*, 2005).

The increase of IL-1β in diabetic rat retinas has been correlated with an increase in BRB permeability. Further, the levels of IL-1β decreased in the retinas of diabetic rats treated with cyclosporine A, an anti-inflammatory drug, which correlated with a decreased in the BRB permeability (Carmo *et al.*, 1999; Carmo *et al.*, 2000). Experimental studies showed that intravitreal administration of IL-1β increased vascular permeability which appears to be mediated by leukocyte adhesion, NF-κB activation and retinal capillary cell death (Bamforth *et al.*, 1997; Kowluru and Odenbach, 2004b). Furthermore, inhibition of caspase-1, the enzyme

responsible for IL-1β production, by minocycline inhibited the diabetes-induced increase in IL-1β and decreased the degeneration of retinal capillaries in diabetic and galactosemic mice (Vincent and Mohr, 2007). TNF-α, another proinflammatory cytokine, has also been implicated in the pathogenesis of diabetic retinopathy. In fact, susceptibility to diabetic retinopathy has been associated with a TNF-α gene polymorphism (Hawrami *et al.*, 1996). Retinal TNF-α levels are increased in diabetic patients, specially in patients with proliferative diabetic retinopathy (Limb *et al.*, 1996; Schram *et al.*, 2005; Demircan *et al.*, 2006; Gustavsson *et al.*, 2008). Elevated levels of TNF-α were also detected in the retinas of diabetic rats (Joussen *et al.*, 2002a; Krady *et al.*, 2005; Behl *et al.*, 2008). Moreover, TNF-α increases the adhesion of leukocyte to the retinal endothelium (Ben-Mahmud *et al.*, 2004) and the permeability of BRB (Saishin *et al.*, 2003). The inhibition of TNF-α, with etanercept, a soluble TNF-α receptor, inhibits NF-κB activation, leukostasis, and the BRB breakdown in the diabetic retina (Joussen *et al.*, 2002a). All together, these evidences indicate that proinflammatory cytokines play an important role in the progression of diabetic retinopathy and therefore can be seen as potential targets for the treatment of this disease.

1.4.2.1 Inflammatory cytokines

Proinflammatory cytokines are known to induce the expression of cytokines, chemokines and adhesion molecules, the recruitment of immune cells to the parenchyma and the activation of immune cells and endogenous glial cells, therefore playing an important role in inflammatory processes (Rothwell and Luheshi, 2000).

Interleukin-1β

IL-1β is a major mediator of the pathogenesis of acute and chronic inflammatory diseases (Rothwell *et al.*, 1997; Tringali *et al.*, 2000). IL-1β upregulates several inflammatory mediators, including IL-1β itself, TNF-α, cyclooxygenase 2 (COX-2), prostaglandins, iNOS and chemokines (Chung and Benveniste, 1990; Sparacio *et al.*, 1992; Chai *et al.*, 1996; Rothwell and Luheshi, 2000). IL-1β is primarily synthesized as an immature 31 kDa precursor called pro-IL-1β. This pro-IL-1β is processed to IL-1β (17.5 kDa) by proteolytic cleavage by the interleukin-1 converting enzyme (ICE or caspase-1) (Thornberry *et al.*, 1992). Cells of the monocyte-macrophage lineage are the main cellular source of IL-1β. However, a wide range of cells produce this cytokine, including microglia, endothelial cells and fibroblasts (Tocci and Schmidt,

1997). Within the retina, endothelial cells, glial and microglial cells constitute the major source of IL-1β (Hangai *et al.*, 1995; Morigiwa *et al.*, 2000; Kowluru and Odenbach, 2004c; Gerhardinger *et al.*, 2005).

IL-1β has been show to bind to two specific transmembrane receptors: interleukin-1 type I receptor (IL-1RI) and interleukin-1 type II receptor (IL-1RII). However, IL-1β elicits responses in cells only through the activation of IL-1RI and the IL-1R accessory protein (IL-1RAcP). This receptor accessory protein is a 66 kDa glycoprotein that does not bind to IL-1β (Wesche *et al.*, 1998) but increases the IL-1RI binding affinity for IL-1 and is essential for signal transduction (Sims *et al.*, 1993; Cullinan *et al.*, 1998). IL-1RII acts as a decoy receptor and does not lead to intracellular signaling processes, thus limiting the action of IL-1β. The relative level of expression of IL-1RI and IL-1RII varies considerably between different cell types. IL-1RI is a 80 kDa glycoprotein predominantly expressed by T lymphocytes and fibroblasts (Dower *et al.*, 1985; Mizel *et al.*, 1987) but also by endothelial cells ((Boraschi *et al.*, 1991)). In contrast, IL-1RII, a 68 kDa glycoprotein, is highly expressed in hematopoietic cells such as monocytes, neutrophils and B lymphocytes (Sims and Dower, 1994). In the retina, IL-1RI is mainly expressed in the inner retina, specially in retinal ganglion cells and Müller cells (Diem *et al.*, 2003; Namekata *et al.*, 2008).

IL-1 receptors belong to the interleukin-1 receptor (IL-1R)/Toll-like receptor (TLR) superfamily, characterized by the presence of an intracellular Toll/IL-1R (TIR) domain. The TIR domain is necessary for the recruitment of other TIR domain containing proteins in the signal transduction cascade (Wesche *et al.*, 1997; Burns *et al.*, 1998). The IL-1R subfamily members, such as IL-1RI, IL-1RII and IL-1RAcP, are defined by the presence of three immunoglobulin-like domains in the extracellular region of the receptors, involved in protein-ligand and protein-protein interactions.

The IL-1β binding to IL-1RI causes the recruitment of IL-1RAcP, and this receptor complex then recruits the intracellular TIR domain-containing adapter protein MyD88 which then recruits the serine/threonine kinases IL-1R-associated kinase (IRAK). IRAK interacts with tumor necrosis receptor associated factor-6 (TRAF6) to activate several downstream protein kinases that ultimately lead to NF-κB and c-Jun N-terminal kinase (JNK) activation, which are involved in survival and inflammatory responses. IL-1RI also activates other MAPK, such as p38 and extracellular signal-regulated activated kinase 1 and 2 (ERK1/2), as well as PI_3K (O'Neill and Greene, 1998; Subramaniam *et al.*, 2004).

Tumor necrosis factor-α

TNF-α, like IL-1β, is a pleiotropic proinflammatory cytokine that plays a central role in inflammation, apoptosis, and immune system development. TNF-α is known to induce the expression of several inflammatory mediators such as COX-2 (Mark *et al.*, 2001), ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1) (Pober *et al.*, 1986; Munro *et al.*, 1989), monocyte chemoattractant protein-1 (MCP-1) and IL-8 (Rollins *et al.*, 1990). TNF-α also induces vascular endothelial dysfunction (Bradley and Pober, 1996; Pober, 1998).

TNF-α is produced initially as a 26 kDa monomeric transmembrane protein that is posteriorly assembled intracellularly to form a noncovalent linked homotrimer, which is inserted in the plasma membrane (Tang *et al.*, 1996). Cleavage of membrane bound TNF-α by the matrix metalloproteinase TNF-α converting enzyme (TACE) releases a 55 kDa soluble trimeric form of TNF-α (Black *et al.*, 1997). Both membrane-associated and soluble forms are active in their trimeric forms. Although cells of the monocyte/macrophage lineage are the main source of TNF-α in an inflammatory condition, a wide range of cells can produce TNF-α, including endothelial cells, muscle cells and fibroblasts (Wajant *et al.*, 2003). Within the retina TNF-α has been shown to produced mainly in the inner layers of the retina, by endothelial cells, Müller cells, microglia and ganglion cells (Cotinet *et al.*, 1997; Tezel *et al.*, 2001; Yoshida *et al.*, 2004; Fuchs *et al.*, 2005; Berger *et al.*, 2008).

The effects of TNF-α on target cells are triggered by the activation of specific membrane glycoprotein receptors: TNF receptor type 1 (TNFR1 or p55; 55 kDa) and TNF receptor type 2 (TNFR2 or p75; 75 kDa) (Wallach *et al.*, 1999). TNFR1 is expressed in most cell types, and can be activated by binding of either soluble TNF-α or membrane-bound TNF-α, with a preference for the soluble form; whereas TNFR2 is expressed primarily by cells of the immune system and endothelial cells, and is preferentially activated by the membrane-bound TNF-α (Grell, 1995; Grell *et al.*, 1998b). Both receptors are also expressed as homotrimers (Idriss and Naismith, 2000; Hector *et al.*, 2007). TNF-α exerts its biological actions primarily through the activation of TNFR1, which in the retina is expressed mainly in the inner layers, especially in the GCL (Tezel *et al.*, 2001; Fuchs *et al.*, 2005). Signaling through TNFR1 is very complex, leading to inflammation, survival and programmed cell death activation. TNFR1 contains a cytoplasmic death domain (DD) characteristic of many members of the TNF superfamily. In resting conditions, TNFR1 is associated with a cytoplasmic protein designated silencer of death domain (SODD), that is thought to prevent constitutive activation of TNFR1 (Jiang *et al.*, 1999). TNF-α

binding to TNFR1 results in SODD dissociation and subsequent binding of TNF receptor associated death domain (TRADD). TRADD acts as a scaffold that allows the recruitment of other adaptor proteins including receptor interacting protein (RIP), a serine-threonine kinase which binds TRADD through its own DD and TNF receptor associated factor 2 (TRAF2), an E3 ubiquitin ligase that does not contain a DD. This complex then leads to activation of NF-κB and JNK signaling to initiate pro-survival signaling, cellular proliferation, and inflammatory responses. Similarly to IL-1RI, TNFRI also activates ERK1/2, p38 and PI_3K signaling pathways (Bradley *et al.*, 1995; Madge and Pober, 2001). TNFR1 can also initiate cell death signaling pathways, in addition to the activation of cell surviving and inflammatory signals through NF-κB and activator protein-1 (AP-1). This involves the binding of Fas-associated DD protein (FADD) to TRADD and the subsequent recruitment of pro-caspase-8. Autocatalytic activation of bound pro-caspase-8 releases activated caspase-8 which triggers the activation of executioner caspases such as caspase-3 to initiate apoptosis (Micheau and Tschopp, 2003; Schneider-Brachert *et al.*, 2004).

The consequences of TNFR2 signaling are less well characterized, but TNFR2 has also been shown to induce cell proliferation (Tartaglia *et al.*, 1991; Grell *et al.*, 1998a), cell survival (Horie *et al.*, 1999; Fontaine *et al.*, 2002; Marchetti *et al.*, 2004) as well as inflammatory responses through the recruitment of TRAF1 and TRAF2 adaptor proteins and subsequent activation of the NF-κB pathway (Rao *et al.*, 1995; Rothe *et al.*, 1995). TNFR2 has also been shown to activate the endothelial/epithelial tyrosine kinase (Etk), a cytosolic kinase involved in cell survival and proliferation. Etk mediates TNF-α-induced PI_3K activation in vascular endothelial cells through Etk-mediated cross-talk with VEGFR2 (Zhang *et al.*, 2003). Other reports suggest its involvement in death signaling. Although TNFR2 lacks the DD it enhances the association between TNF-α and TNFR1 via a ligand passing mechanism (Tartaglia *et al.*, 1993). However, other reports suggest that TNFR2 contributes to cell death independently of TNFR1 *via* interaction with RIP (Pimentel-Muinos and Seed, 1999; Cusson *et al.*, 2002). Overall, TNFR2 activation is believed to initiate primarily proinflammatory and pro-survival signaling.

1.4.2.2 Nuclear factor-κB

IL-1β and TNF-α, through the activation of their receptors, initiate a series of signal transduction events that lead to the activation of NF-κB (Karin and Ben-Neriah, 2000; Ghosh and Karin, 2002). NF-κB was first identified about 20 years ago as a transcription factor that binds to the

enhancer of the kappa light chain gene (the κB site) in B cells (Sen and Baltimore, 1986). Soon thereafter, NF-κB emerged as a major regulator of immune and inflammatory responses (Baldwin, 1996). NF-κB is composed of homo or heterodimmers of different members of the Rel protein family: RelA (p65), c-Rel, RelB, NF-κB1 (p50) and NF-κB2 (p52). NF-κB1 and NF-κB2 are synthesized as precursor molecules, p105 and p100, respectively, and the processing of these precursors is needed to generate mature transcription factors. Only RelA, c-Rel and RelB possess C-terminal transactivation domains and thus only dimmers containing one of these proteins activate efficiently NF-κB-dependent transcription. All Rel proteins share the N-terminal Rel homology domain (RHD) mediating dimerization, nuclear localization and DNA binding.

In resting conditions, NF-κB is constitutively sequestered in the cytosol by the inhibitory NF-κB proteins (IκB), such as IκBα and IκBβ. IκB proteins mask the nuclear localization signal sequence present in NF-kB molecules and thereby prevent their nuclear translocation and activity. The canonical and most common form of NF-κB is the heterodimer of p50 and RelA that is regulated by IκBα. Upon cell activation by IL-1β and TNF-α, multiple signaling pathways converge on the trimeric IκB kinase (IKK) complex, comprising two catalytic subunits, IKKα, IKKβ, and a regulatory subunit IKKγ, also called NEMO (NF-κB essential modulator). The activated IKK complex phosphorylates IκBα on Ser32 and Ser36 leading to IκBα ubiquitination and proteasome-dependent degradation. IκBα degradation releases NF-κB which translocates to the nucleus and activates gene transcription. NF-κB is an important transcription factor controlling the expression of an array of inflammatory response genes including cytokines, chemokines and adhesion molecules (Baldwin, 1996; Neumann and Naumann, 2007).

NF-κB has been shown to be activated in the retinal vasculature of diabetic patients and animal models (Joussen *et al.*, 2002b; Kowluru *et al.*, 2003). Exposure of bovine retinal endothelial cells and pericytes to high glucose has also been shown to induce NF-κB activation (Romeo *et al.*, 2002; Kowluru *et al.*, 2003). The activation of NF-κB in both diabetic rat retinas and endothelial cells and pericytes exposed to high glucose was inhibited by anti-inflammatory drugs, such as aspirin (Joussen *et al.*, 2002a), and antioxidant treatment (Kowluru *et al.*, 2003). Also, the administration of curcumin, known to have antioxidant and anti-inflammatory properties, prevents diabetes-induced retinal oxidative stress, NF-κB activation and the increase in proinflammatory markers, such as IL-1β and VEGF (Kowluru and Kanwar, 2007). In addition, AGE-induced oxidative stress and NF-κB activation, concomitant with retinal capillary endothelial cell apoptosis, has also been prevented by antioxidant therapy (Kowluru, 2005) or

PEDF administration (Yamagishi *et al.*, 2007). These evidences suggest that NF-κB has an important role in the pathogenesis of diabetic retinopathy.

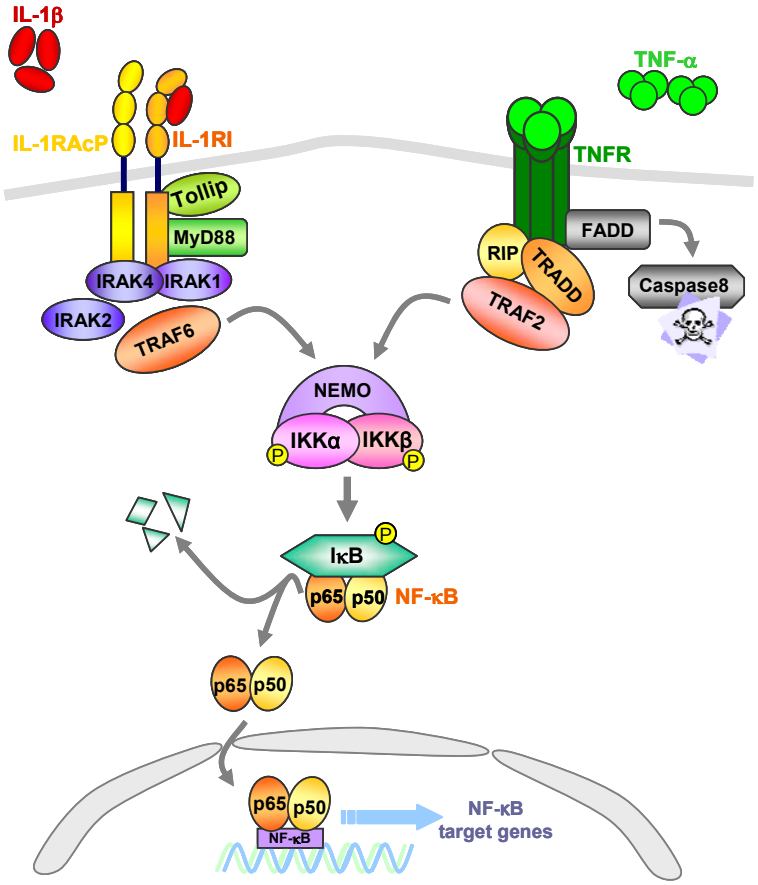

Figure 1.14. Signal transduction pathways of IL-1RI and TNFR1. Upon cell activation by IL-1β and TNF-α, several adaptor proteins are recruited to the receptor complex to converge on the inhibitory NF-κB protein kinase (IKK) complex. The activated IKK complex phosphorylates the inhibitory NF-κB protein (IκB) leading to IκB ubiquitination and proteasome-dependent degradation. IκB degradation releases the transcription factor NF-κB which translocates to the nucleus where it activates the transcription of inflammatory genes.

1.4.3 Anti-inflammatory therapy in diabetic retinopathy

Given the inflammatory nature of diabetic retinopathy, anti-inflammatory drugs constitute an attractive therapeutic tool for the prevention of this disease. Indeed, treatment of diabetic rats with high dose of nonsteroidal anti-inflammatory drugs, such as aspirin and meloxican, a COX-2 inhibitor, or intravitreal injection of the glucocorticoid dexamethasone, reduced retinal ICAM-1 expression and the adhesion of leukocytes to retinal vessels, and inhibited the BRB breakdown (Joussen *et al.*, 2002a; Tamura *et al.*, 2005), clearly indicating that anti-inflammatory therapeutic strategies may be used to treat diabetic retinopathy.

Glucocorticoids are widely used anti-inflammatory drugs and are under investigation for the treatment of diabetic retinopathy and other retinal vascular diseases. Several clinical trials have shown that intravitreal injection of triamcinolone acetonide or dexamethasone reduces macular thicknening and improves visual acuity in patients with diabetic macular edema (Massin *et al.*, 2004; Sutter *et al.*, 2004; Munir *et al.*, 2005; Kuppermann *et al.*, 2007). However, glucocorticoid treatment is often associated with several adverse effects such as cataract formation and elevated intraocular pressure, which leads to glaucoma (Moshfeghi *et al.*, 2003; Ciardella *et al.*, 2004; Gillies *et al.*, 2006). Glucocorticoids have also been used to preserve barrier properties and thereby decrease vascular permeability, particularly in the treatment of brain edema in response to tumor formation (Ruderman and Hall, 1965; Kaal and Vecht, 2004). Recently it has been shown that glucocorticoids reduce retinal endothelial cell permeability concomitant with an increase in claudin-5 and occludin expression, phosphorylation of occludin and promotion of tight junction assembly (Antonetti *et al.*, 2002; Felinski *et al.*, 2008). Further understanding of the mechanisms by which glucocorticoids exert their pro-barrier effects would be beneficial for the identification of new specific targets for controlling retinal vascular permeability.

1.4.3.1 Glucocorticoids

Glucocorticoids are secreted by the cortex of adrenal gland under the regulation of the hypothalamus-pituitary gland axis. Glucocorticoids are essential for normal development and exert influence on various metabolic and immune defense processes. Synthetic glucocorticoids, such as dexamethasone, are well established and widely used immunosuppressive and anti-inflammatory drugs. Glucocorticoids have been proven to exert beneficial effects in different autoimmune diseases and in a plethora of disorders with an inflammatory component, such as chronic allergic diseases, asthma, rheumatoid arthritis, or Crohn’s disease.

Glucocorticoids elicit their biological actions via interaction with the glucocorticoid receptor (GR), a member of the nuclear receptor superfamily of proteins that includes receptors for mineralocorticoids, progesterone, estrogen, thyroid hormones, vitamin D and retinoic acid (Robinson-Rechavi *et al.*, 2001). Due to their lipophilic nature, glucocorticoids rapidly diffuse through the plasma membrane by a passive process and bind directly to the intracellular GR. The GR is maintained inactive in the cytoplasm as a complex with heat shock proteins, including hsp90 and hsp70, a low molecular weight protein p23 and several immunophilins (Dittmar *et al.*, 1997). Upon ligand binding, the GR undergoes a conformational change that allows the dissociation from the multimeric protein complex, thereby exposing its nuclear localization signals and allowing rapid translocation to the nucleus. Once in the nucleus, ligand-receptor complex modulates gene expression either by binding DNA binding-dependent transcription or DNA binding-independent transcription mediated by protein-protein interaction with other transcription factors (Figure 1.11).

Transcriptional activation (transactivation)

GR activates gene transcription by binding as a homodimer to glucocorticoid response elements (GREs) within the promoter region of target genes (Lieberman and Nordeen, 1997; Barnes, 1998). Binding of GR to a GRE results in a conformational change in GR that promotes the recruitment of several coactivators to the GR-DNA complex, such as steroid receptor coactivator-1 (SRC-1) and cyclic adenosine monophosphate (cAMP) response element binding protein (CREB)-binding protein (CBP), and the basic transcription machinery (Barnes, 1998; Kassel *et al.*, 2001). Both the number of GREs and their relative proximity to the TATA box appear to be determinants of the glucocorticoids inducibility of gene expression (Jantzen *et al.*, 1987; Wieland *et al.*, 1990). Glucocorticoids are known to increase the synthesis of several anti-inflammatory proteins, including lipocortin-1, IL-1 receptor antagonist, IL-10 and MAPK phosphatase-1 (MKP-1) (Barnes, 1998; Kassel *et al.*, 2001).

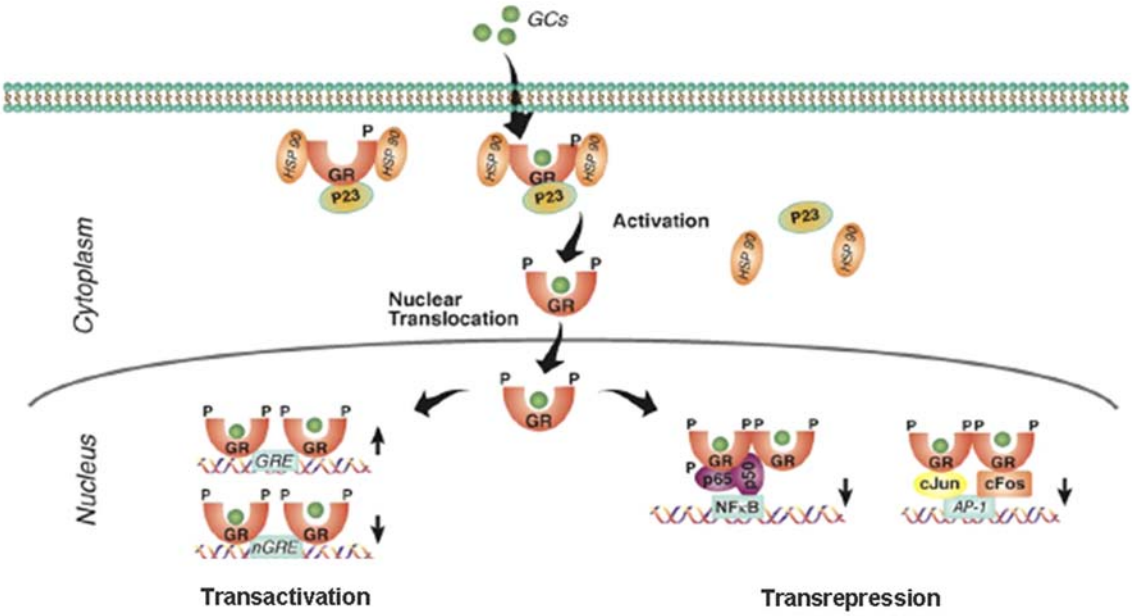

Figure 1.15. The glucocorticoid receptor signaling pathway. The inactive glucocorticoid receptor (GR) is sequestered in the cytoplasm by chaperones until it becomes an activated transcription factor upon binding to ligand. GR dissociates from the multimeric protein complex and translocates to the nucleus. The ligand-receptor complex can stimulate transcriptional responses by binding to glucocorticoids response elements (GREs). In contrast, GR binding to negative GREs (nGREs) results in repressed transcription. (adapted from Smoak and Cidlowski, 2004).

