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Protective effect of calcium dobesilate against inflammation and oxidative/nitrosative stress in the retina of a diabetic rat model

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular. O trabalho foi realizado sob a orientação científica do Investigador Doutor António Francisco Rosa Gomes Ambrósio (Instituto Biomédico de Investigação da Luz e Imagem, Faculdade de Medicina da Universidade de Coimbra) e supervisão do Professor Doutor Paulo Fernando Martins dos Santos (Faculdade de Ciências e Tecnologia da Universidade de Coimbra)

Paula Cristina Antunes Voabil

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Contents

Abbreviations

AGEs - Advanced glycation end products

ANOVA - Analysis of variance

ATP - Adenosine-5'-triphosphate

BCA - Bicinchoninic acid

BRB - Blood-retinal barrier

BSA - Albumin bovine serum

CaD - Calcium dobesilate

cDNA - Complementary deoxyribonucleic acid

CH - Choroid

CNS - Central nervous system

COX-2 - Cyclooxygenase-2

DAG - Diacylglycererol

DAPI - 4`,6-Diamidino-2-phenylindole

DEPC - Diethyl pyrocarbonate

DM - Diabetes *mellitus*

DNA - Deoxyribonucleic acid

DNP - 2,4-Dinitrophenol

DNPH - 2,4-Dinitrophenylhydrazine

dNTP - Deoxyribonucleotide triphosphate

DTT - Dithiothreitol

ECF - Enhanced chemifluorescence

EGTA - Ethylene glycol tetraacetic acid

GCL - Ganglion cell layer

GFAP - Glial fibrillary acidic protein

GFAT - Fructose-6-phosphate amidotransferase

GLUT-1 - Glucose transporter type 1

GSH - Glutathione

G-3-P - Glyceraldehyde-3-phosphate

HbA_{1c} - Hemoglobin A_{1c}

ICAM-1 - Intercellular cell adhesion molecule-1

IGF-I - Insulin-like growth factor I

IL-1β - Interleukin-1 beta

INL - Inner nuclear layer

iNOS - Inducible nitric oxide synthase isoform

IPL – Inner plexiform layer

LY333531 - Ruboxistaurin

MnSOD - Manganese superoxide dismutase

mRNA - Messenger ribonucleic acid

NAD^+ - Nicotinamide adenine dinucleotide (oxidized form)

NADH - Nicotinamide adenine dinucleotide (reduced form)

NAD(P)H - Nicotinamide adenine dinucleotide phosphate (reduced form)