Transcription repression (transrepression)

As opposed to the 'positive' GRE, functional negative GREs (nGRE) have been identified in the promoter regions of several genes including the pro-opiomelanocortin and corticotrophin-releasing hormone genes; the two main peptide hormones of the hypothalamus-pituitary-adrenal axis (Drouin *et al.*, 1998; Malkoski and Dorin, 1999). The direct binding of the GR to the nGREs results in the repression of the expression of the target gene and constitutes an additional mechanism by which glucocorticoids regulate gene transcription. However, the majority of inflammatory genes that are downregulated by glucocorticoids lack nGREs in their promoters. In fact, anti-inflammatory action of glucocorticoids is mostly associated with transrepression of inflammatory genes expression through direct protein-protein interaction with others transcription factors, such as AP-1 (Jonat *et al.*, 1990) and NF-κB (Scheinman *et al.*, 1995), blocking their activity independent of GR binding to DNA (Reichardt *et al.*, 1998). This mechanism leads to the repression of a number of inflammatory proteins that are relevant to

inflammatory diseases such as IL-1β, IL-6, IL-8, TNF-α, iNOS, COX-2, ICAM-1 and VCAM-1 (Almawi and Melemedjian, 2002), all know to be regulated by NF-κB (Baldwin, 1996).

GR represses NF-κB transcriptional activity by physically interacting with the RelA subunit (Ray and Prefontaine, 1994; Scheinman *et al.*, 1995) inhibiting it's binding to DNA. Additionally, glucocorticoids may also repress NF-κB transcriptional activity through induction of IκB (De Bosscher *et al.*, 2003), which could lead to the sequestration of NF-κB in the cytoplasm. Further, it has been demonstrated that glucocorticoids interfere with PI_3K modulation of IKK activity, resulting in reduced NF-κB activity (Leis *et al.*, 2004).

1.5 Objectives of the present study

A growing body of evidences indicates that diabetic retinopathy has several features of a chronic and low-grade inflammatory disease. The inflammatory events occur early in the course of the disease and include increased expression of cytokines and growth factors, microglia activation, increased leukocyte adhesion to retinal vessels and increased retinal vascular permeability. It has also been demonstrated that proinflammatory cytokines, in addition to VEGF, contribute to vascular permeability in diabetic retinopathy. However, the mechanisms by which proinflammatory cytokines alter retinal endothelial permeability have not been explored yet.

Therefore, the main goal of this study was to evaluate the effect of IL-1β and TNF-α in retinal endothelial cell permeability and investigate the molecular mechanisms by which TNF-α increases retinal endothelial cell permeability. Further, the ability of the glucocorticoid dexamethasone and the novel PKCζ inhibitor-1 (PKCζI-1) to prevent TNF-α-induced retinal endothelial cell permeability was also evaluated.

Although retinal endothelial cells appear to be primarily affected by IL-1β and TNF-α, little attention has been given to the regulation of their cognate receptors, such as IL-1RI, which in this case could alter the responsiveness of endothelial cell to IL-1β. In this context, as IL-1RI provides a crucial locus of control of IL-1β activity, we also investigated the effect of high glucose and IL-1β on the regulation of the content of IL-1RI in retinal endothelial cells.

CHAPTER 2

TNF-α signals through PKCζ/NF-κB to alter the tight junction complex and increase retinal endothelial cell permeability

CHAPTER 2

TNF-α signals through PKCζ/NF-κB to alter the tight junction complex and increase retinal endothelial cell permeability

2.1 Abstract

Tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) are elevated in the vitreous of diabetic patients and in diabetic rat retinas, and these findings have been associated with increased retinal vascular permeability and angiogenesis. However, the molecular mechanisms underlying blood-retinal barrier (BRB) deregulation by these cytokines are poorly understood. In this study, the effects of IL-1β and TNF-α on retinal endothelial cell permeability were compared and the molecular mechanisms by which TNF-α increases cell permeability elucidated.

IL-1β and TNF-α increased bovine retinal endothelial cell permeability in a concentration- and time-dependent manner, but TNF-α was more effective. TNF-α decreased the protein and mRNA content of the tight junction proteins *zonula occludens*-1 (ZO-1) and claudin-5, but increased occludin expression, and altered the cellular localization of these tight junction proteins. The glucocorticoid dexamethasone completely prevented TNF-α-induced cell permeability, and this effect was mediated through both transactivation of the glucocorticoid receptor and transrepression of nuclear factor-κB (NF-κB) signaling pathway. Preventing NF-κB activation with an inhibitory NF-κB protein kinase (IKK) chemical inhibitor or adenovirus-mediated overexpression of inhibitory NF-κB protein α (IκBα) also reduced TNF-α-induced cell permeability. Finally, inhibiting protein kinase C ζ (PKCζ) using both a peptide and a novel chemical inhibitor, reduced NF-κB activation, and completely prevented the alterations in the tight junction complex and cell permeability induced by TNF-α in cell culture and rat retinas.

These results suggest that PKCζ may provide a specific therapeutic target for the prevention of vascular permeability in retinal diseases characterized by elevated TNF-α, including diabetic retinopathy.

2.2 Introduction

Retinal microvasculature dysfunction occurs early in the development of diabetic retinopathy, and alterations in the blood-retinal barrier contribute to the formation of lipid exudates, hemorrhages and vasogenic edema (Cunha-Vaz *et al.*, 1975; Frank, 2004; Antonetti *et al.*, 2006). Retinal edema, along with the formation of new blood vessels in the later stages of the disease, is directly associated with visual impairment (Klein *et al.*, 1995a; Moss *et al.*, 1998). Diabetes induces changes in the retinal pigment epithelium, affecting the outer retina, and the retinal blood vessels in the inner retina, although changes in the inner retinal blood vessels appear to occur first in diabetic patients and diabetic animal models (Vinores *et al.*, 1989; Carmo, 1998). The role of VEGF as a factor contributing to the increase in retinal vascular permeability and angiogenesis is well established in both animal models and in humans with proliferative diabetic retinopathy (Aiello *et al.*, 1997; Caldwell *et al.*, 2003).

A growing body of evidence indicates that diabetic retinopathy has several features of a chronic inflammatory disease. The inflammation begins early in the course of the disease and includes increased expression of cytokines and growth factors, microglia activation, increased leukocyte adhesion to retinal vessels and increased vascular permeability (Barber *et al.*, 1998; Carmo *et al.*, 1999; Miyamoto *et al.*, 1999; Rungger-Brandle *et al.*, 2000; Krady *et al.*, 2005). Increased levels of proinflammatory cytokines, such as IL-1β and TNF-α, have been found in the vitreous fluid of diabetic patients (Abu el Asrar *et al.*, 1992; Yuuki *et al.*, 2001; Demircan *et al.*, 2006) and in the retinas of diabetic rats (Carmo *et al.*, 1999; Joussen *et al.*, 2002a; Kowluru and Odenbach, 2004b; Gerhardinger *et al.*, 2005; Krady *et al.*, 2005). The expression of IL-1β decreases in the retinas of diabetic rats treated with cyclosporine A, an anti-inflammatory drug, which correlates with a decrease in the BRB permeability (Carmo *et al.*, 2000). Indeed, intravitreal administration of IL-1β increases vascular permeability, which is associated with increased leukocyte adhesion, NF-κB activation and retinal capillary cell death (Bamforth *et al.*, 1997; Kowluru and Odenbach, 2004b). TNF-α has also been implicated in the pathogenesis of diabetic retinopathy. TNF-α content is increased in the vitreous of diabetic patients, specifically in patients with proliferative diabetic retinopathy (Schram *et al.*, 2005; Demircan *et al.*, 2006; Gustavsson *et al.*, 2008), and in the retinas of diabetic rats (Joussen *et al.*, 2002a; Krady *et al.*, 2005; Behl *et al.*, 2008). Moreover, TNF-α increases leukocyte adhesion to retinal endothelium (Ben-Mahmud *et al.*, 2004) and BRB permeability (Saishin *et al.*, 2003). The inhibition of TNF-α, with etanercept, a soluble TNF-α receptor, inhibits leukostasis, NF-κB activation and BRB

breakdown in the diabetic retina (Joussen *et al.*, 2002a). All together, these data indicate that proinflammatory cytokines, in addition to vascular endothelial growth factor (VEGF), contribute to vascular permeability in diabetic retinopathy. However, the mechanisms by which cytokines alter retinal vascular endothelial permeability have not been addressed yet.

Given the inflammatory nature of diabetic retinopathy, anti-inflammatory drugs constitute an attractive therapeutic option for the treatment of this disease. Indeed, treatment of diabetic rats with high doses of nonsteroidal anti-inflammatory drugs, such as aspirin and meloxican, a cyclooxygenase 2 (COX-2) inhibitor, or intravitreal injection of dexamethasone, reduced the adhesion of leukocytes to retinal vessels, retinal intercellular adhesion molecule-1 (ICAM-1) expression and inhibited the BRB breakdown (Joussen *et al.*, 2002a; Tamura *et al.*, 2005). Glucocorticoids are widely used as anti-inflammatory drugs and are under investigation for the treatment of diabetic retinopathy and other retinal vascular diseases, but their mechanisms of action are not completely elucidated. Further understanding of the mechanisms by which glucocorticoids exert their pro-barrier effects would be beneficial for the development of new specific targets to control retinal vascular permeability. Glucocorticoids have been used to preserve barrier properties and thereby decrease vascular permeability, particularly in the treatment of brain edema in response to tumor formation (Ruderman and Hall, 1965; Kaal and Vecht, 2004). Recently, it has been shown that glucocorticoids reduce retinal endothelial cell permeability concomitant with an increase in claudin-5 and occludin expression, phosphorylation of occludin and promotion of tight junction assembly (Antonetti *et al.*, 2002; Felinski *et al.*, 2008). Several clinical trials have shown that intravitreal injection of triamcinolone acetonide or dexamethasone reduces macular thickening and improves visual acuity in patients with diabetic macular edema (Massin *et al.*, 2004; Sutter *et al.*, 2004; Munir *et al.*, 2005; Kuppermann *et al.*, 2007). However, glucocorticoid treatment is often associated with several adverse effects such as elevated intraocular pressure leading to glaucoma and cataract formation (Moshfeghi *et al.*, 2003; Ciardella *et al.*, 2004; Gillies *et al.*, 2006).

Changes in retinal vascular permeability may result from alterations of the tight junction complex. Tight junctions between endothelial cells of the retinal vessels, in the inner retina, and retinal pigment epithelial cells, in the outer retina, establish a highly selective barrier to water and solutes between the retinal parenchyma and blood circulation. Tight junctions are composed of a combination of more than 40 proteins including the transmembrane proteins occludin, the claudin family and the junction adhesion molecule (JAM) family, several peripheral

membrane-associated proteins, including members of the zonula occludens (ZO) family, and several regulatory proteins (Matter and Balda, 2003). While the transmembrane proteins, namely claudins and occludin, form the seal between adjacent cells and regulate barrier properties, the accessory proteins are multidomain cytoplasmic molecules necessary for the structural support of the tight junction complex, being also involved in signal transduction. To date evidence has been provided for the presence of occludin, claudin-5, ZO-1 and JAM-A in the retinal vascular endothelium (Gardner, 1995; Antonetti *et al.*, 1998; Barber and Antonetti, 2003; Tomi and Hosoya, 2004). Changes in occludin content, localization and phosphorylation occur in response to VEGF, and have been associated with increased endothelial permeability in cell culture and retinal vascular permeability *in vivo* (Antonetti *et al.*, 1998; Antonetti *et al.*, 1999a; Barber and Antonetti, 2003; Harhaj *et al.*, 2006). Recently, multiple VEGF-induced occludin phosphorylation sites have been identified and, one of these sites, Ser490, is VEGF responsive. Also, phosphorylation at this site reduces occludin interaction with ZO-1, which may lead to tight junction disruption (Sundstrom *et al.*, 2009). In addition to occludin, claudin-5 has been shown to be essential for blood-brain barrier function by gene deletion studies (Nitta *et al.*, 2003), and is likely to have a similar function in the BRB. Members of the ZO family are known to interact with the transmembrane proteins (Furuse *et al.*, 1994; Itoh *et al.*, 1999a) and act as scaffolds that link tight junctions to the actin cytoskeleton (Wittchen *et al.*, 1999; Fanning *et al.*, 2002). Recent studies demonstrated that ZO proteins are essential for the formation and organization of tight junction complex assembly (Umeda *et al.*, 2006; Katsuno *et al.*, 2008). Therefore, changes in occludin, claudin-5 and ZO-1 likely contribute to alterations in endothelial cell permeability.

In this study, the effectiveness of IL-1β and TNF-α to increase retinal endothelial cell permeability was compared and the molecular mechanisms by which TNF-α induces endothelial cell permeability were investigated.

2.3 Materials and methods

2.3.1 Reagents

Endothelial cell basal medium MCDB-131, fibronectin, epidermal growth factor, tylosin, microcystin, rhodamine-isothiocyanate (RITC)-conjugated 70 kDa dextran and Hoechst were purchased from Sigma-Aldrich (St. Louis, MO, USA). Endothelial cell growth medium additive (EndoGro) was from Vec Technologies (Rensselaer, NY, USA). Fetal bovine serum (FBS) was

from Hyclone (Logan, UT, USA). Heparin was from Fisher Scientific (Pittsburg, PA, USA). Antibiotic-antimycotic (penicillin, streptomycin, amphotericin B) was from GIBCO (Invitrogen, Carlsbad, CA, USA). Recombinant human TNF-α and IL-1β were purchased from R&D Systems (Minneapolis, MN, USA). Complete mini protease inhibitor cocktail tablets were from Roche (Indianapolis, IN, USA). Polyclonal rabbit anti-ZO-1, polyclonal rabbit anti-claudin-5, polyclonal rabbit anti-occludin and monoclonal mouse anti-occludin were from Zymed (Invitrogen). Anti-IκBα and anti-phosphoIκBα (Ser32) were from Cell Signaling Technology, Inc. (Danvers, MA, USA). Mouse monoclonal anti-actin was from Chemicon (Temecula, CA, USA). Horseradish peroxidase-linked goat anti-rabbit and anti-mouse IgG, alkaline phosphatase-linked goat anti-mouse IgG, enhanced chemifluorescence (ECF) and enhanced chemiluminescence (ECL Plus and ECL Advance) reagents were from Amersham (GE Healthcare, Piscataway, NJ, USA). All immunoblot NUPAGE gels and reagents were from Invitrogen. Goat serum, Cy2-conjugated donkey anti-mouse IgG and Cy3-conjugated donkey anti-rabbit IgG were purchased from Jackson Immunoresearch Laboratories (West Grove, PA, USA). Rabbit polyclonal anti-green fluorescent protein (GFP), Alexa Fluor 674-conjugated goat anti-rat IgG was from Molecular Probes (Invitrogen). The IKK complex inhibitor IKK VII, the phosphoinositide-3 kinase (PI_3K) inhibitor LY294002, the PKCζ pseudosubstrate inhibitor myristoylated, the PKCβ inhibitor and the conventional/novel PKC isoforms inhibitor bisindolylmaleimide I (BIM I) were purchased to Calbiochem (Gibbstown, NJ, USA). The PKCζ inhibitor 1 (PKCζI-1) is one of a series of phenyl-thiophene inhibitors identified from a chemical library screen (ChemBridge Corporation, San Diego, CA, USA).

2.3.2 Primary bovine retinal endothelial cell culture

Primary cultures of bovine retinal endothelial cells (BREC) were isolated as described previously (Antonetti and Wolpert, 2003). BREC were cultured in flasks coated with 1 μg/cm^2 fibronectin in MCDB-131 medium, supplemented with 10% FBS, 10 ng/ml epidermal growth factor, 0.2 mg/ml endothelial cell growth medium additive, 0.09 mg/ml heparin, 0.01 ml/ml antibiotic-antimycotic and 8 μg/ml tylosin, and maintained at 37ºC in a humid atmosphere of 5% CO_2/95% air. For experimentation, cells were used at passages 4 to 7 and plated at a seeding density of 50 x 10^3 cells/cm^2 on fibronectin substrate. When BREC reached confluence, media was changed to MCDB-131 medium supplemented with 1% FBS, 0.01 ml/ml antibiotic-antimycotic and 8 μg/ml tylosin for 1 day. Four hours after changing the medium, BREC were

exposed in a time- (0.5, 6 and 24 h) and concentration-dependent manner to IL-1β (1 to 100 ng/ml) and TNF-α (1 to 10 ng/ml).

2.3.3 Measurement of BREC permeability

To measure cell monolayer permeability, BREC were grown to confluence on 0.4 μm pore transwell filters (Corning Costar, Acton, MA, USA) and treated with IL-1β and TNF-α (added to both apical and basolateral chambers) for the time and concentration described above. After treatments, 70 kDa RITC-dextran was added to the apical chamber of the inserts. After 30, 60, 90, 120, 150, 180 and 210 min, 50 μl aliquots were taken from the basolateral chamber and placed in a 96-well black/clear bottom plate. Samples were also taken form the apical chamber at the last time point and placed in the 96-well plate. The RITC-dextran fluorescence was quantified with a fluorescence imager (Typhoon, GE Healthcare, Piscataway, NJ, USA) and the diffusive permeability (Po) was calculated as described previously (Antonetti *et al.*, 2002), using the following formula:

$$P\text{o} = [(F_A/\Delta t)\, V_A]/(F_L A)$$

where, Po is in centimeters per second; F_A is the basolateral fluorescence; F_L is the apical fluorescence; Δt is change in time; A is the surface area of the filter (in cm^2); and V_A is the volume of the basolateral chamber (in cm^3). The average Po for control conditions was 2.77×10^{-6} cm/s.

2.3.4 Animal model

Male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 150 to 175 g were used to evaluate retinal vascular permeability and tight junction proteins localization in retinal blood vessels. Animals were housed under a 12-hour light/dark cycle with free access to water and a standard rat chow. All experiments were conducted in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research, and were approved and monitored by the Institutional Animal Care and Use Committee (IACUC) at the Penn State College of Medicine. Under anesthesia (66.7 mg ketamine and 6.7 mg xylazine per kg body weight), the animal received an intravitreal injection of TNF-α or vehicle (2.5 μl/eye) with a 5 μl Hamilton syringe

(Hamilton Company, Reno, NV) through a puncture created by a 32-gauge needle. The animals were assessed for retinal permeability with Evans blue assay, 24 hours after receiving the intravitreal injection of either phosphate-buffered saline (PBS) with 1% bovine serum albumine (BSA), 10 ng TNF-α, 280 ng PKCζI-1, or TNF-α plus PKCzI-1. In a separate study, retinas were harvested 4 hours after injection, immunolabeled for tight junction proteins and analyzed by confocal microscopy.

2.3.5 *In vivo* measure of retinal vascular permeability

Retinal vascular permeability was quantified in male Sprague-Dawley rats by measuring albumin leakage from blood vessels into the retina using the albumin binding dye Evans blue following a documented protocol (Xu *et al.*, 2001), with minor modifications. Under anesthesia (66.7 mg ketamine and 6.7 mg xylazine per kg body weight), animals received injection of 45 mg per kg body weight of Evans blue (Sigma, St. Louis, MO) in saline via the femoral vein. After 2 hours, blood was drawn from the inferior vena cava to obtain the levels of Evans blue in plasma. Subsequently, the animal was perfused through the heart for 2 min with a citrate buffer (50 mM, pH 3.5, 37°C) containing 1% paraformaldehyde (Fisher, Pittsburgh, PA). Retinas were then harvested and dried overnight in a Savant Speed-Vac (Thermo Scientific, Waltham, MA). Evans blue was extracted by incubating each retina in 150 µl formamide (Sigma, St. Louis, MO) at 70°C overnight. The extract was transferred to an Ultrafree-MC filter tube (Millipore, Bedford, MA) and centrifuged at 5,000 x g for 2 hours at 4°C. Retina extract and diluted plasma samples were assayed in a Microcell cuvette with a Beckman DU640B spectrophotometer (Beckman, Fullerton, CA). A background-subtracted absorbance was determined by measurig each sample at both 620 nm, the absorbance maximum for Evans blue, and 740 nm, the absorbance minimum. The concentration of dye in each sample was calculated from a standard curve of Evans blue in formamide, with normalization to blood plasma after 2 h circulation and expressed as microliters per g retina (dry weight) per h circulation.

2.3.6 Assessment of cell viability

To assess the effect of cytokine exposure on cell viability, the LIVE viability assay (Molecular Probes, Invitrogen) was used according to manufacturer's instructions. This assay allows the determination of cell viability using the membrane-permeant calcein-acetoxymethyl ester (calcein AM) that is cleaved by live cells to yield green fluorescence. Cells were exposed to 10

ng/ml IL-1β or 5 ng/ml TNF-α for 6 and 24 h and then incubated with 2 μM calcein AM for 30 min at room temperature. The fluorescence was measured with a fluorescence plate reader (SpectraMax Gemini EM, Molecular Devices, CA, USA) with excitation at 485 nm and emission at 530 nm.

2.3.7 Caspase-3/7 activity assay

The Apo-ONE Homogenous caspase-3/7 assay (Promega, Madison, WI, USA) was used to measure caspase-3/7 activity via cleavage of a pro-fluorescent substrate (bis-(N-CBZL-aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide; Z-DEVD-R110). Upon cleavage of the substrate, the product becomes intensely fluorescent, being the fluorescence proportional to caspase-3/7 activity. BREC were plated in 96-well black/clear bottom plates until confluence. After 6 and 24 h treatment with 10 ng/ml IL-1β or 5 ng/ml TNF-α, caspase-3/7 activity was measured according to the manufacturer's instructions. Briefly, equal volumes of assay reagent and sample volume (1:1 ratio) were added directly to the cell culture plates. The plates were incubated for 1 h at room temperature. The fluorescence was measured in a fluorescence plate reader (SpectraMax Gemini EM, Molecular Devices) with an excitation of 485 nm and emission of 530 nm. As a positive control of apoptosis induction, cells were exposed to 100 nM staurosporine for 6 h.

2.3.8 Western blotting

Cells were harvested after the indicated times by washing twice with ice-cold PBS containing phenylmethylsulfonylfluoride (PMSF; 200 μM) and then lysed with Triton-deoxycholate-sodium dodecyl sulphate (SDS) buffer (10 mM Hepes pH 7.5, 100 mM NaCl, 2 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.2% SDS, 1 mM benzamidine) containing a protease inhibitor cocktail tablet (Roche Diagnostics Corporation, Indianapolis, IN, USA), 1 mM Na_3VO_4, 10 mM NaF, 10 mM sodium pyrophosphate and 1 μM microcystin. Lysates were incubated for 15 min at 4ºC, and the insoluble material was pelleted by centrifugation for 10 min at 12,000x g and 4ºC. The supernatants were used to determine the protein concentration by the BioRad Dc Protein colorimetric assay (BioRad, Hercules, CA, USA) and then were denaturated with 4x concentrated NuPAGE LDS Sample Buffer plus 10x concentrated NuPAGE Sample reducing agent and heated for 10 min at 70ºC. Samples were stored at -70ºC until use.

For immunodetection of proteins on nitrocellulose membranes, 50 μg of protein were loaded per lane on 4-12% NuPAGE Bis-Tris gels and separated by electrophoresis using NuPAGE MOPS-

SDS buffer. Following electrophoresis, proteins were transferred electrophoretically to nitrocellulose membranes. The membranes were blocked with 5% non-fat milk in Tris-buffered saline (20 mM Tris, 137 mM NaCl, pH 7.6) containing 0.1% Tween 20 (TBS-T) for 2 h. Membranes were then incubated overnight at 4ºC with the following antibodies (all used at a dilution of 1:1,000): rabbit polyclonal anti-mouse ZO-1, anti-claudin-5, anti-IκBα or anti-GFP, mouse monoclonal anti-occludin or rabbit monoclonal anti-phosphoSer32-IκBα. To control for protein loading, membranes were probed with a mouse monoclonal anti-β-actin antibody (1:10,000). After 5 washes with TBS-T, membranes were incubated with a horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG or an alkaline phosphatase-conjugated anti-rabbit or anti-mouse IgG for 1 h at room temperature. Membranes were washed 5 times and immunoreactive bands were detected by chemiluminescence with ECL Plus or ECL Advance substrate, or chemifluorescence with ECF substrate. Band intensity was quantified by GeneSnap software (Syngene, Cambridge, UK) or ImageQuant 5.0 software (Molecular Dynamics, Sunnyvaley, CA, USA).

2.3.9 RNA extraction and reverse transcription

Total RNA was isolated a using Qiagen RNeasy Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's protocol. Total RNA quality and quantity was assessed using the RNA 6000 Nano LabChip with an Agilent 2100 Expert Bioanalyzer (Agilent, Palo Alto, CA) and NanoDrop ND 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE). The integrity of RNA, expressed as RNA Integrity Number was around 9.6, indicating high-quality, non-degraded RNA. Reverse transcription into cDNA was carried out using the ABI high capacity cDNA Reverse Transcription with RNase inhibitor kit (Applied Biosystems, Foster City, CA) following the manufacturer's protocol. Briefly, 1 µg of total RNA from each sample was reverse-transcribed into cDNA in a 20 µl reaction containing 1X RT-PCR buffer, 1 nM of each dNTP, 20 units of RNase inhibitor, 1x random hexamers, and 50 units of Multiscribe reverse transcriptase Reverse transcription reactions were performed in a thermocycler at 25°C for 10 min, 37°C for 120 min, and 85°C for 5 sec. The cDNA was quantified using a NanoDrop ND 1000 Spectrophotometer, and cDNA samples were then stored at -20ºC until use.

2.3.10 Real-time quantitative PCR

Real-time quantitative PCR (qPCR) analysis was performed using the 7900 HT Sequence Detection System in 384-well optical plates using TaqMan Universal PCR Master Mix, Assay-on-Demand and Assay-by-Design primers and probes (Applied Biosystems, Foster City, CA, USA). Primers/probes used were: CLDN5 (claudin-5; Bt03288088_s1), OCLN (occludin; Bt03255225_m1), IL-8 (interleukin-8; Bt03211907_g1), and bovine ZO-1 specific primers, 5'-AGAAAGATGTTTATCGTCGCATCGT-3' (forward), 5'-ATTCCTTCTCATATTCAAAATGGGTTCTGA-3' (reverse) and FAM 5'-ACCCACATCGGATTCT-3' minor groove binding (MGB) probe. Reaction mixtures consisted of 5 µl 2x Taqman Universal Master Mix with uracil-N-glycosylase, 450 nM unlabeled primers, 125 nM Fam dye-labeled TaqMan MGB probe and 50 ng cDNA reaction product in a 10 µl final volume reaction. PCR conditions were 2 min at 50ºC, 10 min at 95ºC and 40 cycles of 15 sec at 95ºC and 1 min at 60ºC. Relative quantities were calculated using ABI SDS 2.0 RQ software and the $2^{-\Delta\Delta Ct}$ analysis method (Livak and Schmittgen, 2001), with glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Bt03210919_g1) as the endogenous control. For each sample, real-time PCR reactions were performed in triplicate and the average threshold cycle (Ct) was calculated. The relative mRNA expression of several genes in treated samples was determined as fold increase compared with control samples.

2.3.11 Immunocytochemistry

Tight junction protein cellular localization was evaluated by immunocytochemistry. After treatments, cells were washed twice with PBS and fixed with 1% paraformaldehyde for 10 min, permeabilized with 0.2% Triton X-100/PBS for 10 min at room temperature and blocked with 10% goat serum (ZO-1 and claudin-5) or 10% BSA (occludin) in 0.1% Triton X-100/PBS for 1 h. Cells were incubated with a rat anti-ZO-1 monoclonal antibody culture supernatant (1:5), rabbit anti-claudin-5 (1:500) or anti-occludin (1:300) antibodies in the respective blocking solution overnight at 4ºC. After 3 washes with 0.1% Triton X-100/PBS, cells were incubated with Alexa Fluor 647-conjugated anti-rat IgG or Cy3-conjugated anti-rabbit IgG for 1 h, followed by 3 washes with 0.1% Triton X-100/PBS. The nuclei were stained with Hoechst (2 µg/ml) during secondary antibody incubation. Coverslips were mounted onto slides using Aqua Poly/Mount (Aquamount; Polysciences, Warrington, PA) and analyzed by a Leica TCS SP2 AOBS confocal microscope. Ten confocal Z stacks were collected over a depth of 2.56 µm and projected onto

one image. ZO-1, claudin-5 and occludin cell border staining was quantified by a semi-quantitative ranking score system on a scale of 1 to 5: 1 for loss or near complete loss of cell border staining (0-25%), 2 for 25-50% continuous cell border staining, 3 for 50-75% continuous cell border staining, 4 for 75-100% continuous cell border staining, and 5 for completely continuous cell border staining through all plasma membranes. To each image, a 5x5 grid was applied and each square of the grid was assigned a ranking score by two independent observers in a masked fashion. For each image, the average of the ranking score of each square was calculated. For each condition, the results of four images representative of four independent experiments were summed and the frequency of each ranking score was calculated and plotted on a bar chart. The chi-square test of independence was used to determine the statistical differences of the ranking score distribution between conditions.

2.3.12 Immunohistochemistry

ZO-1 and occludin localization in retinal vessels was assessed by immunohistochemistry in whole retinas as described previously (Barber *et al.*, 2000). Briefly, the animals were sacrificed under deep anesthesia followed by decapitation, and both eyes were enucleated immediately. The retinas were dissected in ice-cold PBS and fixed in fresh 2% paraformaldehyde for 10 minutes at room temperature. Whole retinas were blocked and permeabilized in 10% donkey serum with 0.3% Triton in PBS, for 1-2 hours. The retinas were incubated with monoclonal anti-occludin (1:50) and polyclonal anti-ZO-1 (1:50) antibodies in blocking solution for 3 days at 4°C. After this incubation, the retinas were incubated with anti-mouse Cy2-conjugated secondary antibody (1:1,000) and anti-rabbit Cy3-conjugated secondary antibody (1:1,000) for 24 h at 4°C after extensive washing in PBS with 0.3% Triton. After incubation, retinas were washed, mounted in slides with Aqua Poly/Mount and analyzed in a Leica TCS SP2 AOBS confocal microscope.

2.3.13 Adenovirus-mediated IκBα overexpression

IκBα was expressed using the AdEasy adenoviral vector system. The replication-deficient recombinant adenovirus was constructed by homologous recombination between the adenoviral backbone vector (pAdEasy-1) and adenoviral shuttle vector pAdTrack-CMV as described (He *et al.*, 1998; Bobrovnikova-Marjon *et al.*, 2004). Subconfluent cells were transduced with adenovirus expressing IκBα (AdIκBα) or with an empty expression cassette as control

(AdEmpty) at a multiplicity of infection of 20,000 for 6 h in MCDB complete media. Six hours after incubation, cells were washed twice with PBS and MCDB media supplemented with 1% serum was added for 24 h until use for experimentation. Successful viral infection was followed by fluorescence microscopy since the pAdTrack vector contains an enhanced GFP cassette incorporated into the viral vector. Viral proteins expression was also confirmed by immunoblotting with anti-IκBα and anti-GFP antibodies.

2.3.14 Luciferase reporter assay

A 293T/NFκB-luc stable reporter cell line (Panomics-Affymetrix, Fremont, CA, USA) was used to evaluate NF-κB transcription factor activity. These cells maintain a chromosomal integration of a luciferase reporter gene regulated by multiple copies of the NF-κB response element (κB). After treatments, cells were harvested and lysed with Passive Lysis Buffer (Promega, Madison, WI, USA) and the luciferase activity was measured using the Dual-Luciferase Reporter Assay System according to the manufacturer's instructions. Luciferase activity was normalized to the total protein content of each sample.

2.3.15 Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). Data were analyzed using Students *t*-test, chi-square test of independence or one-way analysis of variance (ANOVA) followed by Dunnet's or Bonferroni's *post* test, as indicated in figure legends. A value of $p<0.05$ was considered significant. Prism 4.0 (GraphPad Software, San Diego, CA) was used for all statistical analysis.

2.4 Results

2.4.1 IL-1β and TNF-α increase BREC permeability

In order to investigate the effect of IL-1β and TNF-α on retinal vascular permeability, confluent BREC cultured on transwell filters were exposed to increasing concentrations of IL-1β and TNF-α in a time-dependent manner. Cell monolayer permeability (*P*o) to 70 kDa RITC-dextran was determined over a 3.5 h period by adding the fluorescent dextran to the apical chamber and quantifying the accumulation of dextran in the basolateral chamber. IL-1β significantly increased BREC monolayer permeability at a concentration of 10 ng/ml (220.3 ± 34.2% of the control;

Figure 2.1A), and this effect was statistically significant after 24 h treatment (Fig. 2.1B). Similarly, TNF-α treatment increased BREC permeability in a concentration- and time-dependent manner. However, TNF-α was more effective than IL-1β and significantly increased the permeability at a lower concentration (5 ng/mL, 365.3 ± 47.8% of the control, Figure 2.1C) and at a shorter time-point (6 h; 337.7 ± 43.2% of the control, Figure 2.1D). For further experiments IL-1β was used at 10 ng/ml and TNF-α at 5 ng/ml.

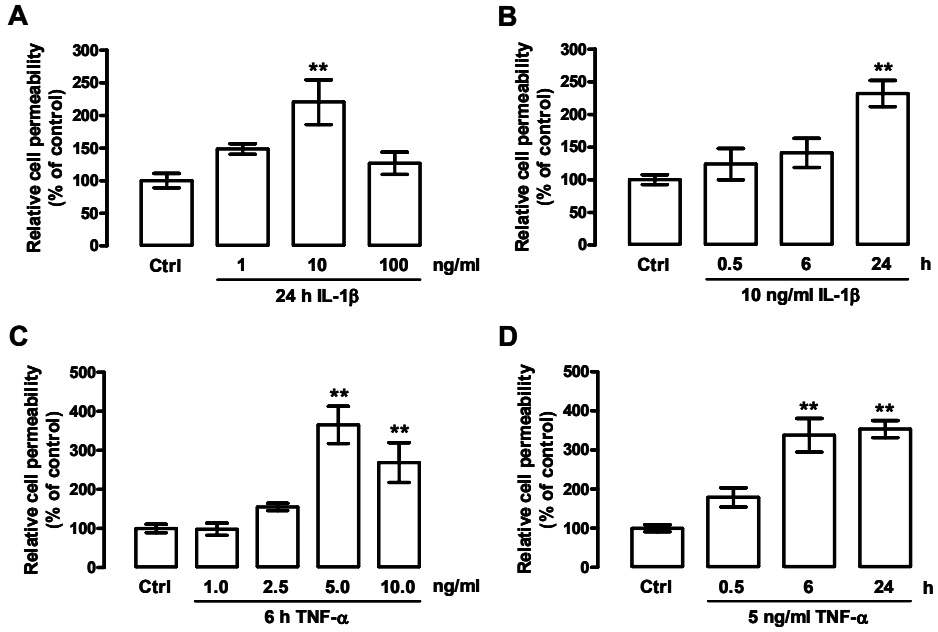

Figure 2.1. IL-1β and TNF-α increase retinal endothelial cell permeability. BREC were grown to confluence on transwell filters and then exposed to IL-1β or TNF-α in a concentration- and time-dependent manner. The monolayer permeability to 70 kDa dextran was measured as described in Methods. **A.** Cells were treated with 1, 10 and 100 ng/ml IL-1β for 24 h. **B.** Cells were treated with 10 ng/ml IL-1β for 0.5, 6 and 24 h (n=12). **C.** Cells were treated with 1, 2.5, 5 and 10 ng/ml TNF-α for 6 h. **D.** Cells were treated with 5 ng/ml TNF-α for 0.5, 6 and 24 h. The results represent the mean ± SEM of, at least 4 independent experiments, and are expressed relatively to control (Ctrl). $^{**}p<0.01$, significantly different from control as determined by ANOVA followed by Dunnett's *post* test.

2.4.2 Effect of cytokines on BREC viability

To insure that the increase in cell permeability induced by IL-1β and TNF-α was not due to cell death, the effect of these cytokines on BREC viability was evaluated in cells exposed to 10 ng/ml IL-1β or 5 ng/ml TNF-α for 6 and 24 h. BREC viability was assessed with calcein AM

uptake using the LIVE viability assay. IL-1β treatment had no effect on cell viability. TNF-α-induced a significant decrease of cell viability after 24 h treatment (72.8 ± 2.0% of the control), but no effect was observed after 6 h treatment (Figure 2.2A). The effect of IL-1β and TNF-α on caspase-3/7 activity, a marker of apoptosis, was also evaluated. Staurosporine, a protein kinase inhibitor, was used as a positive control to induce apoptosis in BREC. IL-1β had no effect on caspase-3/7 activity while TNF-α induced a significant increase in caspase-3/7 activity (178.7 ± 8.7% of the control), but again only after 24 h exposure (Figure 2.2B). Therefore, the increase in cell permeability induced by TNF-α after 6 h exposure was not due to an increase in cell death. Since the effect of TNF-α was more robust than IL-1β, the molecular mechanisms underlying TNF-α-induced retinal endothelial cell permeability were further investigated.

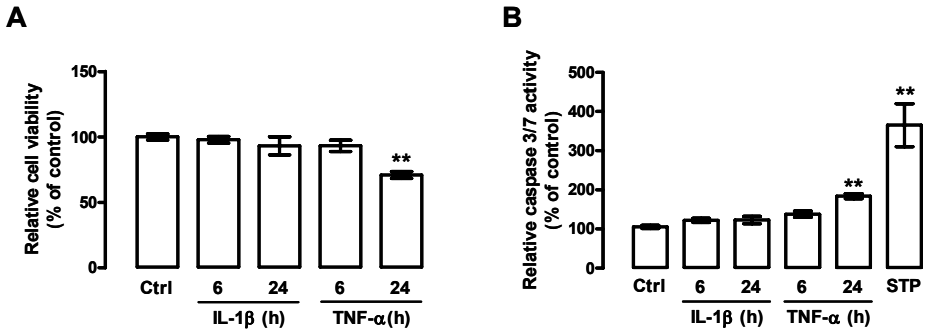

Figure 2.2. Effect of IL-1β and TNF-α on retinal endothelial cell viability. BREC were treated with 10 ng/ml IL-1β or 5 ng/ml TNF-α for 6 and 24 h. **A.** Relative cell viability was measured by calcein AM cleavage by live cells. **B.** Effect of IL-1β and TNF-α on retinal endothelial cell apoptosis. Caspase-3/7 activation was measured by the cleavage of Z-DEVD-R110 substrate for these caspases. As a positive control of apoptosis induction, cells were treated with 100 nM staurosporine (STP) for 6 h. The results represent the mean ± SEM of 8 independent experiments and are expressed relatively to control (Ctrl). $^{**}p<0.01$, significantly different from control as determined by ANOVA followed by Dunnett's *post* test.

2.4.3 TNF-α alters tight junction proteins expression

To determine the effect of TNF-α on the expression of specific tight junction proteins, BREC were exposed to TNF-α for 0.5 and 6 h and the protein content of ZO-1, claudin-5 and occludin was determined by Western blotting. TNF-α significantly decreased ZO-1 protein levels (58.2 ± 6.5% of the control), after 6 h exposure (Figure 2.3A). Claudin-5 protein content was rapidly reduced after 0.5 h of TNF-α treatment (70.8 ± 7.0% of the control, Figure 2.3B), and 6 h of TNF-α exposure further downregulated claudin-5 protein content to 57.6 ± 8.7% of the control. In contrast, TNF-α increased occludin protein content (130.6 ± 6.6% of the control, Figure 2.3C).

To determine if alterations in protein content were due to changes in mRNA expression, total mRNA content was evaluated by qPCR 6 h after TNF-α exposure. TNF-α significantly decreased ZO-1 (74.6 ± 3.3% of the control; Figure 2.3D) and claudin-5 (80.9 ± 5.7% of the control; Figure 2.3E) mRNA content, but induced a 2-fold increase in occludin mRNA (Figure 2.3F).

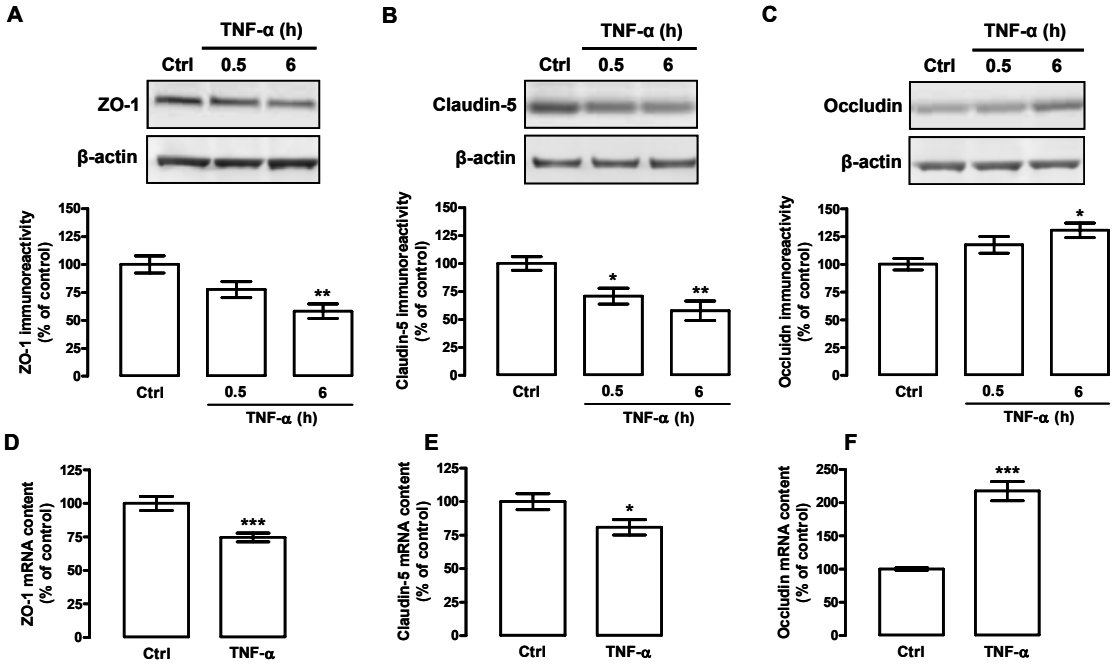

Figure 2.3. Effect of TNF-α on tight junction proteins content. BREC were treated with 5 ng/ml TNF-α for 0.5 and 6 h. Whole cell extracts were assayed for **(A)** ZO-1, **(B)** claudin-5 and **(C)** occludin immunoreactivity by Western blotting. Representative Western blots for each tight junction protein and β-actin (loading control) are presented above each respective graph. The results are normalized to β-actin and represent the mean ± SEM of, at least, 5 independent experiments and are expressed as the relative amount compared to control (Ctrl). $^{*}p<0.05$, $^{**}p<0.01$, significantly different from control as determined by ANOVA followed by Dunnet's *post* test. Total RNA was isolated and the transcript levels of **(D)** ZO-1, **(E)** claudin-5 and **(F)** occludin were analyzed by qPCR. GAPDH was used as an endogenous control. Results represent the mean ± SEM of 8 independent experiments and are expressed as the relative amount compared to control conditions. $^{*}p<0.05$, $^{***}p<0.001$, significantly different from control as determined by Student's *t*-test.

2.4.4 TNF-α alters tight junction protein localization at the cell border

A proper assembly of tight junction proteins at the cell border is essential to form a barrier to the flux of water and solutes. To investigate whether TNF-α alters the tight junction complex at the

cell membrane, the cellular localization of the tight junction proteins was determined by immunocytochemistry and confocal microscopy. As shown in Figure 2.4A, in the control condition, ZO-1, claudin-5 and occludin immunoreactivity appeared as a near continuous staining at the cell border. Upon TNF-α treatment, a loss of both ZO-1 and claudin-5 immunostaining was observed leading to a fragmented border staining, although the effect on ZO-1 was more pronounced. Also, a diffuse cytoplasmic distribution of claudin-5 and occludin was observed in TNF-α-treated cells. After TNF-α treatment, occludin staining increased and it was irregularly distributed at the cell plasma membrane. Cell border localization was quantified for each of the tight junction proteins in a masked fashion by a 5 tier ranking system as described in the Material and Methods section. TNF-α significantly altered the junctional localization of occludin, claudin-5 and ZO-1 compared to non-treated cells ($p<0.0001$, chi-square test of independence, Figure 2.4B).

2.4.5 Dexamethasone prevents TNF-α-induced BREC permeability

Dexamethasone is a potent anti-inflammatory glucocorticoid that increases endothelial cell barrier properties by increasing occludin and claudin-5 transcription and redistributing tight junction proteins to the plasma membrane (Antonetti *et al.*, 2002; Felinski *et al.*, 2008). In order to determine whether dexamethasone treatment prevents the increase in BREC permeability in response to TNF-α exposure, BREC were grown on transwell filters until confluence and then treated with 50 ng/ml dexamethasone for 18 h before the addition of 5 ng/ml TNF-α for an additional 6 h. As shown in Figure 2.5A, TNF-α treatment led to a significant increase in diffusive permeability of 70kDa dextran (208.9 ± 26.0% of the control), and pretreatment with dexamethasone completely prevented TNF-α-induced increase in BREC monolayer permeability (60.4 ± 7.1 % of the control).

To investigate if the protective effect of dexamethasone against TNF-α-induced cell permeability is dependent on transcriptional transactivation by the glucocorticoid receptor, BREC were pre-treated with RU486, a glucocorticoid receptor antagonist, which has been shown to specifically inhibit the glucocorticoids receptor transactivation of gene expression (Mahajan and London, 1997). Cells were treated with 5 μM RU486 1 h prior the addition of 50 ng/ml dexamethasone for 24 h. After 18 h of dexamethasone addition, cells were treated with TNF-α for 6 h and the permeability to 70 kDa dextran across endothelial cell monolayers was measured as described.