NF-κB - Transcriptional nuclear factor-kappa B

NO - Nitric oxide

OCT - Optimal cutting temperature gel

ONL - Outer nuclear layer

p38 MAPK - p38 Mitogen-activated protein kinase

PBS - Phosphate-buffered saline

PCR - Polymerase chain reaction

PEDF - Pigment epithelium-derived growth factor

PKC - Protein kinase C

PKC-β - Protein kinase C-beta isoform

PVDF - Polyvinylidene fluoride

RAGE - Receptor for advanced glycation end products

RNA - Ribonucleic acid

ROS - Reactive oxygen species

RT-PCR - Reverse transcription polymerase chain reaction

SDS - Sodium dodecyl sulfate

SEM - Standard error of mean

SOD - Superoxide dismutase

STZ - Streptozotocin

TBS-T - Tris-buffered saline -Tween

TCA - Trichloroacetic acid

TFA - Trifluoroacetic acid

TNF-α - Tumor necrosis factor-α

UDP-GlcNAc - Uridine diphosphate N-acetylglucosamine

VEGF - Vascular endothelial growth factor

Resumo

A retinopatia diabética é uma das principais complicações da diabetes *mellitus*, sendo considerada uma das principais causas de perda de visão e cegueira em adultos em idade activa nos países desenvolvidos. O dobesilate de cálcio (*CaD*) é um derivado sintético do sulfonato de benzeno, utilizado no tratamento da retinopatia diabética. Clinicamente, a sua eficácia foi demonstrada, principalmente, através da correcção da excessiva permeabilidade vascular na retina em pacientes diabéticos e em diabetes experimental. Contudo, os mecanismos moleculares e celulares dos fármacos envolvidos nesses efeitos não estão ainda completamente esclarecidos. Os efeitos protectores do *CaD* podem estar relacionados com as suas propriedades antioxidantes ou, possivelmente, com uma acção anti-inflamatória. Assim, este estudo teve como principal objectivo elucidar o efeito do tratamento com o *CaD* na inflamação e no stress oxidativo/nitrosativo induzidos na retina pela diabetes, utilizando para isso um modelo animal de diabetes.

Ratos *Wistar* foram divididos em 4 grupos: controlos, animais tratados com *CaD*, diabéticos (2 meses de duração da diabetes) e diabéticos tratados com *CaD* (100 mg/kg/dia; administrado oralmente durante as duas últimas semanas). Devido à natureza inflamatória da retinopatia diabética, a reactividade glial foi avaliada nas células de Müller e nos astrócitos na retina. A expressão da proteína glial fibrilar acídica (*GFAP*) aumentou na retina aos 2 meses de diabetes. Curiosamente, o tratamento com *CaD*, por si só, fez aumentar os níveis de *GFAP* na retina. No que diz respeito à expressão das citocinas pró-inflamatórias, foi prestada particular atenção à interleucina-1β (*IL-1β*) e ao factor de necrose tumoral-α (*TNF-α*). Os níveis de expressão destas citocinas pró-inflamatórias aumentaram nas retinas dos animais diabéticos e o tratamento com *CaD* preveniu esse aumento. Além disso, o stress oxidativo/ nitrosativo foi avaliado através da detecção de grupos carbonilo oxidados e de resíduos de nitrotirosina. Tal com esperado, a diabetes aumentou o stress oxidativo/nitrosativo na retina, o qual foi inibido pelo tratamento com *CaD*, sugerindo assim que o efeito protector do *CaD* se mostra inegavelmente relacionado com suas as propriedades antioxidantes.

Em conclusão, estes resultados suportam resultados anteriores, mostrando que o *CaD* exerce efeitos protectores na retina, principalmente inibindo o stress oxidativo/nitrosativo e a inflamação. Particularmente, neste trabalho, foi demonstrada pela primeira vez a inibição da produção de citocinas pró-inflamatórias pelo *CaD* na

retina. É também provável, que os efeitos protectores do *CaD* sejam essencialmente devido às suas propriedades antioxidantes.

Abstract

Diabetic retinopathy is one of the most common complications of diabetes *mellitus* and is a leading cause of acquired blindness in working-age adults in developed countries. Calcium dobesilate (CaD) is a synthetic benzene sulfonate derivative used in the treatment of diabetic retinopathy. Its clinical effectiveness has been demonstrated mainly through a correction of the excessive vascular permeability in the retina of diabetic patients and in experimental diabetes. However, its molecular and cellular mechanisms of action are not completely elucidated. The protective effects of CaD might be due to its antioxidant properties or possibly they could also be related with an anti-inflammatory activity. Therefore, the main goal of the present study was to clarify the effects of the treatment with CaD on the inflammation and oxidative/nitrosative stress induced by diabetes in the retina of an animal model.

Wistar rats were divided into four groups: controls, animals treated with CaD, diabetic (2 month diabetes duration) and diabetic treated with CaD (100 mg/kg/day; orally given, during the last 2 weeks). Given the inflammatory nature of diabetic retinopathy, glial reactivity was evaluated in Müller cells and astrocytes. Glial fibrillary acidic protein (GFAP) immunoreactivity was upregulated in the retina at 2 months of diabetes. Interestingly, CaD per se also enhanced GFAP levels in the retina. Regarding the expression of pro-inflammatory cytokines, a particular attention was given to interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α). The expression levels of these pro-inflammatory cytokines were elevated in the retinas of diabetic animals, and CaD was able to prevent this upregulation. Oxidative/nitrosative stress was analysed by the detection of oxidized carbonyl groups and tyrosine nitration. As expected, diabetes increased the oxidative/nitrosative stress in the retina, which was inhibited by treatment with CaD, thus suggesting that the protective effect of CaD appeared undeniably related with its antioxidant properties.