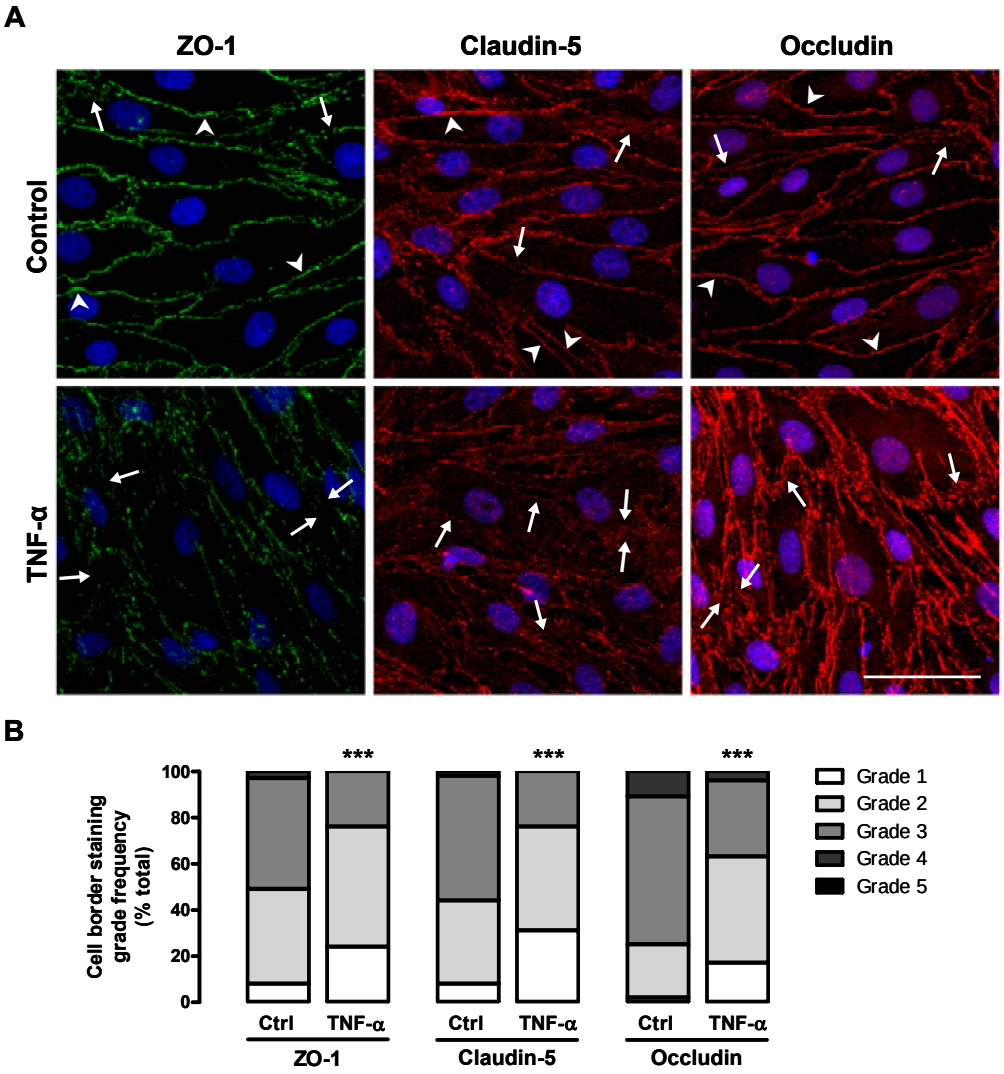

Figure 2.4. TNF-α disrupts tight junction proteins localization at the cell border. BREC were treated with 5 ng/ml TNF-α for 6 h. **A.** Cells were immunolabeled for ZO-1, claudin-5 and occludin, and 10 confocal Z-stacks were taken through 2.56 µm and projected into one image. Arrowheads indicate continuous staining at cell borders. Arrows indicate loss and/or discontinuous staining in tight junctions between cells. These results are representative of four independent experiments. Scale bar, 50 µm. **B.** ZO-1, claudin-5 and occludin staining at the cell border was quantified as described in Methods. The results represent the frequency of each ranking grade of the 4 independent experiments. $^{***}p<0.0001$, significantly different from control (Ctrl) as determined by chi-square test of independence.

A 1 h pretreatment of RU486 before the addition of dexamethasone significantly reduced the protective effect of dexamethasone on TNF-α-induced cell permeability (157.1 ± 13.6% of the control; Figure 2.5B). RU486 alone had no effect on cell permeability and did not alter the TNF-α response but did prevent the dexamethasone-induced reduction in cell permeability as previously described (Felinski *et al.*, 2008). These results show that dexamethasone's protective effect is partially due to the glucocorticoid receptor transactivation but also suggests that transrepression of TNF-α-responsive transcription factors mediated by the glucocorticoid receptor also contributes to the inhibition of TNF-α-induced cell permeability.

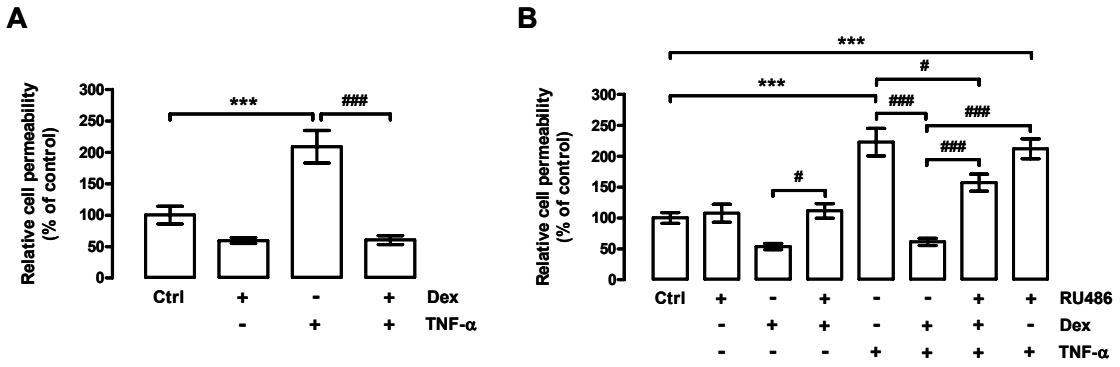

Figure 2.5. Dexamethasone prevents TNF-α-induced cell permeability through transactivation of the glucocorticoid receptor. A. BREC were grown to confluence on transwell filters and treated with 50 ng/ml dexamethasone (Dex) 18 h before TNF-α treatment (5 ng/ml, 6 h). **B.** Cells were treated with 5 μM RU486 1 h before dexamethasone (Dex) treatment. The monolayer permeability to 70 kDa dextran was measured as described in Methods. The results represent the mean ± SEM of, at least, 7 independent experiments and are expressed relatively to control (Ctrl). $^{***}p<0.001$, significantly different from control as determined by ANOVA followed by Dunnett's *post* test. $^{\#}p<0.05$, $^{\#\#\#}p<0.001$, significantly different, as determined by ANOVA followed by Bonferroni's *post* test.

2.4.6 Dexamethasone prevents TNF-α-induced alterations in tight junction proteins

The effect of dexamethasone on TNF-α-induced changes on tight junction protein content and cellular localization was also evaluated. Dexamethasone alone significantly increased ZO-1, claudin-5 and occludin protein content to 123.3 ± 6.2%, 154.7 ± 12.2% and 201.9 ± 15.0% of the control, respectively (Figure 2.6). TNF-α decreased ZO-1 and claudin-5 protein content and these effects were completely prevented by dexamethasone pretreatment (Figure 2.6A and B) while dexamethasone and TNF-α treatment yielded an additive 3-fold increase in occludin

protein content (Figure 2.6C). Dexamethasone increased ZO-1, claudin-5 and occludin staining at the cell border and prevented the TNF-α-induced fragmentation of these tight junction proteins (Figure 2.7A and B).

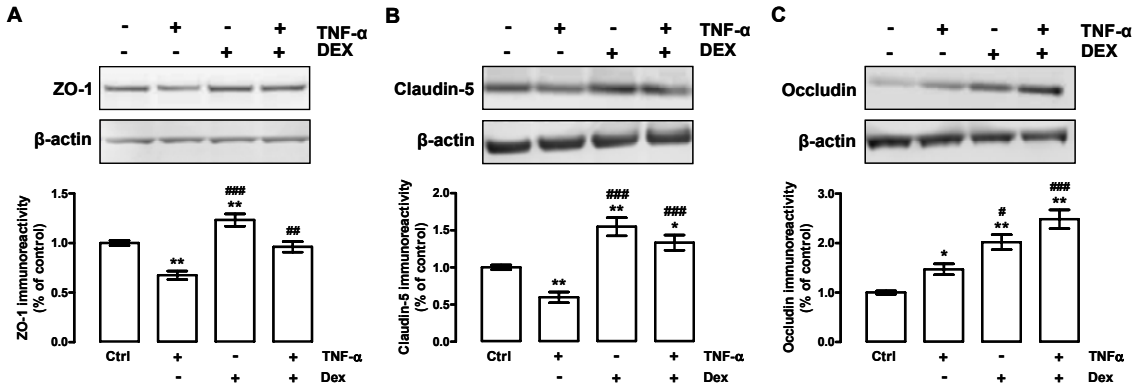

Figure 2.6. Dexamethasone prevents TNF-α-induced alteration in the tight junction complex. Confluent BREC were treated with 50 ng/ml dexamethasone 18 h before TNF-α treatment (5 ng/ml, 6 h). Whole cell extracts were assayed for **(A)** ZO-1, **(B)** claudin-5 and **(C)** occludin immunoreactivity by Western blotting as described in Methods. Representative Western blots for each tight junction protein and β-actin (loading control) are presented above each respective graph. The results are normalized to β-actin and represent the mean ± SEM of, at least, 5 independent experiments and are expressed as the relative amount compared to control. $^{*}p<0.05$, $^{**}p<0.01$, significantly different from control as determined by ANOVA followed by Dunnett's *post* test; $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$, significantly different from TNF-α, as determined by ANOVA followed by Bonferronni's *post* test.

2.4.7 NF-κB inhibition reduces TNF-α-induced cell permeability

Glucocorticoids act to suppress inflammation by repression of proinflammatory transcription factors such as AP-1 (Jonat *et al.*, 1990) and NF-κB (Scheinman *et al.*, 1995), which leads to an inhibition of proinflammatory genes expression (Almawi and Melemedjian, 2002). As NF-κB is central to TNF-α signal transduction, the involvement of NF-κB on the TNF-α-induced cell permeability was investigated. Since NF-κB is regulated primarily by the IKK complex, the effect of the inhibitor IKK VII, a potent and specific inhibitor of IKK complex, on TNF-α-induced cell permeability was investigated. As a control for inhibitor IKK VII effectiveness, the effect of IKK VII on TNF-α-induced IκBα phosphorylation by IKK was evaluated. BREC were exposed to 1 μM IKK VII 30 min before the addition of TNF-α. Cells were harvested 5 min after the TNF-α treatment and phosphorylated IκBα and IκBα protein levels were analyzed by Western blotting.

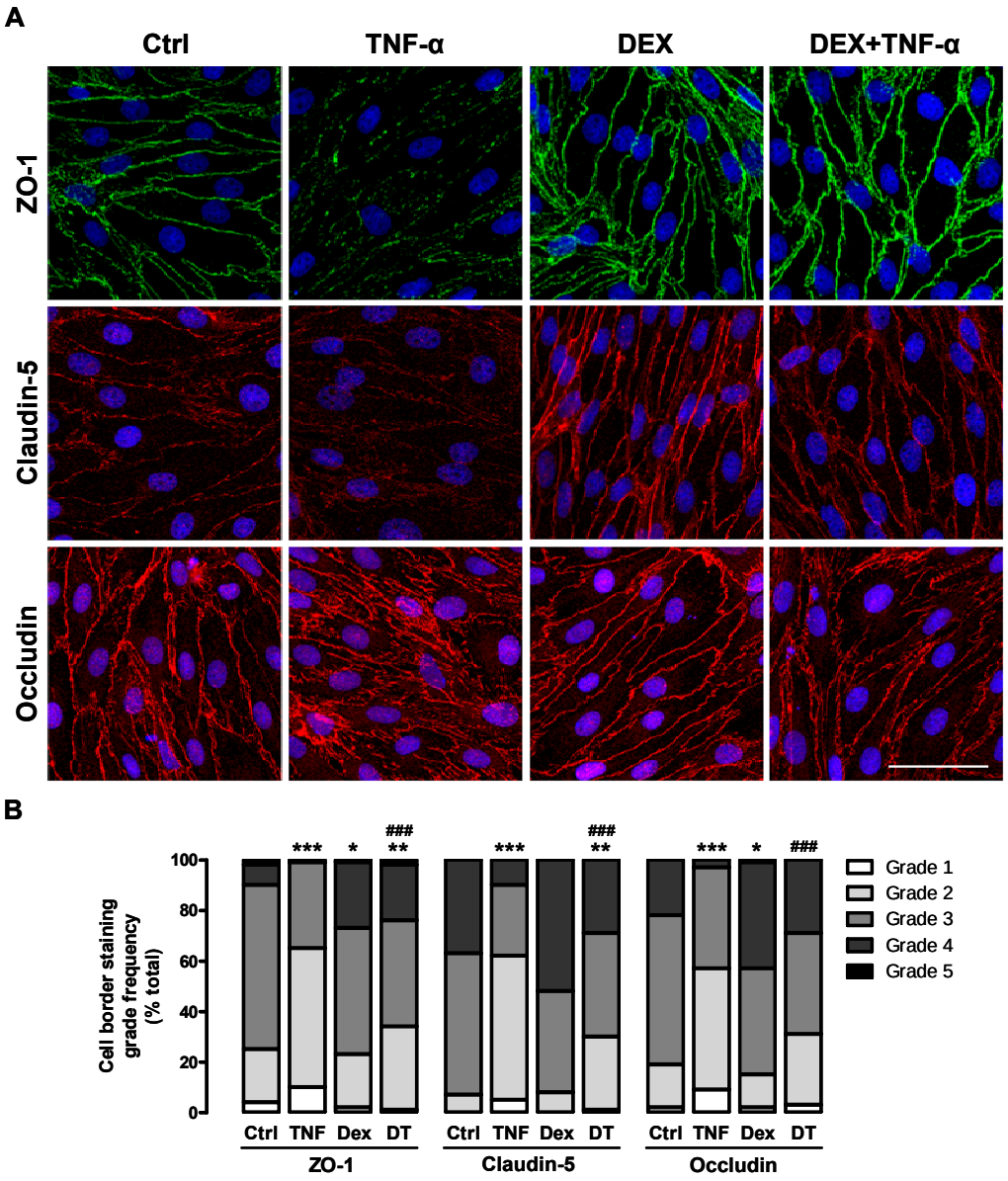

Figure 2.7. Dexamethasone prevents TNF-α-induced disruption of the tight junction complex at the cell border. Confluent BREC were treated with 50 ng/ml dexamethasone 18 h before TNF-α treatment (5 ng/ml, 6 h). **A.** Cells were immunolabeled for ZO-1, claudin-5 and occludin and 10 confocal Z-stacks were taken through 2.56 µm and projected into one image. These results are representative of 4 independent experiments. Scale bar, 50 µm. **B.** ZO-1, claudin-5 and occludin staining at the cell border was quantified as described. The results represent the frequency of each ranking score of the 4 independent experiments. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.0001$, significantly different from control. $^{\#\#\#}p<0.0001$ significantly different from TNF-α as determined by chi-square test of independence. Ctrl, Control; Dex, dexamethasone; TNF, TNF-α, DT, Dex+TNF-α.

TNF-α treatment resulted in marked IκBα phosphorylation in retinal endothelial cells (Figure 2.8A). IKK VII pretreatment effectively blocked the phosphorylation of IκBα induced by TNF-α. Subsequently, the effect of IKK VII on retinal endothelial cell permeability was evaluated. BREC were exposed to 1 μM IKK VII for 30 min before the addition of TNF-α. After 6 h of TNF-α treatment, cell permeability was measured. The increase in cell permeability induced by TNF-α (275.0 ± 46.7% of the control) was significantly reduced by IKK VII (160.5 ± 21.7% of the control; Figure 2.8B), suggesting that NF-κB activation is involved in TNF-α regulation of retinal endothelial cell permeability. To further evaluate the contribution of NF-κB to TNF-α-induced cell permeability, the effect of adenovirus-mediated overexpression of IκBα on cell permeability was evaluated. BREC were transduced with AdEmpty or AdIκBα for 30 h. The transduction efficiency of the adenoviral vectors was evaluated by fluorescence microscopy since both viral vectors encode GFP. More than 80% of the cells expressed GFP after 30 h of adenoviral infection. Western blotting was used to confirm the adenovirus-mediated expression of GFP and IκBα 30 h after adenoviral infection. GFP expression was similar in both AdEmpty and Ad IκBα-transduced cells, while IκBα was heavily expressed in AdIκBα-transduced cells compared to AdEmpty-transduced cells (Figure 2.8C). Most inflammatory cytokine genes that play an important role in the inflammatory response, such as IL-8, contain NF-κB binding sites in their promoter region. The ability of IκBα overexpression to inhibit NF-κB activation was evaluated by examining the TNF-α-induced expression of IL-8 by qPCR. In both nontransduced and AdEmpty-transduced cells, IL-8 mRNA levels were significantly increased (by 18- and 16-fold, respectively), after 2 h of TNF-α stimulation (Figure 2.8D). This robust increase in IL-8 expression was significantly reduced (by 41%) in AdIκBα-transduced cells, demonstrating that adenovirus-mediated overexpression of IκBα effectively blocked NF-κB activation by TNF-α in retinal endothelial cells.

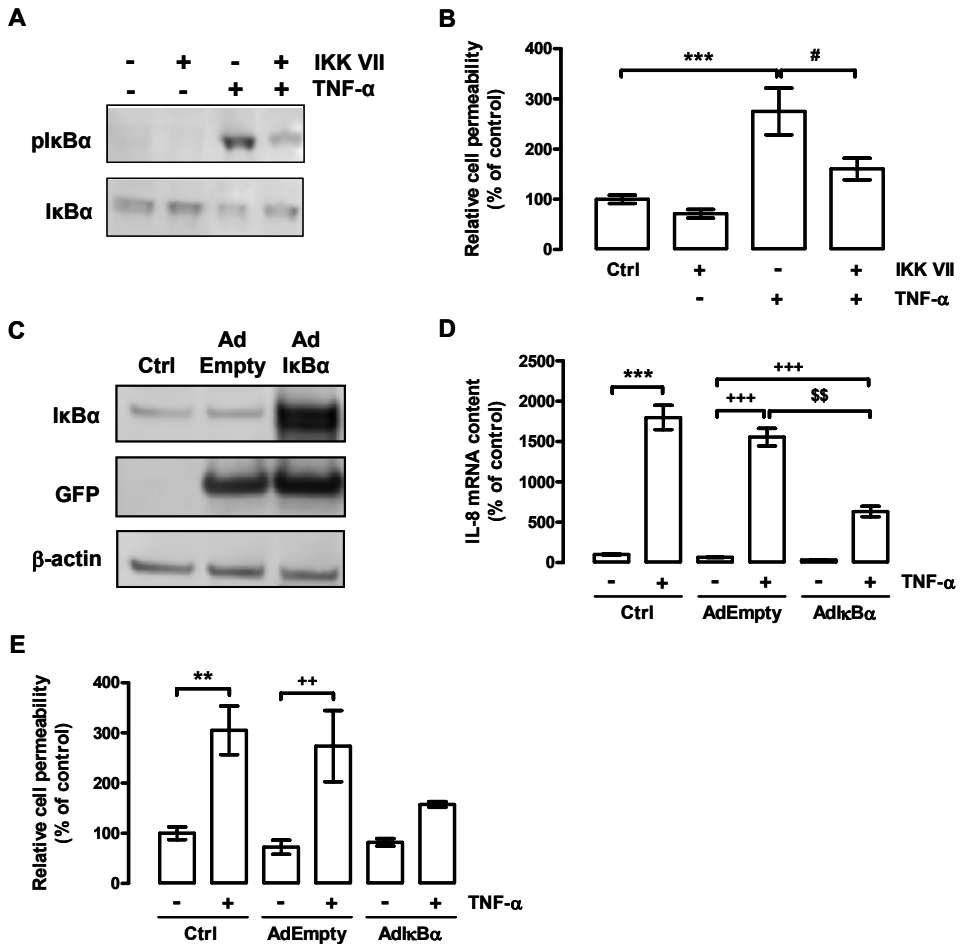

Figure 2.8. Effect of NF-κB inhibition on TNF-α-induced cell permeability. A. Cells were incubated with 1 μM IKK VII, an IKK inhibitor, 30 min before the addition of 5 ng/ml TNF-α for 5 min. Whole cell lysates were assayed for phophorylated IκBα (Ser32/Ser36) and total IκBα immunoreactivity by Western blotting as described. Representative Western blots are shown. **B.** Cells were grown to confluence on transwell filters and then treated with 1 μM IKK VII 30 min before TNF-α addition (5 ng/ml, 6 h). The monolayer permeability to 70 kDa dextran was measured as described. (**C**-**E**) Adenovirus-mediated overexpression of IκBα. BREC were transduced with AdEmpty or AdIκBα as described. **C.** Whole lysates of BREC were used to detect IκBα, GFP and β-actin (loading control) by Western blotting as described. Representative Western blots are shown. **D.** After 28 h of adenovirus transduction, cells were exposed to 5 ng/ml TNF-α for 2 h. Total RNA was isolated and the transcript levels of IL-8 were analyzed by qPCR. **E.** Cells were grown to confluence on transwell filters and after 24 h of adenovirus transduction cells were treated with 5 ng/ml TNF-α for 6 h. The monolayer permeability to 70 kDa dextran was measured. The results represent the mean ± SEM of, at least, 3 independent experiments and are expressed relatively to control. $^{***}p<0.001$, significantly different from control as determined by ANOVA followed by Dunnett's *post* test. $^{\#}p<0.05$ significantly different from TNF-α, $^{++}p<0.01$, $^{+++}p<0.001$, significantly different form AdEmpty, $^{\$\$}p<0.01$, significantly different form AdEmpty+TNF-α, as determined by ANOVA followed by Bonferroni's *post* test.

Subsequently, the effect of IκBα overexpression on TNF-α-induced cell permeability was determined. BREC grown in transwell filters were transduced with AdEmpty and AdIκBα adenovirus for 30 h. TNF-α was added 24 h after infection, for 6 h, and cell permeability was measured. In AdEmpty-transduced cells, TNF-α induced a significant increase in cell permeability (273.6 ± 71.0% of the control; Figure 2.8E). This effect was similar to the one observed in non-transduced cells (Figure 2.7E). IκBα overexpression reduced TNF-α-induced cell permeability to 157.5±5.7% of the control. These observations suggest that NF-κB activation is necessary for a substantial part of the TNF-α-induced changes in BREC permeability.

2.4.8 PKCζI-1 inhibits NF-κB activation induced by TNF-α

The regulation of NF-κB activity occurs at several levels, including cytosolic-nuclear shuttling and modulation of its transcriptional activity (Neumann and Naumann, 2007). It has been demonstrated that the atypical PKCζ isoform has a critical role in the activation of NF-κB pathway (Leitges *et al.*, 2001; Duran *et al.*, 2003). To determine if PKCζ modulates NF-κB activation by TNF-α in retinal endothelial cells, the effect of PKCζI-1, a novel PKCζ inhibitior with litle or no inhibitory activity on PKCβ or PKCδ (Antonetti *et al.*, 2008), on TNF-α-induced IL-8 mRNA expression was evaluated. Confluent cells were treated with 10 μM PKCζI-1 30 min before the addition of TNF-α for 2 h. Total mRNA was isolated and IL-8 mRNA was determined by qPCR. As shown in Figure 2.9A, TNF-α induced a significant 14-fold increase in IL-8 transcripts, which was significantly reduced to 6-fold by PKCζI-1. Next, the effect of PKCζI-1 on TNF-α-induced NF-κB reporter activity was investigated in 293/NF-κB/luc stable reporter cell line. Cells were treated with 10 or 50 μM PKCζI-1 30 min before the addition of TNF-α for 6 h, and the luciferase activity was determined in whole cell lysates. The results shown in Figure 2.9B indicate that PKCζI-1 significantly decreased TNF-α-induced NF-κB-responsive luciferase expression in these cells. These observations suggest that pharmacological inhibition of PKCζ reduces NF-κB activation in response to TNF-α, consistent with the previously published role of PKCζ in NF-κB activation.

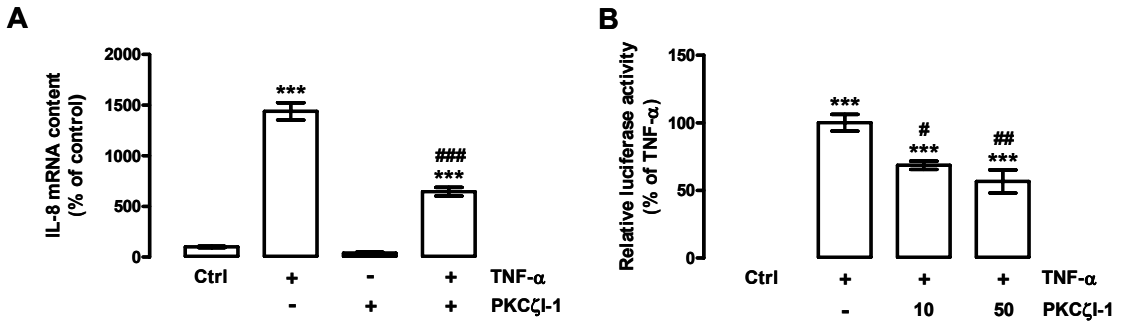

Figure 2.9. Inhibition of PKCζ inhibits NF-κB activation by TNF-α. A. BREC were treated with 10 μM PKCζI-1, a PKCζ inhibitor, 30 min before the addition of 5 ng/mL TNF-α for 2 h. Total RNA was isolated and the transcript levels of IL-8 were analyzed by qPCR. The results represent the mean ± SEM of 6 independent experiments and are expressed relatively to control. **B.** 293/NF-κB/luc cells, with a κB-dependent luciferase reporter gene, were treated with 10 or 50 μM PKCζI-1 30 min prior to the addition of TNF-α for 6 h. Cells were harvested and luciferase activity was determined in whole cell lysates. The results represent the mean ± SEM of 4 independent experiments and are expressed relatively to TNF-α. $^{***}p<0.001$, significantly different from control as determined by ANOVA followed by Dunnett's *post* test. $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$, significantly different form TNF-α, as determined by ANOVA followed by Bonferroni's *post* test.

2.4.9 TNF-α-induced cell permeability requires PKCζ

The mechanism of PKCζ activation is not completely understood, but it is known to be a downstream effector of the PI_3K pathway (Chou *et al.*, 1998), which has been shown to be activated by TNF-α in vascular endothelial cells (Zhang *et al.*, 2003). Therefore, the effect of PI_3K/PKCζ pathway inhibition on retinal endothelial cell permeability was investigated. Confluent BREC were treated with 2 μM LY294002, a PI_3K inhibitor, 30 min before the treatment with TNF-α. After six hours, the monolayer permeability to 70 kDa dextran was measured. The increase in cell permeability induced by TNF-α (348.2 ± 49.3% of the control; Figure 2.10A) was significantly inhibited in the presence of LY294002 (196.4 ± 26.4% of the control; Figure 2.10A). Next, it was investigated whether PKCζ inhibition could prevent the increase in cell permeability induced by TNF-α treatment. BREC were exposed to 10 μM PKCζI-1 or the myristoylated pseudosubstrate inhibitor of PKCζ (PKCζpep; 250 nM) 30 min prior to the addition of TNF-α. Both PKCζI-1 and PKCζpep completely suppressed the increase in cell permeability induced by TNF-α (Figure 2.10B and C).

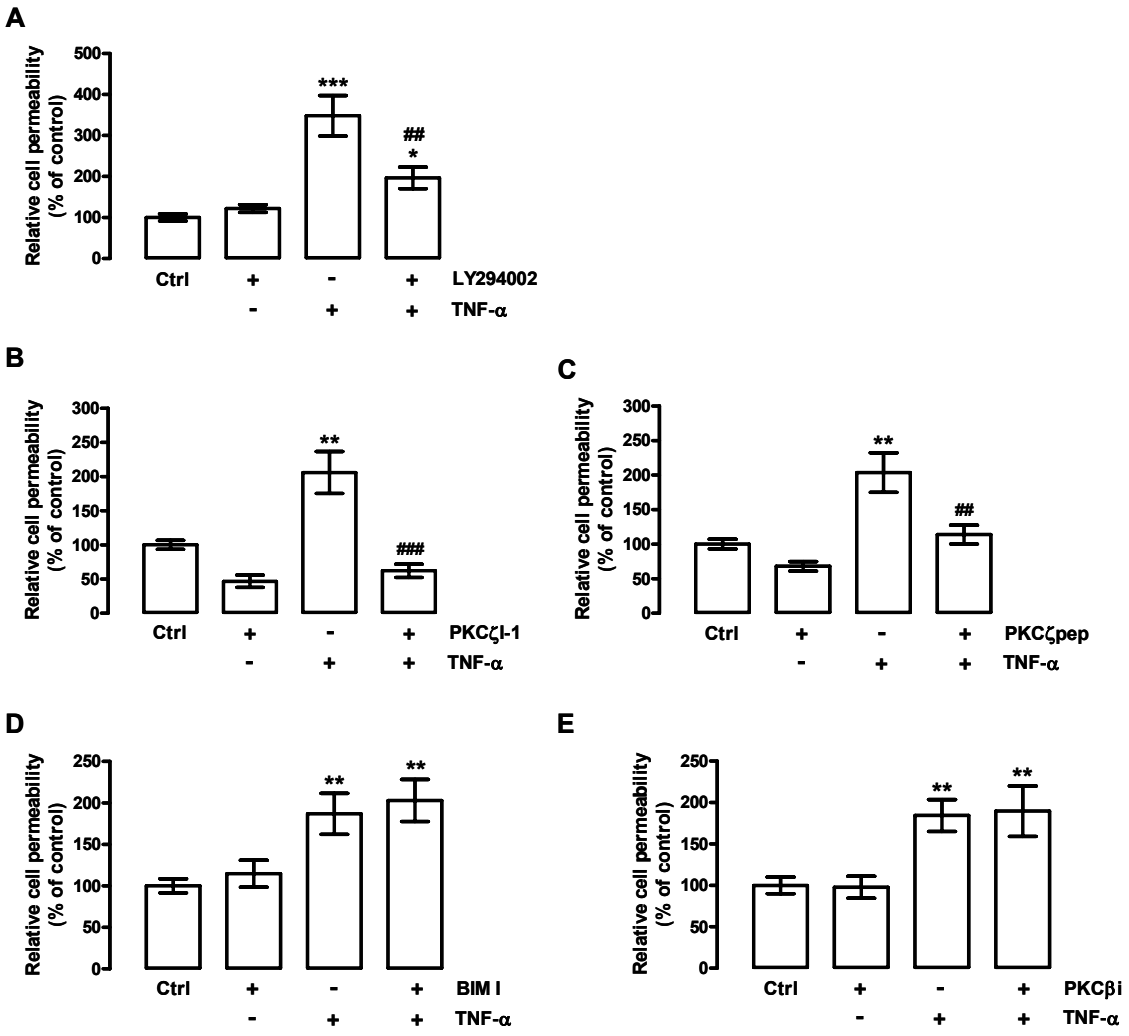

Figure 2.10. TNF-α increases cell permeability in a PKCζ-dependent manner. BREC were grown to confluence on transwell filters and then treated with (**A**) 2 μM LY294002, (**B**) 10 μM PKCζI-1, (**C**) 250 nM PKCζ pseudosubstrate inhibitor (PKCζpep), (**D**) 5 μM BIM I or (**E**) 30 nM PKCβ inhibitor. All inhibitors were added to the cell culture medium 30 min before TNF-α addition (5 ng/ml, 6 h). The monolayer permeability to 70 kDa dextran was measured as described. Results represent the mean ± SEM of, at least 5 experiments and are expressed relatively to control (Ctrl). $^{**}p<0.01$, significantly different from control as determined by ANOVA followed by Dunnett's *post* test. $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$, significantly different form TNF-α, as determined by ANOVA followed by Bonferroni's *post* test.

Since conventional PKC isoforms contribute, in part, to the VEGF-induced permeability in retinal endothelial cells (Aiello *et al.*, 1997; Harhaj *et al.*, 2006), the contribution of conventional and novel PKC isoforms for TNF-α-induced cell permeability was also evaluated. BREC were

treated with 5 μM BIM I (a selective inhibitor of conventional and novel PKC isoforms) or with 30 nM PKCβ inhibitor (a specific inhibitor of PKCβ, analog to LY333531) 30 min before TNF-α treatment. These inhibitors had no effect on TNF-α-induced cell permeability (Figures 2.10D and E). These results suggest that PKCζ, but not conventional and novel PKC isoforms, mediate TNF-α-induced increase in retinal endothelial cell permeability.

2.4.10 PKCζI-1 inhibits the TNF-α-induced alterations in tight junction proteins

To further evaluate the contribution of PKCζ on TNF-α-induced cell permeability, the effect of PKCζ inhibition on tight junction protein content and cellular localization was determined in retinal endothelial cells. Confluent cells were treated with 10 μM PKCζI-1 30 min before the treatment with TNF-α for 6 h. The protein content of ZO-1, claudin-5 and occludin was not affected by treatment with PKCζI-1. However, the decrease in ZO-1 and claudin-5 protein levels induced by TNF-α was effectively prevented by PKCζI-1 (Figures 2.11A and B). Surprisingly, in the presence of PKCζI-1, TNF-α still caused an increase in occludin protein content, which actually was greater than the effect of TNF-α alone (Figure 2.11C).

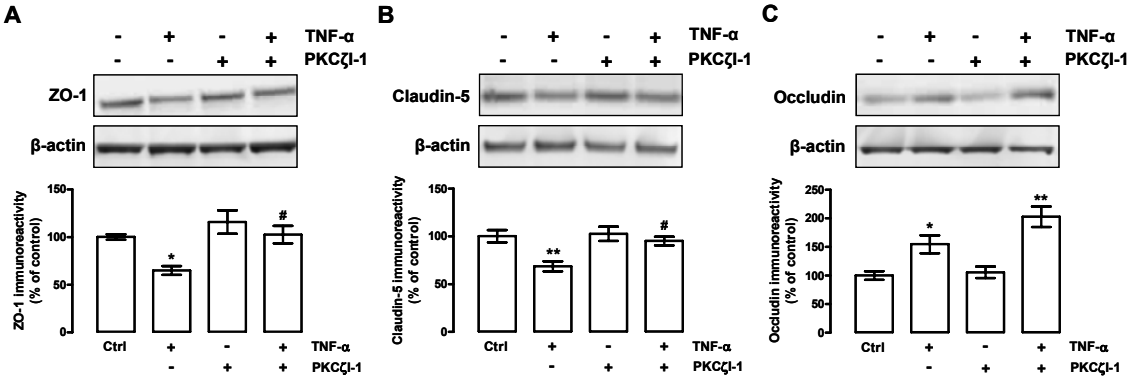

Figure 2.10. PKCζI-1 prevents TNF-α-induced alterations in the tight junction protein content. Confluent BREC were treated with 10 μM PKCζI-1 30 min before TNF-α treatment (5 ng/ml, 6 h). Whole cell extracts were assayed for **(A)** ZO-1, **(B)** claudin-5 and **(C)** occludin immunoreactivity by Western blotting as described. Representative Western blots for each tight junction protein and β-actin (loading control) are presented above each respective graph. The results are normalized to β-actin and represent the mean ± SEM of, at least, 8 independent experiments and are expressed as the relative amount compared to control (Ctrl). $^{*}p<0.05$, $^{**}p<0.01$, significantly different from control as determined by ANOVA followed by Dunnett's *post* test. $^{\#}p<0.05$, significantly different from TNF-α, as determined by ANOVA followed by Bonferroni's *post* test.

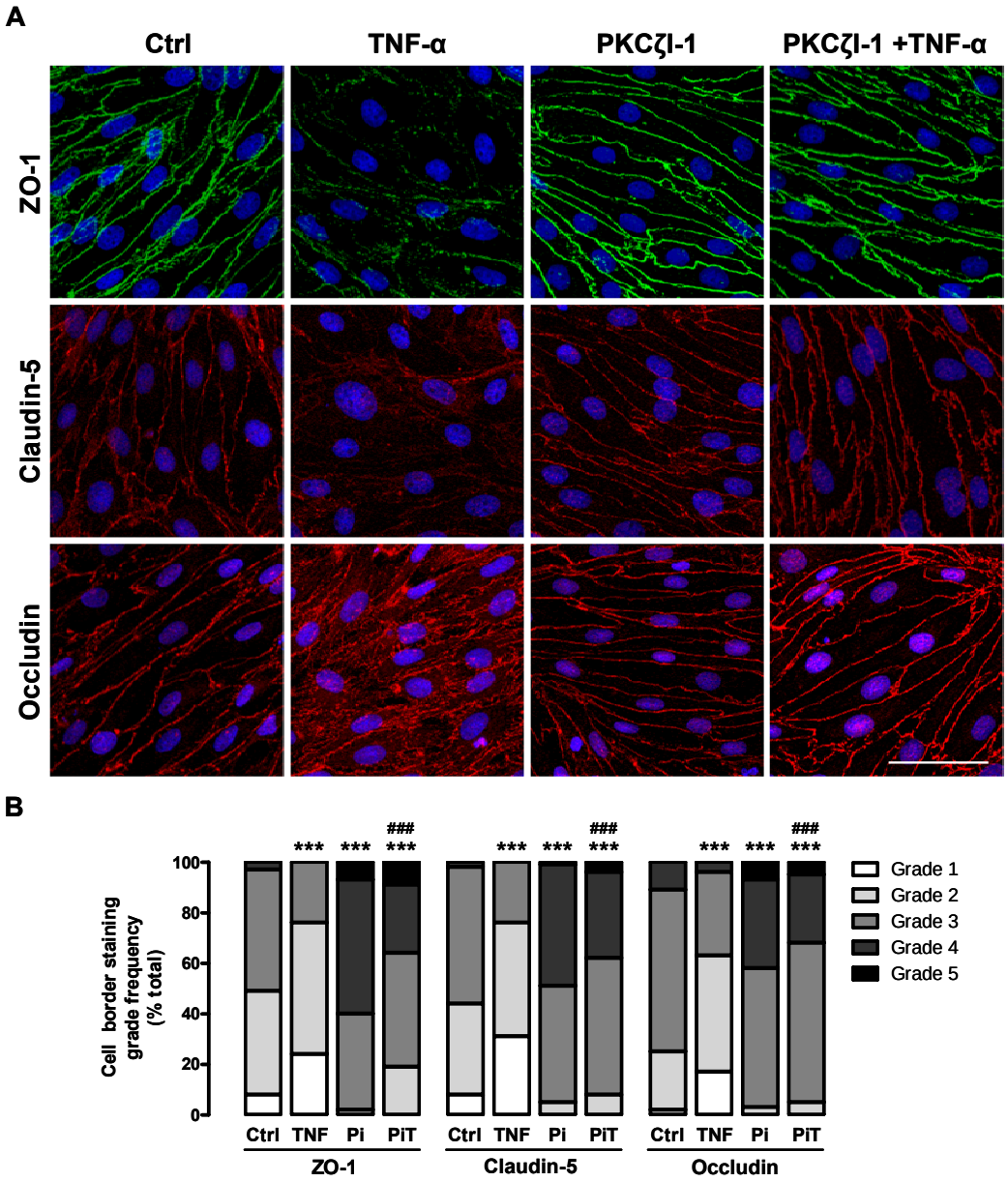

Figure 2.12. PKCζI-1 prevents tight junction complex disruption induced by TNF-α. Confluent BREC were treated with 10 µM PKCζI-1 30 min before TNF-α treatment (5 ng/ml, 6 h).**A.** Cells were immunolabeled for ZO-1, claudin-5 and occludin and 10 confocal Z-stacks were taken through 2.56 µm and projected into one image. These results are representative of 4 independent experiments. Scale bar, 50 µm. **B.** ZO-1, claudin-5 and occludin staining at the cell border was quantified as described. The results represent the frequency of each ranking score of the 4 independent experiments. $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.0001$, significantly different from control (Ctrl). $^{\#\#\#}p<0.0001$ significantly different from TNF-α, as determined by chi-square test of independence.

2.4.11 PKCζI-1 prevents TNF-α-induced BRB permeability *in vivo*

The effect of PKCζI-1 on TNF-α-induced BRB permeability was evaluated *in vivo* by the Evans blue assay. The injection of TNF-α in the vitreous induced a 2-fold increase in the accumulation of Evans blue as compared to vehicle-injected (PBS) animals (Figure 2.13). PKCζI-1 alone had no effect on the accumulation of Evans blue, but completely prevented TNF-α-induced accumulation of the dye. To determine whether increased retinal vascular leakage, observed in TNF-α-injected eyes, was caused by alterations in the localization of tight junction proteins in the retinal blood vessels, retina whole flat mounts were immunolabeled for ZO-1 and occludin proteins after injection of the cytokine or cytokine with PKCζI-1. In PBS-injected rat eyes, ZO-1 and occludin immunoreactivity was intense and localized at the junctions of the cell membranes of endothelial cells in retinal vessels. In TNF-α-injected eyes, changes in ZO-1 immunostaining were particularly evident, which became markedly reduced and intermittently absent from the cell borders. These alterations were prevented by co-treatment with PKCζI-1 (Figure 2.14). Consistent with the results obtained with BREC in culture, the occludin protein content was not reduced with TNF-α but displayed regions with disorganized cell border labeling that were reversed by PKCζI-1 treatment.

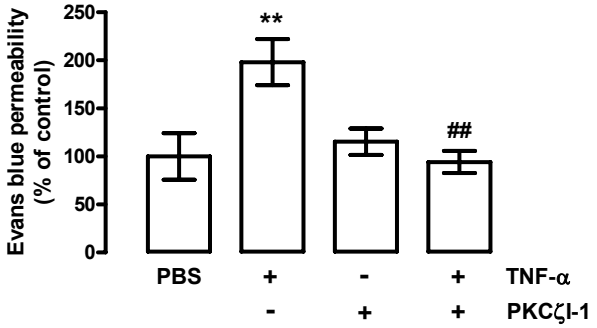

Figure 2.13. PKCζI-1 prevents TNF-α-induced retinal vascular permeability *in vivo*. Animals eyes were injected with PBS with 0.1% BSA, TNF-α (10 ng) (B,E); PKCζI-1 (280 ng) or with both PKCζI-1 and TNF-α (C,F) Evans blue leakage was evaluated 24 h after intravitreous injections. The results represent the mean ± SEM (n=7-8 animals per group) and are expressed relatively to control (PBS-injected eyes). $^{**}p<0.01$, significantly different from control as determined by ANOVA followed by Dunnett's *post* test. $^{\#\#}p<0.01$, significantly different from TNF-α, as determined by ANOVA followed by Bonferroni's *post* test.

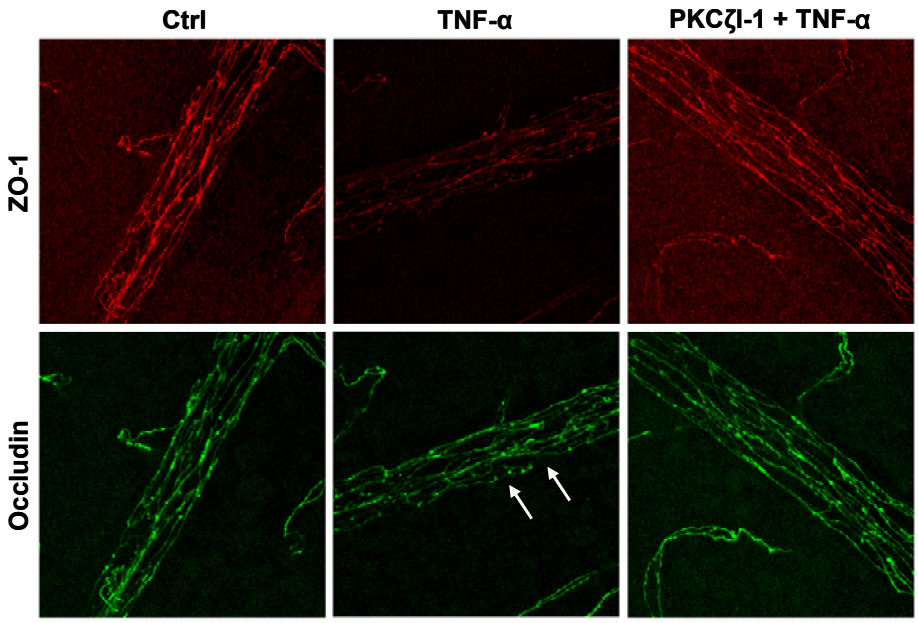

Figure 2.14. PKCζI-1 prevents the alterations in tight junction proteins induced by TNF-α *in vivo*. Animal eyes were injected with PBS with 0.1% BSA (Ctrl), TNF-α (10 ng); PKCζI-1 (280 ng) or with both PKCζI-1 and TNF-α. Whole retinas were immunolabeled for ZO-1 and occludin as described in Methods section. Images were obtained on a Leica TCS SP2 AOBS confocal microscope and are presented as a maximum projection. Arrows indicate loss and/or discontinuous cell border staining.

2.5 Discussion

It is well documented that alterations in the BRB occur early in the development of diabetic retinopathy (Cunha-Vaz *et al.*, 1975; Vinores *et al.*, 1989; Carmo, 1998). Diabetic retinopathy has been characterized as a low-grade chronic inflammatory disease (Carmo *et al.*, 1999; Miyamoto *et al.*, 1999; Carmo *et al.*, 2000; Joussen *et al.*, 2002a; Krady *et al.*, 2005). Elevated levels of proinflammatory cytokines, particularly IL-1β and TNF-α, have been identified in the vitreous of diabetic patients with retinopathy in patients with proliferative diabetic retinopathy (Abu el Asrar *et al.*, 1992; Demircan *et al.*, 2006). Also, increased levels of IL-1β and TNF-α in diabetic rat retinas have been correlated with increased BRB permeability (Carmo *et al.*, 2000; Joussen *et al.*, 2002a). However, the role of IL-1β and TNF-α on retinal microvascular barrier function has not been addressed. The results presented herein show that IL-1β and TNF-α increase retinal endothelial cell permeability and that TNF-α acts through PKCζ/NF-κB to reduce the expression and alter the distribution of the tight junction proteins claudin-5 and ZO-1. Moreover, glucocorticoid treatment completely prevented the TNF-α-induced increase in retinal

endothelial cell permeability, and this effect was mediated through both transactivation of the glucocorticoid receptor and transrepression of the NF-κB signaling pathway. Pharmacological inhibition of PKCζ reduced NF-κB activation and prevented TNF-α-induced retinal endothelial cell permeability both *in vitro* and *in vivo*.