In conclusion, these results further support previous findings showing that CaD can exert potent protective effects in the retina, namely inhibiting oxidative/nitrosative stress and inflammation. In particular, the inhibition of the production of pro-inflammatory cytokines in the retina by CaD was demonstrated by the first time in this work. It is also likely that the protective effects of CaD are mainly due to its antioxidant properties.

Chapter 1 Introduction

1.1 - The retina

Retina, a light sensitive nerve tissue that lies at the back of the eye, is a multi-layer tissue spanning from the retinal pigmented epithelium to the vitreous body. It is constituted by four major cell types (Figure 1): neurons (photoreceptors, and ganglion, bipolar, horizontal, and amacrine cells), macroglia (Müller cells and astrocytes), microglia (resident macrophages) and microvascular cells (endothelial cells and pericytes; Lorenzi & Gerhardinger, 2001).

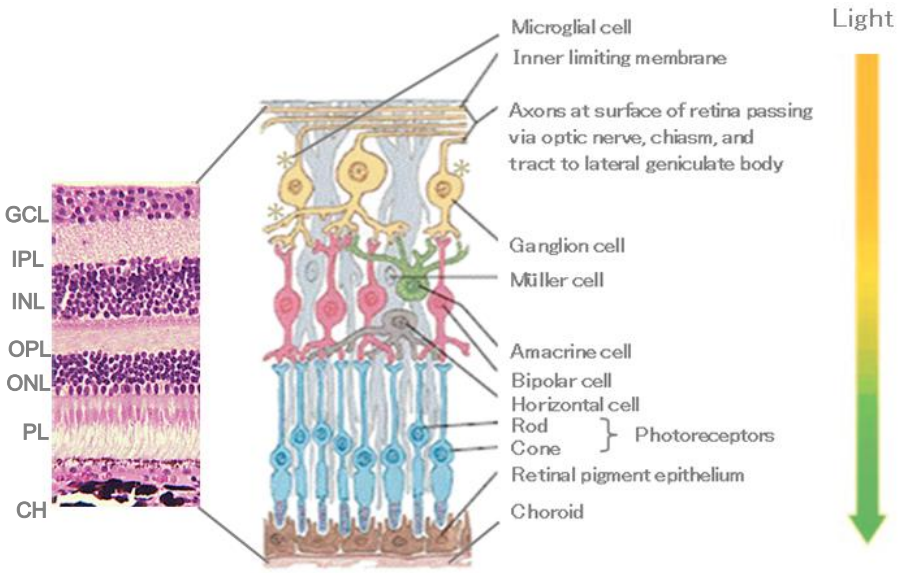

Figure 1. The anatomy of the retina. **(A)** Histological cross section the retina showing the retinal layers (Adapted from www.thalamus.wustl.edu/Course/). **(B)** Schematic representation of a retinal cross section showing the different cell types of the retina (Adapted from Hansen and Koeppen, 2002). Legend: GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; PL, photoreceptors layer; CH, choroid.

Photoreceptors detect light and convert light signals into electrical impulses along the organized layers of retinal neurons for transmission to the brain via the optic nerve, composed by ganglion cells axons. Macroglial cells provide nutritional and regulatory support serving as an interface between neurons and vascular cells. Microglial cells provide immunomodulatory functions in retina. They interact with all other retinal cells

to monitor the local environment, react to stress such as infection and trauma, releasing pro-inflammatory cytokines, and clear apoptotic and necrotic cells by phagocytosis (Madsen-Bouterse & Kowluru, 2008). The retinal vessels are present in the inner half of the neural retina, and the diameter of the capillary network varies in different parts of the retina. The retinal capillary wall is surrounded by the basement membrane, a connective tissue on which a single layer of vascular endothelial cells and pericytes lie on. In summary, each cell type works in a highly coordinated fashion to allow the conduction of the visual stimuli to the brain.