The increase in retinal vascular permeability is associated with changes in the expression or distribution of the tight junction proteins. The content of occludin and ZO-1 decreases in the retinas of diabetic animals (Antonetti *et al.*, 1998; Barber *et al.*, 2000; Leal *et al.*, 2007). Also, VEGF treatment reduces occludin content and induces a rapid phosphorylation of occludin and ZO-1 in rat retinas and cultured BREC, concomitant with increased cell permeability (Antonetti *et al.*, 1998; Antonetti *et al.*, 1999a; Harhaj *et al.*, 2006). As TNF-α, a potent permeabilizing agent, is also associated with the pathogenesis of DR, the hypothesis that TNF-α increases retinal endothelial cell permeability through alterations in the tight junction complex, and the mechanisms involved, were investigated. Indeed, TNF-α downregulated ZO-1 and claudin-5 protein and mRNA expression, and decreased the junctional localization of both proteins, which associated with an increase in cell permeability. These results are consistent with previous publications demonstrating reduced claudin-5 gene expression after TNF-α treatment, concomitant with an increase in cell permeability, in brain capillary endothelial cells (Forster *et al.*, 2008; Burek and Forster, 2009). However, in these same studies a decrease in occludin was observed after TNF-α treatment, which contrast the increase in occludin observed in the present study. Further, TNF-α reduced occludin promoter activity in a human intestinal epithelial cell line (43 do paper). The cause for this difference is unclear but may relate to the use of cell lines versus primary cultures or differences in epithelial and endothelial cell types. Regardless, TNF-α consistently reduces both claudin-5 and ZO-1 expression, two tight junction proteins essential for barrier properties.

The effect of TNF-α and VEGF on retinal endothelial permeability is distinct. While VEGF treatment decreases occludin expression in retinal endothelial cells (Antonetti *et al.*, 1999a; Harhaj *et al.*, 2006), TNF-α induced an increase in both occludin protein and transcript expression. It is well established that expression of occludin correlates with the degree of barrier properties (Hirase *et al.*, 1997; Mitic and Anderson, 1998). Recent studies demonstrate that occludin becomes phosphorylated on multiple sites after VEGF treatment in a conventional PKC-dependent manner (Harhaj *et al.*, 2006), and Ser490 has recently been identified as a phosphorylation site necessary for VEGF-induced permeability (Sundstrom *et al.*, 2009).

Phosphorylation on Ser490 allows ubiquitination and subsequent endocytosis of occludin (Murakami *et al.*, 2009). Subsequent degradation of occludin by proteasome accounts for the decreased occludin content after VEGF treatment. TNF-α did not lead to an increase in occludin phosphorylation on Ser490 (data not shown) but rather led to an increase in occludin expression. Further, inhibition of conventional or novel PKC isoforms did not prevent TNF-α-induced cell permeability. These data demonstrate that VEGF and TNF-α alter the retinal barrier properties by distinct molecular mechanisms.

Identification of therapies that target common elements of VEGF and TNF-α signaling may provide a means to control the BRB in a number of retinopathies. Glucocorticoids have been used to preserve barrier properties and thereby decrease vascular permeability, particularly in the treatment of brain edema in response to tumor formation (Ruderman and Hall, 1965; Kaal and Vecht, 2004). The glucocorticoid receptor can activate or repress gene expression, depending on the response element sequence and promoter context (Lieberman and Nordeen, 1997; Barnes, 1998). In this study, dexamethasone treatment prevented the selective downregulation of ZO-1 and claudin-5 protein content and the concomitant increase in retinal endothelial permeability induced by TNF-α. Further, dexamethasone redistributed tight junction proteins towards the cell border although the effect on ZO-1 was more dramatic. Glucocorticoids have been shown to regulate tight junction organization and therefore cell permeability. In retinal endothelial cells, occludin and claudin-5 expression is upregulated in response to glucocorticoid treatment (Antonetti *et al.*, 2002; Felinski *et al.*, 2008). In mammary epithelial cells, glucocorticoids induce tight junction reorganization through a Ras-dependent mechanism (Woo *et al.*, 1999; Guan *et al.*, 2002), which is dependent on the glucocorticoid-mediated downregulation of fascin (Wong *et al.*, 1999), an actin-bundling protein that binds β-catenin. Glucocorticoids also induce the expression of β-catenin and stabilize a nonphosphorylated form of β-catenin at the cell periphery through glycogen synthase kinase-3 (GSK-3) degradation (Guan *et al.*, 2004; Failor *et al.*, 2007), leading to the reorganization of adherens junctions and subsequent tight junction formation. It is unclear if endothelial cells also utilize the same pathways for recruitment of tight junctions to the plasma membrane. However, glucocorticoids effectively completely prevented TNF-α induced permeability in BREC and promoted tight junction organization at the plasma membrane for claudin-5, ZO-1 and occludin.

The data presented here suggest that glucocorticoid receptor activation inhibits TNF-α-induced cell permeability through both transactivation and transrepression mechanisms. Inhibition of IKK

and adenovirus-mediated overexpression of IκBα blocked the increase in BREC permeability induced by TNF-α treatment. Together, these data demonstrate that TNF-α-induced retinal endothelial cell permeability is mediated, at least in part, by NF-κB. The mechanisms by which NF-κB may regulate cell permeability and the tight junction complex are largely unknown. NF-κB putative binding sites and several E-box sequences were identified within the claudin-5 promoter sequence (Burek and Forster, 2009). Recent studies demonstrated that NF-κB induces the expression of Snail and Slug transcription factors (Criswell and Arteaga, 2007; Julien *et al.*, 2007; Strippoli *et al.*, 2008), which repress E-cadherin, occludin, and claudin family members gene expression by binding to specific E-box sequences during epithelial-mesenchymal transition (Batlle *et al.*, 2000; Ikenouchi *et al.*, 2003; Martinez-Estrada *et al.*, 2006). Raf-1 mediated epithelial cell transformation is associated with induction of the transcriptional repressor Slug that inhibits occludin expression also through the occludin promoter E-box (Wang *et al.*, 2007). These reports suggest the possibility that a similar regulation by transcriptional repressors might also play a role in claudin-5 and ZO-1 expression in retinal endothelial cells. Interestingly, TNF-α upregulates the occludin transcript levels in retinal endothelial cells, suggesting that NF-κB may act as a positive regulator of occludin expression. Supporting such a mechanism is the presence of a putative NF-κB binding site within the occludin promoter (Mankertz *et al.*, 2000), and E-box sequences have been shown to repress occludin expression (Wang *et al.*, 2007). However, in this work, inhibiting NF-κB activity through PKCζI-1 did not prevent the occludin increase after TNF-α treatment suggesting an alternative mechanism of activation.

PKCζ has been shown to play a critical and selective role in the regulation of NF-κB. The inhibition of PKCζ with either pseudosubstrate inhibitors, anti-sense oligonucleotides or the transfection of kinase-dead dominant negative mutants of PKCζ dramatically impairs NF-κB activation (Diaz-Meco *et al.*, 1993; Dominguez *et al.*, 1993; Lozano *et al.*, 1994; Folgueira *et al.*, 1996). Also, PKCζ binds to IKKs *in vivo* and *in vitro* and specifically modulates IKKβ (Lallena *et al.*, 1999). Moreover, studies demonstrate that the atypical PKC binding protein p62/ZIP binds to receptor interactive protein (RIP) leading to the formation of a TNF-R/TRAF2/RIP/p62/PKCζ/IKK complex (Sanz *et al.*, 1999). These data position PKCζ upstream in the NF-κB pathway. Emerging evidence also suggests a second level of regulation of NF-κB activity by PKCζ independent of IkB degradation. In endothelial cells, it has been shown that the transcriptional activity of NF-κB is dependent on the phosphorylation of p65 subunit by PKCζ

(Anrather *et al.*, 1999). In embryonic fibroblast from PKCζ-deficient mice, the loss of PKCζ severely impairs NF-κB transcriptional activity as well as the phosphorylation of p65 in response to TNF-α (Leitges *et al.*, 2001). Further, PKCζ associates with and directly phosphorylates p65 *in vitro*, suggesting that PKCζ regulates p65 phosphorylation. Recent data demonstrate that PKCζ targets Ser311 *in vivo*, which is a critical PKCζ-dependent p65 phosphorylation site, essential for NF-κB transcriptional activity (Duran *et al.*, 2003). Ser311 is phosphorylated by PKCζ upon TNF-α stimulation, leading to an enhanced interaction of p65 with the cAMP response element binding (CREB) binding protein (CBP) and its recruitment to κB-dependent promoters. Importantly, in PKCζ-deficient cells, Ser311 phosphorylation in response to TNF-α is severely abrogated as well as the interaction of p65 with CBP and kb-dependent expression of IL-6. The observations that TNF-α-induced increase in IL-8 expression and NF-κB-dependent luciferase reporter expression upon TNF-α treatment are inhibited by the PKCζI-1 further support that PKCζ is important for NF-κB transcriptional activity.

The mechanism of activation of PKCζ remains to be fully clarified, but it has been shown to be an important downstream target of PI_3K, which through PDK-1 activation phosphorylates PKCζ at Thr410 (Chou *et al.*, 1998). PKCζ has also been shown to be activated by TNF-α (Lozano *et al.*, 1994; Muller *et al.*, 1995; Anrather *et al.*, 1999). TNF-α is known to activate PI_3K in endothelial cells through activation of the endothelial/epithelial tyrosine kinase (Etk). Etk is a novel regulator of epithelial cell junctions and mediates the TNF-α-induced PI_3K activation in vascular endothelial cells through transactivation of Etk and VEGFR2 (Zhang *et al.*, 2003). Interestingly, VEGFR2 activation by TNF-α fails to activate phospholipase C γ (PLCγ), which is known to activate classical PKC isoforms, such as PKCβ, an important mediator of VEGF-induced retinal endothelial vascular permeability (Aiello *et al.*, 1997; Harhaj *et al.*, 2006). In this study, PI_3K inhibition partially blocked TNF-α-induced cell permeability. Further, both pseudosubstrate inhibitor and the newly identified chemical inhibitor of PKCζ, PKCζ I-1, completely prevented the effects of TNF-α on cell permeability and tight junction complex, whereas classical PKC inhibitors failed. Targeting PKCζ reduced both NF-κB activation and likely inhibited additional signaling pathways that contribute to the regulation of the tight junction complex and retinal endothelial cell permeability induced by TNF-α. These data suggest that PKCζ has an important role in the regulation of TNF-α-induced retinal vascular permeability. Further, PKCζI-1 is also effective at blocking VEGF-induced permeability (Antonetti *et al.*, 2008). Although VEGF and TNF-α alter the tight junction complex by a distinct mechanisms, inhibition

of PKCζ is a common target for blocking both VEGF- and TNF-α-induced cell permeability. Therefore, targeting PKCζ may provide a specific therapeutic target for the prevention of vascular permeability in retinal diseases with elevated TNF-α and VEGF, including diabetic retinopathy.

CHAPTER 3

High glucose and interleukin-1β downregulate interleukin-1 type I receptor in retinal endothelial cells by enhancing its degradation through a lysosome-dependent mechanism

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3.1 Abstract

Diabetic retinopathy has been considered a low-grade chronic inflammatory disease. The production of interleukin-1β (IL-1β) in the retina is increased, and this finding has been correlated with an increase in blood-retinal barrier (BRB) permeability, suggesting that IL-1β might have an important role in the pathogenesis of diabetic retinopathy. However, in this context, no attention has been given to interleukin-1 type I receptor (IL-1RI), which is the receptor responsible for IL-1β-triggered effects. Therefore, the effect of high glucose and IL-1β on the IL-1RI regulation in retinal endothelial cells was investigated. A time-dependent downregulation of IL-1RI protein levels was detected in retinal endothelial cells exposed (1 – 24 h) to high glucose, mannitol or IL-1β. Long-term exposure (7 days) to high glucose or mannitol also decreased IL-1RI protein content. IL-1RI downregulation was due to its activation by IL-1β, since it was inhibited by the presence of anti-IL-1RI or anti-IL-1β antibodies. Moreover, IL-1RI downregulation was prevented by lysosome inhibitors, chloroquine and ammonium chloride, but not by proteasome inhibitors, MG132 and lactacystin. It was also found that IL-1RI translocates to the nucleus after high glucose or IL-1β treatment. In conclusion, the results indicate that high glucose, probably due to osmotic stress, and IL-1β downregulate IL-1RI in retinal endothelial cells. The downregulation of IL-1RI is triggered by its activation and is due, at least partially, to lysosomal degradation. High glucose and IL-1β also enhance the translocation of IL-1RI to the nucleus.

3.2 Introduction

Diabetic retinopathy is one of the most common complications of diabetes and a leading cause of vision impairment and blindness in developed countries, affecting 90% of patients over 20 years duration of diabetes (Aiello *et al.*, 1998; Cunha-Vaz, 2000). In the last decade, several important findings have improved our knowledge about the pathogenesis of diabetic retinopathy, however, the molecular mechanisms underlying this disease have not been completely elucidated yet and the treatments available are not effective.

Hyperglycemia is considered the major factor for the initiation and progression of diabetic retinopathy. Prolonged hyperglycemia results in microvascular dysfunction, including basement membrane thickening of retinal vessels, loss of pericytes and endothelial cells and BRB breakdown (DCCT Research Group, 1993; Cunha-Vaz, 2004).

A growing body of evidence indicates that diabetic retinopathy has several features that resemble a chronic inflammatory disease. Increased leukocyte adhesion to retinal vessels, which has been associated with increased expression of adhesion molecules and vascular permeability, are features commonly linked to inflammatory processes and also appear to play an important role in the progression of diabetic retinopathy (Antonetti *et al.*, 1998; Carmo, 1998; Miyamoto *et al.*, 1999; Joussen *et al.*, 2001b; Leal *et al.*, 2007). Increased levels of cytokines, such as IL-1β and tumor necrosis factor-alpha (TNF-α), have been found in the vitreous fluid of diabetic patients (Abu el Asrar *et al.*, 1992; Demircan *et al.*, 2006; Patel *et al.*, 2008). Moreover, it has been demonstrated that the expression of several genes involved in inflammatory processes and the levels of proinflammatory cytokines are increased in the retina of diabetic rats (Carmo *et al.*, 1999; Joussen *et al.*, 2001a; Joussen *et al.*, 2002a; Kowluru and Odenbach, 2004b; Gerhardinger *et al.*, 2005; Krady *et al.*, 2005).

In diabetic rat retinas, the increase in IL-1β levels was correlated with an increase in BRB permeability and treatment with cyclosporine A, an anti-inflammatory drug, decreased both IL-1β levels and vascular permeability (Carmo *et al.*, 1999; Carmo *et al.*, 2000). Experimental studies also showed that intravitreal administration of IL-1β increases vascular permeability, which appears to be mediated by leukocyte adhesion, nuclear factor-κB (NF-κB) activation and retinal capillary cell death (Bamforth *et al.*, 1997; Kowluru and Odenbach, 2004b), suggesting that this inflammatory cytokine might play an important role in the pathogenesis of diabetic retinopathy. However, the role of IL-1β in the development of diabetic retinopathy still remains to be clarified.

IL-1β is a proinflammatory cytokine that upregulates several inflammatory mediators, including IL-1β itself, TNF-α, cyclooxygenase 2 (COX-2), prostaglandins, inducible nitric oxide synthase (iNOS) and chemokines (Chung and Benveniste, 1990; Sparacio *et al.*, 1992; Chai *et al.*, 1996; Rothwell and Luheshi, 2000). IL-1β elicits responses in cells through the activation of the cell surface IL-1RI receptor, although IL-1β can also bind to interleukin-1 type II receptor (IL-1RII), which acts as a "decoy" receptor and does not lead to intracellular signaling processes, thus limiting the action of IL-1β. IL-1 binds first to IL-1RI and this complex then recruits the 66 kDa interleukin-1 receptor accessory protein (IL-1RAcP), which increases the binding affinity for IL-1 and is essential for signal transduction (Sims *et al.*, 1993; Cullinan *et al.*, 1998).

IL-1RI is a 80 kDa glycoprotein that was first described in T lymphocytes and fibroblasts (Dower *et al.*, 1985; Mizel *et al.*, 1987). It has been demonstrated that vascular endothelial cells express IL-1RI (Thieme *et al.*, 1987; Boraschi *et al.*, 1991; Konsman *et al.*, 2004). However, it has not been reported the presence of IL-1RI in retinal endothelial cells. Although retinal endothelial cells are an important target for IL-1β (Mantovani *et al.*, 1997; Pober, 1998), very little is known about the mechanisms that regulate IL-1RI expression, particularly under stress conditions, which could alter the responsiveness of these cells to IL-1β. As IL-1RI provides a crucial locus of control of IL-1β activity, and considering that this cytokine appears to play an important role in the pathogenesis of diabetic retinopathy, it is important to investigate the IL-1RI regulation in retinal endothelial cells. Thus, the purpose of this study was to investigate the effect of high glucose and IL-1β on IL-1RI regulation in retinal endothelial cells and elucidate the mechanisms involved.

3.3 Materials and Methods

3.3.1 Reagents

Low glucose Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from GIBCO (Invitrogen, Barcelona, Spain). Collagen type I was purchased from Biochrom AG (Berlin, Germany). Recombinant rat IL-1β, rabbit polyclonal anti-IL-1RAcP, mouse anti-lamin B and the alkaline phosphatase anti-rat IgG secondary antibodies were purchased from Chemicon (Temecula, CA, USA). The rat monoclonal anti-IL-1RI was purchased from R&D Systems (Minneapolis, IL, USA). The Complete Mini protease inhibitor cocktail tablets were from Roche Diagnostics (Mannheim, Germany). The bicinchoninic acid (BCA) protein assay kit was from Pierce (Rockford, IL, USA). The polyvinylidene difluoride (PVDF) membranes, alkaline

phosphatase anti-rabbit and anti-mouse IgG secondary antibodies and the enhanced chemifluorescence (ECF) reagent were obtained from Amersham Biosciences (Buckinghamshire, UK). Other reagents used in immunoblotting experiments were purchased from Bio-Rad (Hercules, CA, USA). The proteasome inhibitors, MG132 and lactacystin, were purchased from Boston Biochem (Cambridge, MA, USA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Merck (Darmstadt, Germany).

3.3.2 Cell culture and treatments

A conditionally immortalized retinal capillary endothelial cell line (TR-iBRB2), which was established from retinal capillaries isolated from transgenic rats carrying the temperature-sensitive Simian vacuolating virus 40 (SV-40) large T antigen gene, was used in this study. TR-iBRB2 cells have typical characteristics of primary retinal endothelial cells in culture, including spindle-shaped morphology, expression of factor VIII, vascular endothelial growth factor receptor 2 (VEGF-R2) and p-glycoprotein, among others (Hosoya *et al.*, 2001). The cells were grown on collagen type I–coated flasks in low glucose (5.5 mM) DMEM, supplemented with 10% FBS and antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin) at 33ºC with 5% CO_2.

To investigate the effect of high glucose on IL-1RI protein content, endothelial cells were exposed to 30 mM glucose for short-term (1, 3, 6, 12 and 24 h) and long-term (7 days) periods. TR-iBRB2 cells were also exposed to 24.5 mM mannitol (plus 5.5 mM glucose), which was used as an osmotic control. In addition, endothelial cells were exposed, in a time-dependent manner (1, 3, 6, 12 and 24 h), to 10 ng/ml IL-1β. In order to investigate if IL-1RI protein expression changes were triggered by IL-1RI receptor activation, cells were exposed simultaneously to high glucose, mannitol or IL-1β and either anti-IL-1β (1 µg/ml) or anti-IL-1RI (0.5 µg/ml) antibodies. Also, to investigate whether protein degradation was involved in IL-1RI downregulation, cells were exposed to either proteasome inhibitors, MG132 (10 µM) and lactacystin (10 µM), or lysosome inhibitors, chloroquine (100 µM) and ammonium chloride (NH_4Cl; 20 mM).

3.3.3 Cell lysates and fractioning

Cell lysates

After treatments, endothelial cells were washed twice with ice-cold phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) and then lysed with RIPA buffer: 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.5%

deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 1 mM dithiothreitol (DTT), supplemented with a complete mini protease inhibitor cocktail tablet. The lysates were incubated on ice for 30 min and sonicated six times (for 1 s each) to completely disrupt cells, and then stored at -80ºC until use. After thawing, the samples were centrifuged at 14,000 rpm for 10 min at 4ºC and supernatants (enriched in cytosolic and membrane proteins) were used to determine the protein concentration by the BCA colorimetric assay. Finally, the samples were denaturated with 6x concentrated electrophoresis sample buffer (0.5 M Tris-HCl, pH 6.8, 30% glycerol, 10.4% SDS, 0.6 M DTT, 0.02% bromophenol blue), boiled for 5 min at 95ºC and stored at -20ºC until use.

Total cell extracts

Cells were washed with ice-cold PBS and directly resuspended in Laemmli buffer to obtain the total protein fraction.

Cytosolic/membrane and nuclear protein fraction

Cytosolic/membrane and nuclear protein fractions were prepared according to the method described by Levrand and colleagues (Levrand *et al.*, 2005), with minor modifications. Briefly, endothelial cells were washed twice with ice-cold PBS and lysed with a hypotonic buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.4% NP-40, 1 mM DTT), supplemented with a complete mini protease inhibitors tablet. The lysates were incubated on ice for 30 min and centrifuged at 14,000 rpm for 5 min at 4ºC. The supernatant containing the cytosolic/membrane fraction was collected and stored at -80ºC until use. The resulting pellet was ressuspended in high-salt extraction buffer (20 mM HEPES, pH 7.9, 420 mM NaCl, 1 mM EDTA, 10% glycerol, 1 mM DTT), supplemented with a complete mini protease inhibitors tablet. The nuclear suspension was agitated for 1 h at 4ºC and centrifuged at 14,000 rpm for 5 min at 4ºC. For Western blotting analysis, both cytosolic/membrane and nuclear extracts were denaturated with 6x concentrated electrophoresis sample buffer and boiled for 5 min at 95ºC.

3.3.4 Western blotting

For immunodetection of IL-1RI and IL-1RAcP, equal amounts of total protein (50 or 100 µg for IL-1RI detection from whole cell lysates or cytosolic/membrane and nuclear fractions, respectively; 30 µg for IL-1RAcP detection), were loaded per lane and separated by electrophoresis on 4-9% sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE). After

electrophoretic transfer to PVDF membranes, the membranes were blocked with 5% non-fat milk in Tris-buffered saline (20 mM Tris, 137 mM NaCl, pH 7.6) containing 0.1% Tween 20 (TBS-T) for 1 h. Membranes were then incubated with a rat monoclonal anti-IL-1RI (1:500) or rabbit polyclonal anti-IL-1RAcP (1:2,000) antibody for 2 h in TBS-T containing 1% non-fat milk. To control for protein loading, membranes were also incubated with mouse anti-β-actin (1:20,000), mouse anti-β-tubulin (1:300,000) or mouse anti-lamin B1 (1:100) antibodies. After 5 washes with washing buffer (TBS-T containing 0.5% nonfat milk), the membranes were incubated with the alkaline phosphatase anti-rat, anti-rabbit or anti-mouse IgG secondary antibodies (1:20,000) for 1 h in TBS-T containing 1% non-fat milk. After incubation, the membranes were washed 5 times in washing buffer and protein immunoreactive bands were visualized using the enhanced chemifluorescence (ECF) substrate. Fluorescence was detected on an imaging system (STORM 860 gel and blot image system, Molecular Dynamics, Amersham Biosciences, Uppsala, Sweden). The optical density of the bands was quantified with the Image Quant 5.0 Software (Molecular Dynamics, Amersham Biosciences, Uppsala, Sweden).

3.3.5 Statistical analysis

The results are expressed as mean±standard error of the mean (SEM). Data were analyzed using one-way analysis of variance (ANOVA) followed by Dunnett's or Bonferroni's post test. Prism 4.0 (GraphPad Software, San Diego, CA) was used for all statistical analysis.

3.4 Results

3.4.1 High glucose decreases IL-1RI protein levels in retinal endothelial cells

Retinal endothelial cells were exposed to 30 mM glucose (short- and long-term exposures) to investigate the effect of high glucose (hyperglycemic condition) on IL-1RI protein levels, which were evaluated by Western blotting. Endothelial cells exposure to mannitol (24.5 mM + 5.5 mM glucose) was used as a control for osmolarity. Short-term exposure (1-24 h) to high glucose downregulated IL-1RI protein levels in a time-dependent manner. After 6 h exposure, high glucose induced a significant decrease in IL-1RI protein levels (82.2±4.8% of control), and the downregulation of IL-1RI persisted after 24 h exposure (71.3±2.9% of control; Figure 3.1A). Similarly, short-term exposure to mannitol decreased IL-1RI protein levels. After 24 h exposure, IL-1RI protein levels decreased to 75.3±3.9% of control (Figure 3.1B). It was also analyzed the

effect of a chronic exposure (7 days) to high glucose and mannitol on IL-1RI protein content. Long-term exposure to high glucose and mannitol induced a significant decrease (84.2±4.8% and 75.2±2.7% of control, respectively) in IL-1RI protein levels (Figure 3.1C).

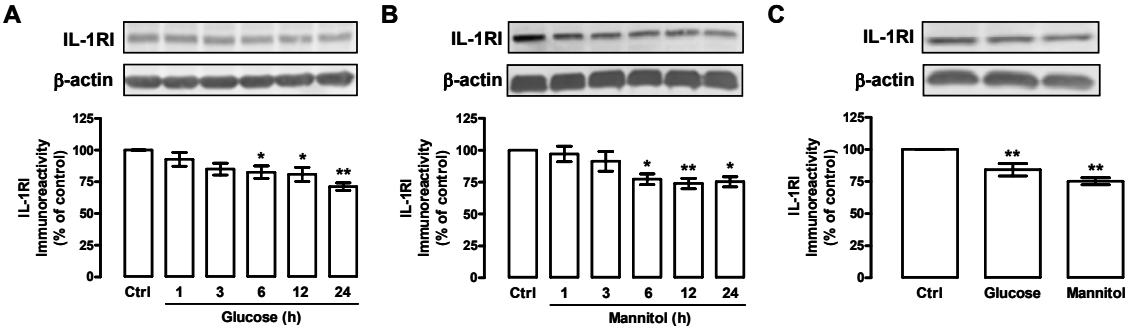

Figure 3.1. Effect of high glucose and mannitol on IL-1RI protein levels in retinal endothelial cells. Cells were exposed to 30 mM glucose **(A)**, or 24.5 mM mannitol (plus 5.5 mM glucose) **(B)** for 1, 3, 6, 12 and 24 h, or for 7 days **(C)**. IL-1RI immunoreactivity was analyzed by Western blotting in cell lysates, enriched in membrane and cytosolic fractions. Representative Western blots for IL-1RI and β-actin (loading control) are presented above the graphs. The intensity of the bands was determined by quantitative densitometric analysis. The results represent the mean±SEM of, at least, four independent experiments and are expressed as percentage of control. *p<0.05, **p<0.01; significantly different from control as determined by ANOVA followed by Dunnett's *post* test.

3.4.2 IL-1β downregulates IL-1RI protein levels in retinal endothelial cells

Since the levels of IL-1β have been shown to be increased in the retinas of diabetic animals (Carmo *et al.*, 1999; Kowluru and Odenbach, 2004b), the effect of IL-1β on IL-1RI protein expression in retinal endothelial cells was also evaluated. Endothelial cells were exposed to 10 ng/ml IL-1β for 1, 3, 6, 12 and 24 h. IL-1β induced a rapid downregulation of IL-1RI (81.1±8.6% of control after 1 h exposure; Figure 3.2). This donwregulation was still observed after 3, 6 and 12 h exposure to IL-1β (72.7±5.0%, 70.2±3.3% and 81.4±3.9% of control, respectively), but not after 24 h exposure (93.2±6.7% of control, p<0.05).

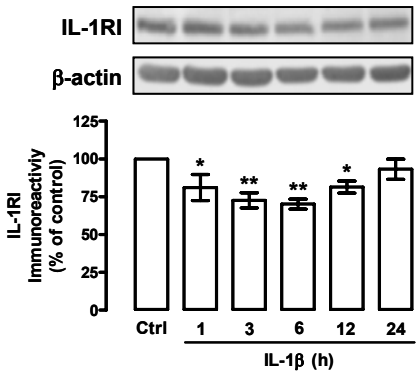

Figure 3.2. Effect of IL-1β on IL-1RI protein levels in retinal endothelial cells. Cells were exposed to 10 ng/ml IL-1β for 1, 3, 6, 12 and 24 h. IL-1RI immunoreactivity was analyzed by Western blotting in cell lysates, enriched in membrane and cytosolic fractions. Representative Western blots for IL-1RI and β-actin (loading control) are presented above the graphs. The intensity of the bands was determined by quantitative densitometric analysis. The results represent the mean±SEM of, at least, seven independent experiments and are expressed as percentage of control. *$p<0.05$, **$p<0.01$; significantly different from control as determined by ANOVA followed by Dunnett's *post* test.

3.4.3 IL-1RAcP protein content in retinal endothelial cells is not affected by high glucose and IL-1β

IL-1RAcP is an essential component of the IL-1RI complex, being required for the activation of the signal transduction cascade upon ligand binding to IL-1RI. Since high glucose, mannitol and IL-1β decreased IL-1RI protein levels in retinal endothelial cells, we also evaluated whether IL-1RAcP protein content could be affected under these conditions. Short- (1-24 h) and long-term (7 days) exposure to high glucose and mannitol did not change IL-1RAcP protein levels in endothelial cells (Figure 3.3A-C). The protein content of IL-1RAcP was also unchanged by IL-1β treatment (Figure 3.3D).

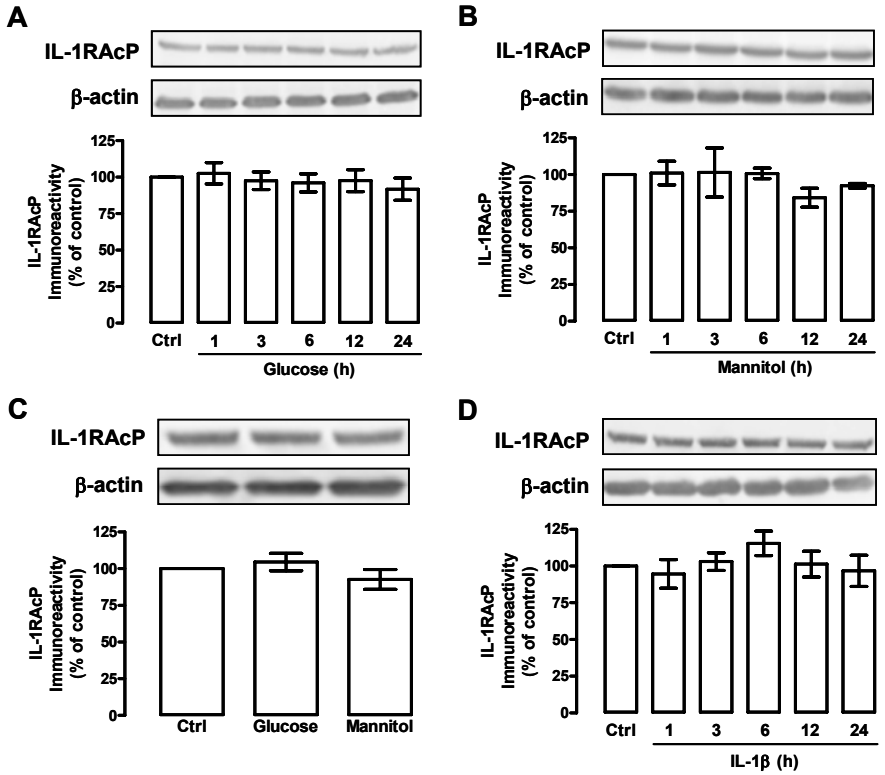

Figure 3.3. Effect of high glucose, mannitol and IL-1β on IL-1RAcP protein levels in retinal endothelial cells. Cells were exposed to 30 mM glucose (**A**), 24.5 mM mannitol (plus 5.5 mM glucose) (**B**) or 10 ng/ml IL-1β (**C**) for 1, 3, 6, 12 and 24 h. Cells were also exposed to high glucose and mannitol for 7 days (**D**). IL-1RAcP immunoreactivity was analyzed by Western blotting in cell lysates, enriched in membrane and cytosolic fractions. Representative Western blots for IL-1RAcP and β-actin (loading control) are presented above the graphs. The intensity of the bands was determined by quantitative densitometric analysis. The results represent the mean±SEM of, at least, four independent experiments and are expressed as percentage of control.

3.4.4 IL-1β- and high glucose-induced IL-1RI downregulation is prevented by anti-IL-1β or anti-IL-1RI treatment

To assess if IL-1RI downregulation induced by high glucose or mannitol was triggered by the activation of IL-1RI receptor, endothelial cells were co-treated, for 24 h, with high glucose or mannitol and either anti-IL-1β (1 μg/ml) or anti-IL-1RI (0.5 μg/ml) antibodies. The presence of the neutralizing antibodies prevented the decrease in IL-1RI content induced by high glucose and mannitol (Figure 3.4A). Using an ELISA assay, we measured the content of IL-1β in the extracellular medium of endothelial cell cultures, to analyze whether IL-1β levels were increased

in high glucose- and mannitol-treated cells, but the levels of IL-1β were below the detection limit of the kit (data not shown). We also investigated whether the activation of IL-1RI by IL-1β was mediating the downregulation of IL-1RI induced by exposure to IL-1β. For that, endothelial cells were exposed simultaneously, for 6 h, to IL-1β and anti-IL-1β (1 μg/ml) or anti-IL-1RI (0.5 μg/ml) antibodies. As shown in Figure 3.4B, both anti-IL-1β and anti-IL-1RI antibodies prevented the IL-1β-induced decrease in IL-1RI protein levels (110.5±7.7% and 114.5±11.1% of control, respectively).

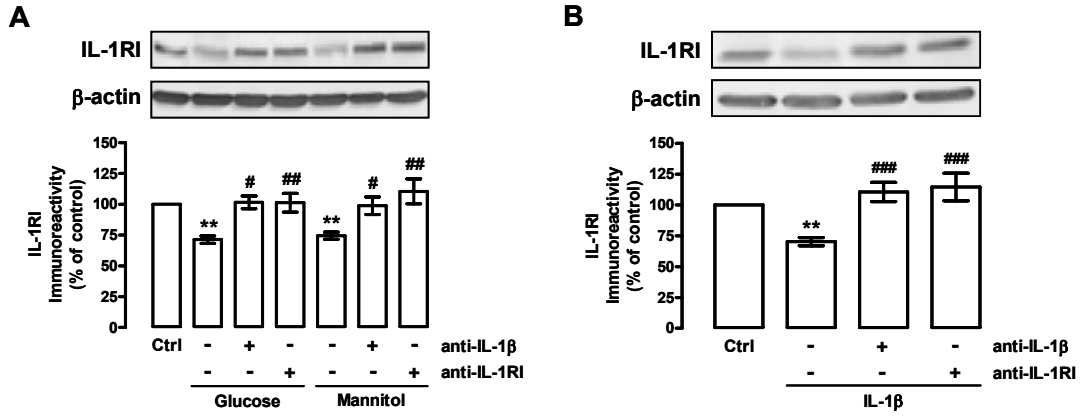

Figure 3.4. Effect of IL-1β and IL-1RI blockage on IL-1RI downregulation induced by high glucose, mannitol and IL-1β. A. Endothelial cells were co-incubated with 30 mM glucose or 24.5 mM mannitol (plus 5.5 mM glucose) with anti-IL-1β (1 μg/ml) or anti-IL-1RI (0.5 μg/ml) antibodies for 24 h. **B.** Cells were treated with anti-IL-1β or anti-IL-1RI antibodies for 30 min before the addition of 10 ng/ml IL-1β (IL-1β) for 6 h. IL-1RI immunoreactivity was analyzed by Western blotting in cell lysates, enriched in membrane and cytosolic fractions. Representative Western blots for IL-1RI and β-actin (loading control) are presented above the graphs. The intensity of the bands was determined by quantitative densitometric analysis. The results represent the mean±SEM of, at least, four independent experiments and are expressed as percentage of control. *p<0.05, **p<0.01, significantly different from control as determined by ANOVA followed by Dunnett's *post* test. #p<0.05, ##p<0.01, ###p<0.001, significantly different from high glucose, mannitol or IL-1β, as determined by ANOVA followed by Bonferroni's *post* test.

3.4.5 IL-1RI downregulation is due to lysosomal degradation

The degradation of receptors, mediated by the proteasome and lysosome pathways, represents an important mechanism to control their amount at cell surface and thus modulate the biological activity of cytokines. To determine the role of proteasome and lysosome in the IL-1RI downregulation, cells were pre-treated with proteasome inhibitors, MG132 (10 μM) and lactacystin (10 μM), or lysosome inhibitors, chloroquine (100 μM) and NH_4Cl (20 mM), for 30

min, before the incubation with high glucose, mannitol or IL-1β for 6 h. The proteasome inhibitors MG132 and lactacystin were unable to prevent the decrease in IL-1RI protein content induced by high glucose or mannitol (Figure 3.5A and 3.5B). Also, when cells were exposed to IL-1β in the presence of MG132 or lactacystin, the IL-1RI protein levels were similar to those found in cells treated only with IL-1β (Figure 3.5C). On the other hand, chloroquine and NH_4Cl alone significantly increased IL-1RI protein content to 138.6±8.3% and 160.8±7.0% of control, respectively (Figure 3.5C-E). Moreover, the IL-1RI downregulation induced by high glucose was prevented by pre-treatment with either chloroquine or NH_4Cl (140.8±2.8% and 118.9±1.8% of control, respectively; Figure 3.5D). Similarly, lysosome inhibitors prevented mannitol-induced IL-1RI downregulation (Figure 3.5E). As shown in Figure 3.5F, chloroquine and NH_4Cl also prevented the downregulation of IL-1RI induced by IL-1β (120.8±6.8% and 131.8±9.2% of control, respectively). These results indicate that IL-1RI is degraded by the lysosome and not by the proteasome.

3.4.6 IL-1RI translocates to the nucleus after its activation

Previous reports have shown that after IL-1β binding to its receptor, the receptor complex is internalized and translocates to the nucleus (Qwarnstrom *et al.*, 1988; Heguy *et al.*, 1991). The endothelial cell extracts used herein were enriched in membrane and cytosolic proteins. For instance, when we performed Western Blotting against lamin B, a nuclear envelope protein that has been used as a nuclear marker, we could not detect any signal (data not shown). Therefore, assuming that our cell lysates are virtually devoid of nuclear proteins, we hypothesized that translocation of the IL-1RI to the nucleus may also account for the decrease in IL-1RI protein levels.

To better understand the intracellular trafficking of IL-1RI, we also prepared total, cytosolic/membrane and nuclear protein extracts, in addition to cell lysates enriched in cytosolic and membrane fraction obtained with the RIPA buffer, from endothelial cells exposed to high glucose or mannitol for 24 h or to IL-1β for 3 and 6 h. As shown in Figure 7, IL-1RI protein content in total cell extracts did not change in cells exposed to high glucose, mannitol or IL-1β. On the other hand, there was a decrease in IL-1RI protein levels in the cytosolic/membrane fraction. These results are consistent with the observed downregulation of IL-1RI detected in RIPA cell extracts of high glucose-, mannitol- and IL-1β-treated cells.

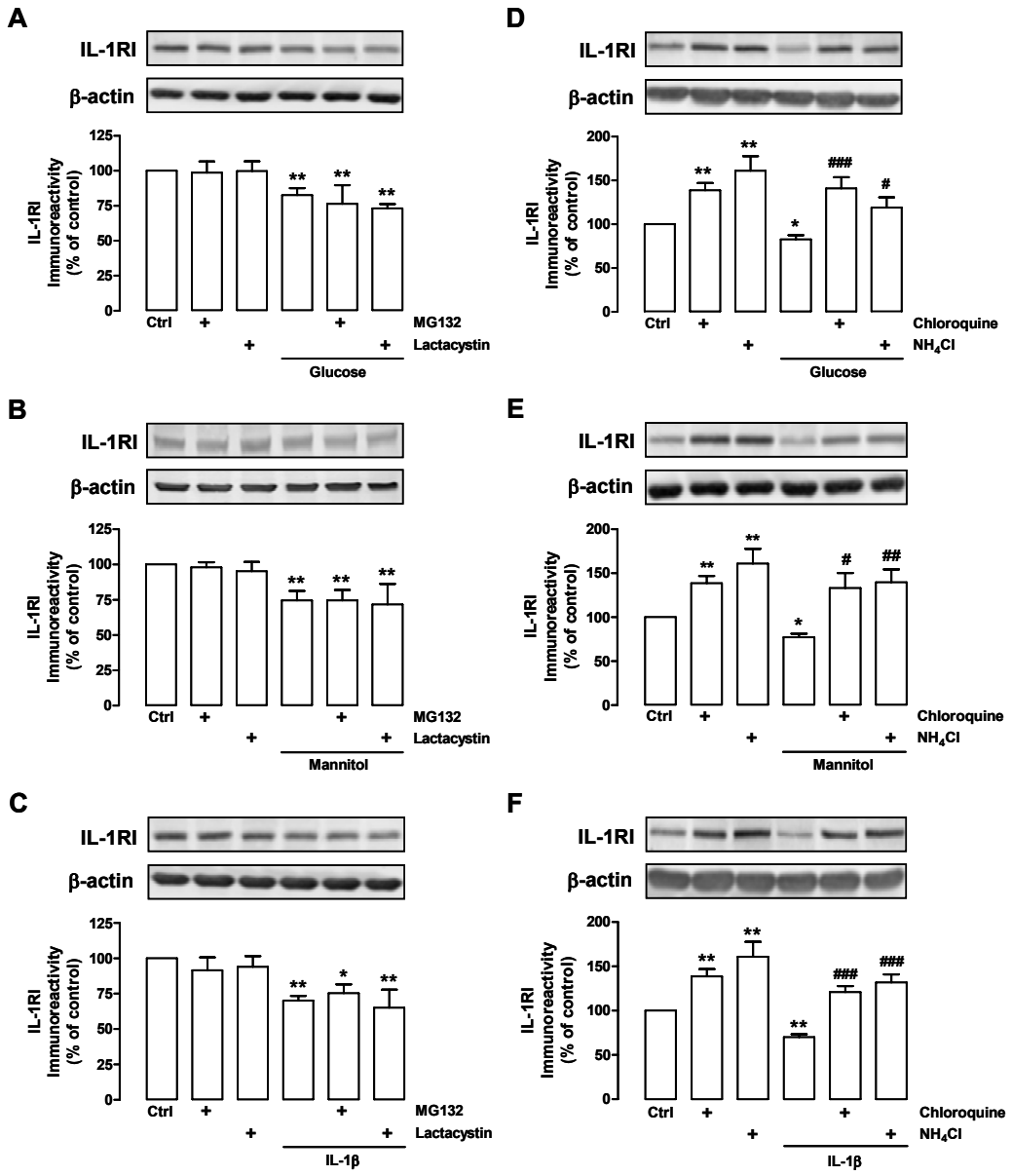

Figure 3.5. Lysosome inhibitors prevent high glucose- and IL-1β-induced IL-1RI downregulation. Cells were exposed to 30 mM glucose (**A**, **D**), 24.5 mM mannitol (plus 5.5 mM glucose; **B**, **E**) or 10 ng/ml IL-1β (**C**, **F**) in the presence of proteasome inhibitors, MG132 (10 μM) and lactacystin (10 μM) (**A**, **B**, **C**), or lysosome inhibitors, chloroquine (100 μM) and NH_4Cl 20 mM (**D**, **E**, **F**), for 6 h. IL-1RI immunoreactivity was analyzed by Western blotting in cell lysates, enriched in membrane and cytosolic fractions. Representative Western blots for IL-1RI and β-actin (loading control) are presented above the graphs. The intensity of bands was determined by quantitative densitometric analysis. The results represent the mean±SEM of, at least, three independent experiments and are expressed as percentage of control.

*p<0.05, **p<0.01, significantly different from control as determined by ANOVA followed by Dunnett's *post* test. #p<0.05, ###p<0.001, significantly different from high glucose or IL-1β, as determined by ANOVA followed by Bonferroni's *post* test.

The results also show that IL-1RI is present in nuclear fractions of retinal endothelial cells in basal conditions (Figure 3.6). Both high glucose and mannitol induced an accumulation of IL-1RI in nuclear fractions (122.4±5.3% and 127.0±4.8% of control, respectively), as shown in Figure 3.6A and B. Also, in nuclear extracts of IL-1β-treated cells, we detected a significant increase in IL-RI protein content. After 3 h treatment, IL-1RI protein content increased to 140.1±6.9% of the control. This increase was still observed after 6 h exposure to IL-1β (Fig. 7B).