1.2 - Diabetes *mellitus*

Diabetes *mellitus* (DM) is a metabolic disorder characterized by chronic high blood glucose levels (hyperglycemia) that result from defects in insulin secretion, resistance to insulin action, or both. In a normal condition, blood glucose levels are tightly controlled by insulin, a hormone produced by pancreatic β-cells of the islets of Lagerhans. When the concentration of glucose in the blood is elevated insulin is released from the pancreas to normalize the glucose levels. In diabetes, impaired insulin action leads to chronic hyperglycemia inducing abnormalities in protein, lipid and carbohydrate metabolism. Thus, DM is often associated with complications, such as cardiovascular diseases, retinopathy, nephropathy and peripheral and autonomic neuropathy (Donnelly et al., 2000).

Although diabetes can be classified into four etiological categories: (1) type 1, (2) type 2, (3) diabetes due to other specific mechanisms or conditions, and (4) gestational diabetes *mellitus* (Kuzuya et al., 2002), the two main forms of the disease are: type 1 and type 2. Type 1 diabetes most commonly appears during the childhood and is characterized by loss of insulin production, due to autoimmune-mediated destruction of pancreatic β-cell islets (Zimmet et al., 2001). Type 2 diabetes is caused by insulin resistance and/or abnormal insulin secretion. This form of diabetes is strongly related with sedentary lifestyle, overly rich nutrition and obesity, appearing more associated with older age (Zimmet et al., 2001).

Diabetes has become one of the most challenging health problems of the 21st century. Projections show that in the year 2030 there will be 366 millions of diabetic cases all over the world (Wild et al., 2004), over 5.4% of the total population.

1.3 - Diabetic retinopathy

Diabetic retinopathy is one of the common complications of diabetes and is the leading cause of vision impairment and acquired blindness among the working population of developed countries (Stitt, 2003; Antonetti et al., 2006; Kowluru & Chan, 2007).

Diabetic retinopathy affects both type 1 and type 2 diabetic patients, but the incidence of the disease differs. This public health problem is present in nearly all persons who had type 1 diabetes for 20 years and in nearly 80% of those with type 2 diabetes for similar duration (Klein et al., 1984). In general, longer duration of diabetes increases the prevalence and progression of diabetic retinopathy increasing the risk of vision loss.

1.3.1 - Classification of diabetic retinopathy

Since long time that diabetic retinopathy is recognized as a disease of the intraretinal blood vessels. Efforts have been made to classify the retinal microvascular damage according to the lesions observed during the course of the disease. Thus, taking into account the classification proposed by Cunha-Vaz, diabetic retinopathy can be divided into five major clinical stages: 1) Pre-clinic diabetic retinopathy (Figure 2A) is characterized by the absence of detected ophthalmologic alterations in the retina. However, more sensible diagnostic techniques allow the detection of some alterations in this early stage, such as microaneurysms, capillary occlusion and pericyte and endothelial cell degeneration (Cunha Vaz, 2006); (2) In the non-proliferative diabetic retinopathy stage (Figure 2B), microaneurysms, intraretinal hemorrhages and lipid exudates become visible as well as a certain level of retinal edema; (3) Macular edema

is the result of plasma leakage from small blood vessels that exist in the central portion of the retina (macula). Macula is responsible for detailed central vision and because of this increase in the content of fluids, visual acuity might be severely disturbed in diabetic patients; (4) The pre-proliferative stage (Figure 2C) is characterized by an increase in the number and size of the intraretinal hemorrhages that is accompanied by retinal venous dilation and increase in retinal ischemic signs, such as cotton-wool spots; (5) Finally, in proliferative retinopathy (Figure 2D) the ischemia and hypoxia caused by occlusion of capillaries induce the formation of new blood vessels (neovascularization), which are very fragile and may form elsewhere in the retina (Cunha-Vaz, 2006). These vessels can extend into the vitreous cavity of the eye and hemorrhage into the vitreous. Fibrous proliferation on the retina could also lead to traction retinal detachment, and ultimately to blindness (Klein & Klein, 1997; Frank, 2004).