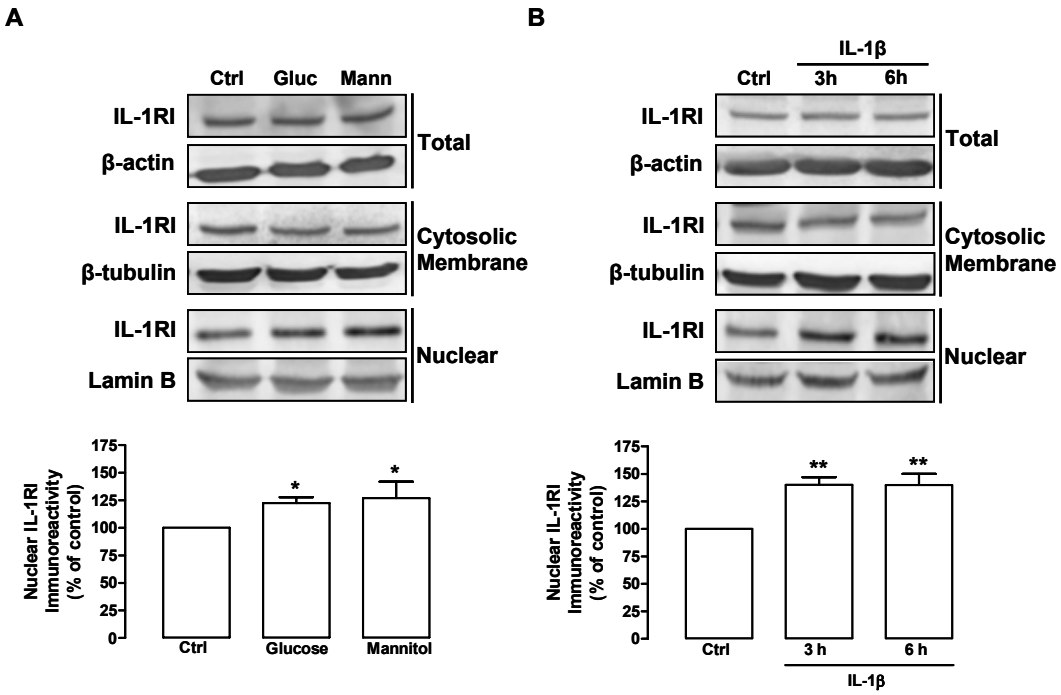

Figure 3.6. High glucose and IL-1β induce IL-1RI translocation to the nucleus. Endothelial cells were exposed to 30 mM glucose or 24.5 mM mannitol (plus 5.5 mM glucose) for 24 h (A), or to 10 ng/ml IL-1β for 3 and 6 h (B). IL-1RI immunoreactivity was analyzed in total, cytosolic/membrane and nuclear extracts by Western blotting. Representative Western blots for IL-1RI and β-actin, β-tubulin or lamin B (loading controls for the total, cytosolic/membrane and nuclear fractions, respectively) are presented above the graphs. The intensity of the bands in nuclear extracts was determined by quantitative densitometric analysis. The results represent the mean±SEM of, at least, six independent experiments and are expressed as percentage of control. *p<0.05, **p<0.01; significantly different from control as determined by ANOVA followed by Dunnett's post test.

3.5 Discussion

In this study, we show that high glucose and IL-1β downregulate IL-1RI protein content in retinal endothelial cells. The downregulation of IL-1RI in retinal endothelial cell extracts, enriched in membrane and cytosolic fractions, is due, at least partially, to lysosomal, but not proteosomal, degradation. However, the translocation and accumulation of IL-1RI in the nucleus might also account for the observed downregulation.

Hyperglycemia is considered a major risk factor for the development of diabetic retinopathy and retinal vasculature is a prime target of hyperglycemic damage (Cunha-Vaz, 2004). In the past years, a growing body of evidence indicates that diabetic retinopathy has characteristics of a low grade chronic inflammatory disease, which includes the increased production of cytokines, such as IL-1β and TNF-α, increased expression of adhesion molecules and increased vascular permeability (Carmo, 1998; Miyamoto *et al.*, 1999; Joussen *et al.*, 2001b; Demircan *et al.*, 2006). Moreover, the increase of IL-1β levels was correlated with an increase in the blood-retinal barrier permeability (Carmo *et al.*, 1999; Carmo *et al.*, 2000), suggesting that this cytokine might have an important role in the pathogenesis of DR. Although retinal endothelial cells are affected by IL-1β (Bamforth *et al.*, 1997; Kowluru and Odenbach, 2004c), no attention has been given to its receptor, IL-1RI, which mediates IL-1β proinflammatory action (Boraschi *et al.*, 1991).

We used a retinal endothelial cell line (TR-iBRB2 cells) as an in vitro model, which has been described as a good model for endothelial cells of the inner blood-retinal barrier (Hosoya *et al.*, 2001). To mimic hyperglycemic conditions, endothelial cells were exposed to elevated glucose. Cells were also exposed to mannitol, which was used as an osmotic control. In addition, we also exposed TR-iBRB2 cells to IL-1β to mimic an inflammatory condition.

High glucose decreased IL-1RI protein content in retinal endothelial cells. However, exposure to mannitol induced similar effects, suggesting that the IL-1RI downregulation was due to an increase in osmolarity, instead of any direct effect of glucose itself. This observation is in accordance with previous findings by Rosette & Karin (Rosette and Karin, 1996), who demonstrated that high osmolarity can result in clustering and internalization of surface receptors for epidermal growth factor (EGF), IL-1 and TNF without the presence of the specific ligands, probably by inducing alterations in plasma membrane or conformational changes in membrane proteins structure. Our results also show that the downregulation of IL-1RI occurs relatively early after exposure to high glucose or mannitol (6 h), and that IL-1RI downregulation

is maintained when cells are under those conditions for longer periods (7 days), what seems to be a compensatory mechanism against osmotic stress.

IL-1β treatment also induced a downregulation of IL-1RI protein levels in a time-dependent manner in these cells, suggesting that retinal endothelial cells reduce their responsiveness to IL-1β by decreasing IL-1RI. Indeed, previous studies have shown that IL-1β induces a downregulation in the IL-1RI mRNA and/or protein expression in T cells (Ye *et al.*, 1992), epithelial cells (McGee *et al.*, 1996) and fibroblasts (Takii *et al.*, 1994), supporting our findings with TR-iBRB2 cells. However, and contrary to what was found in high glucose- and mannitol-treated cells, the downregulation of IL-1RI is transient when cells are directly exposed to IL-1β, probably because permanent elevated glucose induces higher levels of stress.

Contrarily to IL-1RI, the protein levels of IL-1RAcP, which is an essential component of the IL-1RI complex, remained unchanged, suggesting that this protein is more resistant to stress conditions and that adaptive changes to IL-1 triggered responses are regulated essentially at the IL-1RI level.

The downregulation of IL-1RI protein content induced by high glucose, mannitol or IL-1β was completely prevented by anti-IL-1β and anti-IL-1RI antibodies, indicating that IL-1RI activation by IL-1β is a necessary step in this process. Thus, this observation suggests that retinal endothelial cells can produce and release IL-1β, which in turn activates IL-1RI receptor, inducing its own downregulation. We measured IL-1β levels in the culture medium by ELISA in cell cultures treated with high glucose and mannitol, but IL-1β levels were below the detection levels of the kit (data not shown). However, it has been claimed that fewer than 10 ligand-occupied receptors per cell are required to induce an efficient activation of the IL-1β/IL-1RI signaling cascade (Sims and Dower, 1994; Dinarello, 1996), which supports the observations that both antibodies, anti-IL-1β and anti-IL-1RI, prevented IL-1RI downregulation induced by high glucose and mannitol. Moreover, in the diabetic retina, microglia and Müller cells are activated and are responsible for the production of high levels of pro-inflammatory cytokines that in turn will activate IL-1RI present in endothelial cells (Rungger-Brandle *et al.*, 2000; Zeng *et al.*, 2000; Gerhardinger *et al.*, 2005; Krady *et al.*, 2005).

IL-1β is a pleiotropic pro-inflammatory cytokine that regulates a wide range of immunological and inflammatory processes. Therefore, cellular responsiveness to this cytokine must be tightly regulated. As IL-1β only signals through the activation of the IL-1RI, an important mechanism to regulate the IL-1β-induced intracellular events is by controlling receptor cell surface levels.

Herein, retinal endothelial cells downregulate IL-1RI protein content in response to high glucose and IL-1β, probably as a negative feedback mechanism to regulate the receptor availability and thus limiting cell sensitivity to IL-1β. The observation that high glucose and IL-1β induce a significant decrease in IL-1RI protein levels led us to hypothesize that such decrease could result from an increase in IL-1RI protein degradation. The results demonstrate that IL-1RI was degraded via the lysosomal pathway and not by the proteasome pathway, since the decrease in IL-1RI content induced by high glucose and IL-1β was completely blocked by the lysosome inhibitors, chloroquine and NH_4Cl. On the contrary, the proteasome inhibitors, MG132 and lactacystin, did not prevent the IL-1RI downregulation.

As mentioned above, the downregulation of IL-1RI protein levels appears to be mainly due to its degradation in the lysosome. However, several reports have shown that IL-1RI mediates IL-1 trafficking into the nucleus. The translocation of receptor-bound IL-1 to the nucleus was claimed to contribute to or to be essential for gene transactivation (Qwarnstrom *et al.*, 1988; Curtis *et al.*, 1990; Heguy *et al.*, 1991). Since the endothelial cell extracts used in this work were enriched in membrane and cytosolic proteins, we hypothesized that the decrease in IL-1RI protein levels could also be due, at least partially, to its translocation to the nucleus. In fact, we found that high glucose, mannitol and IL-1β increased the IL-1RI protein levels in the nuclear fraction. A similar effect was observed in human fibroblasts (Qwarnstrom *et al.*, 1988) and chinese hamster ovary (CHO) cell line (Heguy *et al.*, 1991), where after IL-1β binding, the receptor complex is internalized and translocates to the nucleus. These findings suggest that under high glucose or inflammatory conditions, in retinal endothelial cells, IL1-RI receptors are activated by IL-1β and a fraction of these receptors is internalized and goes into the nucleus where they might control gene expression. Other receptors are directed for lysosomal degradation, which is the way cells use to regulate the processes linked to IL1-RI activation, preventing an overactivation of IL-1β-triggered signaling pathways.

In summary, we show that high glucose, by osmotic stress, and IL-1β downregulate IL-1RI content in retinal endothelial cells. The downregulation of IL-1RI is triggered by its activation and is due, at least partially, to lysosomal degradation and translocation and accumulation in the nucleus. Increasing evidence suggest that IL-1β might play an important role in endothelial cell dysfunction and BRB breakdown in diabetes. It was reported that intravitreous injection of IL-1β or in vitro exposure of retinal endothelial cells to this cytokine induces endothelial cell degeneration (Kowluru and Odenbach, 2004c), which seems to be mediated by NF-κB (Kowluru

and Odenbach, 2004b; Kowluru and Odenbach, 2004c). In addition, increased levels of IL-1β in rat retinas correlate with BRB breakdown, which is prevented by the treatment with cyclosporine A, an anti-inflammatory drug (Carmo *et al.*, 2000). Furthermore, inhibition of caspase-1, the enzyme responsible for the cleavage of the pro-IL-1β into IL-1β, by minocycline, prevented the diabetes-induced increase in IL-1β and the degeneration of retinal capillary endothelial cells in diabetic rats (Vincent and Mohr, 2007). Likewise, inhibition of the IL-1β signaling pathway by using IL-1RI-deficient mice protected the animals from diabetes-induced retinal pathology (Vincent and Mohr, 2007), suggesting that IL-1RI may play a key role in the development of diabetic retinopathy. Since IL-1β might play an important role in diabetic retinopathy and IL-1RI provides a crucial locus of control of IL-1 activity, blocking the activation of IL-1RI should be taken into account as a possible therapeutic strategy for the treatment of diabetic retinopathy.

CHAPTER 4

General discussion

CHAPTER 4

General discussion

Diabetic retinopathy is a leading cause of vision impairment and blindness in working-age adults in the western countries (Aiello *et al.*, 1998). In the past years, several important findings have improved our knowledge about the pathogenesis of diabetic retinopathy. However, the disease is still not curable, and the available treatments are not very effective against the progression of the disease.

Retinal microvasculature dysfunction is one of the earliest 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 directly associated with visual loss (Klein *et al.*, 1995a; Aiello, 1997; Moss *et al.*, 1998; Caldwell *et al.*, 2003). VEGF is a potent permeabilizing agent and its contribution to the BRB breakdown is well established (Aiello, 1997; Caldwell *et al.*, 2003).

Over the last decade, increasing evidence indicates that low-grade chronic inflammatory processes play an important role in the pathogenesis of diabetic retinopathy. The increase in cytokine and growth factor levels, increased leukostasis and BRB permeability, and the activation of microglia are common characteristics of diabetic retinopathy and occur very early in the course of the disease (Carmo *et al.*, 1999; Miyamoto *et al.*, 1999; Carmo *et al.*, 2000; Joussen *et al.*, 2002a; Krady *et al.*, 2005). For instance, increased levels of the proinflammatory cytokines IL-1β and TNF-α have been found in the vitreous of diabetic patients, particularly in patients with proliferative diabetic retinopathy (Abu el Asrar *et al.*, 1992; Demircan *et al.*, 2006), and in the retinas of diabetic animals, which correlated with increased BRB permeability (Carmo *et al.*, 2000; Joussen *et al.*, 2002a; Kowluru and Odenbach, 2004c; Krady *et al.*, 2005). However, the contributions and the molecular mechanisms underlying the increase in retinal vascular permeability induced by these inflammatory mediators have not been elucidated yet. Therefore, the main goal of this study was to investigate the effect of IL-1β and TNF-α on retinal endothelial cell permeability, and the molecular mechanisms underlying the increase in cell permeability induced by TNF-α, which was found to be more potent permeabilizing factor than

IL-1β. Additionally, the regulation of the (IL-1RI) content in endothelial cells exposed to elevated glucose or IL-1β was also investigated.

The increase in retinal vascular permeability has been associated with alterations in the content and cellular localization of the tight junction proteins. Both diabetes and VEGF reduce the content of occludin (Antonetti *et al.*, 1998) and cause a redistribution of the protein in retinal endothelial cells (Barber *et al.*, 2000; Barber and Antonetti, 2003). In addition, VEGF induces occludin phosphorylation, which correlates with its cellular redistribution and increased cell permeability (Antonetti *et al.*, 1998; Antonetti *et al.*, 1999b; Harhaj *et al.*, 2006). More recently, multiple occludin phosphorylation sites have been identified and, one of these sites, Ser490, is VEGF-responsive. In fact, phosphorylation at this site reduces occludin interaction with ZO-1, which my lead to tight junction disruption (Sundstrom *et al.*, 2009). A decrease in claudin-5 and ZO-1 content has also been correlated with increased retinal vascular permeability in diabetic rats (Jung *et al.*, 2001; Leal *et al.*, 2007; Ambrosio *et al.*, 2009; Bucolo *et al.*, 2009; Klaassen *et al.*, 2009). VEGF treatment also reduces claudin-5 (Klaassen et al., 2009) and ZO-1 (Ghassemifar et al., 2006; Peters et al., 2007) protein and mRNA levels in retinal endothelial cells. In this study, TNF-α induced a marked downregulation of ZO-1 and claudin-5 expression and a decrease in the content of these tight junction proteins at the cell membrane. However, TNF-α induced an increase in occludin expression, but its localization at the cell border was discontinuous. The alterations in the tight junction complex correlated with an increase in cell permeability. These results demonstrate that both TNF-α and VEGF alter the tight junction complex to induce cell permeability, but through different molecular mechanisms. Further understanding of the mechanisms by which both permeabilizing factors regulate retinal vasculature barrier properties may provide a common specific therapeutic target for the prevention of vascular permeability.

Glucocorticoids exert major anti-inflammatory effects and have been found to reduce retinal edema and improve visual acuity in diabetic patients (Massin *et al.*, 2004; Sutter *et al.*, 2004). However, glucocorticoids treatment is often associated with several adverse effects such as elevated intraocular pressure, leading to glaucoma and cataract formation (Moshfeghi *et al.*, 2003; Ciardella *et al.*, 2004; Gillies *et al.*, 2006). Further understanding of the mechanisms by which glucocorticoids exert their pro-barrier effects would be beneficial for the development of new specific targets to control retinal vascular permeability.

Also, it has been shown that glucocorticoids improve retinal endothelial cell properties through an increase in claudin-5 and occludin expression, and tight junction assembly, a process mediated through transactivation of the glucocorticoid receptor (Antonetti *et al.*, 2002; Felinski *et al.*, 2008). In this study, dexamethasone prevented the downregulation of ZO-1 and claudin-5 protein content and the increase in retinal endothelial permeability induced by TNF-α. The anti-inflammatory action of glucocorticoids is mostly associated with transrepression of inflammatory transcription factors, such as NF-κB (Scheinman *et al.*, 1995). In fact, the inhibition of NF-κB reduced the permeability induced by TNF-α, suggesting a role for NF-κB in cell permeability regulation, and therefore in the pathogenesis of diabetic retinopathy. In fact, several compounds that interfere with NF-κB activity, and these include antioxidants, glucocorticoids and non-steroidal anti-inflammatory drugs, also inhibit the expression of iNOS and ICAM-1, leukostasis and the increase in the retinal vasculature permeability. These main factors contribute to the BRB breakdown and the development of diabetic retinopathy (Joussen *et al.*, 2002a; Hammes *et al.*, 2003; Kowluru and Abbas, 2003; Kowluru and Kanwar, 2007; Zheng *et al.*, 2007).

The mechanisms by which NF-κB regulates cell permeability and the tight junction complex are largely unknown. Recently, it has been shown that PKCζ is essential for NF-κB activation in response to TNF-α (Leitges *et al.*, 2001; Duran *et al.*, 2003). In this study, NF-κB activation in response to TNF-α treatment was inhibited by a novel PKCζ inhibitor PKCζI-1, further supporting a role for PKCζ in NF-κB activation. Moreover, inhibition of PKCζ effectively inhibited both the changes in the tight junction complex and the increase in endothelial cell permeability induced by TNF-α. It is important to mention that PKCβ inhibitors that block VEGF-induced cell permeability (Harhaj *et al.*, 2006), failed to block TNF-α-induced cell permeability, but PKCζ inhibitors effectively prevent both VEGF (Antonetti *et al.*, 2008) and TNF-α-induced retinal endothelial cell permeability. In addition, PKCζ inhibition completely prevented the BRB breakdown *in vivo* induced by both permeabilizing factors. VEGF and TNF-α appear to alter the tight junction complex by distinct mechanisms, but these results show that PKCζ is a common target for blocking both VEGF and TNF-α induced cell permeability, and therefore may be considered a specific therapeutic target for the prevention of vascular permeability in retinal diseases characterized by elevated TNF-α and VEGF levels, such as diabetic retinopathy. Noteworthy, the PKCβ inhibitor ruboxistaurin was shown to reduce retinal vascular permeability in diabetic patients (Strom *et al.*, 2005). Although ruboxistaurin failed to inhibit the progression of proliferative diabetic retinopathy, it is the only therapy found to have a significant beneficial

effect in visual acuity in a clinical trial on patients with diabetic macular edema (Aiello *et al.*, 2006b; PKC-DME Study Group, 2007). Therefore, inhibiting both isoforms in a combined therapy could potentially have a more effective effect in inhibiting BRB breakdown and the progression of the disease.

Retinal endothelial cells are a primary target of IL-1β and TNF-α action. Regarding IL-1β, it has been shown that this cytokine plays an important role in endothelial cell dysfunction and BRB breakdown. It was reported that intravitreous injection of IL-1β or exposure of retinal endothelial cells to this cytokine *in vitro* induces degeneration of retinal capillary endothelial cells (Kowluru and Odenbach, 2004c), which seems to be mediated by NF-κB (Kowluru and Odenbach, 2004b; Kowluru and Odenbach, 2004c). In addition, increased levels of IL-1β in rat retinas correlate with BRB breakdown, which is prevented by the treatment with cyclosporine A, an anti-inflammatory drug (Carmo *et al.*, 2000). Furthermore, inhibition of caspase-1, the enzyme responsible for the cleavage of the pro-IL-1β into IL-1β, by minocycline, prevented the diabetes-induced increase in IL-1β and the degeneration of retinal capillary endothelial cells in diabetic rats (Vincent and Mohr, 2007). Likewise, inhibition IL-1β signaling pathway by using IL-1RI-deficient mice protected the animals from diabetes-induced retinal pathology (Vincent and Mohr, 2007). It is clear that IL-1RI may play a key role in the development of diabetic retinopathy, however, very little is known about its regulation under conditions associated to diabetes. Since IL-1β activity is regulated primarily by IL-1RI (Sims *et al.*, 1993; Cullinan *et al.*, 1998), the effect of high glucose, to mimic hyperglycemic conditions, and IL-1β on the regulation of IL-1RI content in retinal endothelial cells was also investigated.

High glucose decreased IL-1RI protein content in retinal endothelial cells. However, exposure to mannitol induced similar effects, suggesting that the downregulation of IL-1RI was due to an increase in osmolarity rather than a direct effect of glucose. Indeed, it has been shown that high osmolarity induces IL-1β receptor clustering and internalization without the presence of the ligand, probably through alterations in the receptor conformation (Rosette and Karin, 1996). IL-1β also induced a downregulation in the IL-1RI protein levels, suggesting that retinal endothelial cells alter their responsiveness to this cytokine by reducing IL-1RI availability. IL-1RI downregulation was dependent on its activation by IL-1β and, was mediated, at least in part, by lysosomal degradation. In fact, a recent study demonstrated that after IL-1β binding, IL-1RI is rapidly internalized and ubiquitinated, which targets the IL-1RI to lysosomes for degradation (Brissoni *et al.*, 2006). In addition, it was also observed that IL-1RI is translocated and

accumulated in the nucleus, after high glucose and IL-1β treatment. Previous studies have also reported the internalization and translocation of IL-1RI to the nucleus, suggesting that this receptor might mediate IL-1β trafficking into the nucleus, where it may be involved in gene transcription (Qwarnstrom *et al.*, 1988; Curtis *et al.*, 1990; Heguy *et al.*, 1991). These results suggest that high glucose, probably due to osmostic stress, and IL-1β downregulate IL-1RI protein content as a mechanism to regulate IL-1RI availability and therefore, prevent the overactivation of IL-1β-triggered signaling pathways. Therefore, targeting this receptor may provide a beneficial therapeutic approach for the treatment of this disease.

In summary, the results presented in this study provide a better understanding of the role of proinflammatory cytokines on retinal endothelial cell barrier function and provide supporting evidence that IL-1β and TNF-α contribute to the BRB breakdown. Moreover, the results support the fact that targeting cytokine receptors or specific downstream targets, such as PKCζ, may provide potential novel therapeutic targets for the treatment of vascular permeability in ocular diseases characterized by elevated levels of cytokines, such as diabetic retinopathy.

CHAPTER 5

Main conclusions

CHAPTER 5

Main conclusions

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

- IL-1β and TNF-α increase retinal endothelial permeability. However, TNF-α is more effective, yielding increased permeability at a lower concentration and shorter time-point.

- TNF-α downregulates ZO-1 and claudin-5 expression, but not occludin, and alters the tight junction proteins organization at the plasma membrane, which correlates with the increase in cell permeability.

- The glucocorticoid dexamethasone completely prevents the tight junction disruption and the increase in cell permeability induced by TNF-α. The protective effect of dexamethasone is mediated by both the transcriptional activation of the glucocorticoid receptor and transrepression of NF-κB.

- Inhibition of NF-κB activation partially reduced TNF-α-induced cell permeability, suggesting that endothelial cell barrier properties might be regulated by NF-κB.

- Inhibition of PKCζ, but not conventional PKC isoforms, completely prevents the alterations in the tight junction complex and cell permeability induced by TNF-α, in part by reducing NF-κB activation.

- High glucose and IL-1β decrease the content of the IL-1β receptor, IL-1RI, in retinal endothelial cells.

- The downregulation of IL-1RI is triggered by its activation and is due, at least partially, to lysosomal degradation. The translocation and accumulation of IL-1RI in the nucleus, after high glucose and IL-1β treatment, may also contribute to its reduced availability.

In summary, these results suggest that proinflammatory cytokines target retinal endothelial cells and alter retinal vascular barrier properties, supporting their involvement in the BRB breakdown. Further, targeting cytokine receptors or specific downstream targets, such as PKCζ, may be considered as potential novel therapeutic approaches for the treatment of ocular diseases characterized by increased retinal vascular permeability, where cytokines play important roles.

CHAPTER 6

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