The greatest risk of vision loss resulting from these changes usually occurs in the later phases of the disease with the increase of vascular permeability, progressive vascular occlusion and retinal neovascularization.

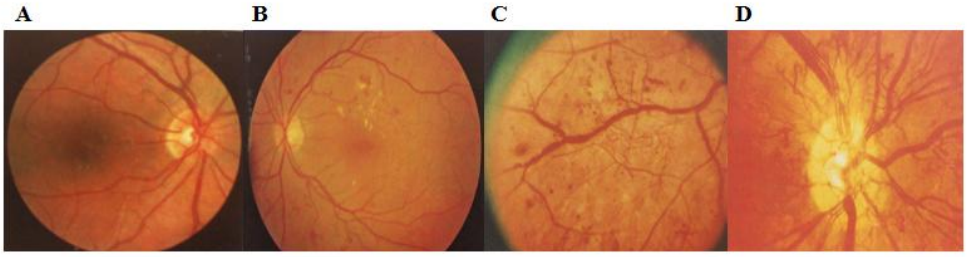

Figure 2. Eye fundus photographs of diabetic patients. **(A)** Eye fundus of a diabetic patient without alterations. **(B)** Non-proliferative diabetic retinopathy. **(C)** Pre-proliferative diabetic retinopathy. **(D)** Proliferative diabetic retinopathy (Adapted from Cunha-Vaz, 1972).

1.3.2 - Histopathology of diabetic retinopathy

In addition to the alterations in retinal vessels, there are evidences that neuroretinal dysfunction occurs early in diabetic patients, even before any sign of vasculopathy could be detected by the commonly used clinical observation methods. These findings suggest that diabetes can compromise the function of neurons in the retina before the alterations in blood–retinal barrier (BRB). The dysfunction of macroglial cells may be a

factor that contributes to this event. However, at this point, it is not known which cell type respond first to the diabetes-induced injury. Nowadays, the mostly certain concept is that vascular and neural cell defects are interdependent. Vascular-neural dysfunction is a concept that considerate all three cellular elements of the retina (neurons, glial and vascular cells). This multifactorial concept assumes that these cells interact under the physiological constraints of diabetes and finally evidence features of diabetic retinopathy. At a cellular level, diabetes alters the function and structure of all retinal cell types.

1.3.2.1 - Retinal microvascular dysfunction

Diabetic retinopathy begins with biochemical and cellular alterations that are not clinically evident. Vascular alterations during this initial stage of the disease include alterations in blood flow, death of retinal pericytes, basement membrane thickening and subtle increase in vascular permeability.

The BRB is essential for the homeostasis of the retina and its breakdown is an early event, remaining impaired during the course of the disease, which can be easily seen in diabetic patients and also in animals. The blood vessels of the retina have tight junctions that protect them from leaking and provide a barrier that is critical for the normal function of the retina. However, in diabetes, tight junctions disassembly make capillary vessels become leaky, allowing plasma or blood to extravasate into the retina, thus resulting in the swelling of the retina (Harhaj & Antonetti, 2004). Pericytes that wrap themselves around the endothelial cells in capillaries and provide support to them, start to disappear resulting in a well know feature in the early histology of the disease designed pericyte ghost. Throughout time, endothelial cells, one of the functional elements that maintain the blood-retinal barrier, also begin to degenerate (Mizutani et al., 1996). These cells delimit the inner side of the capillaries wall, so their absence result in empty spaces, leading to capillary closure and formation of acellular, nonperfused vessels (Mizutani et al., 1996; Caldwell et al., 2003; Madsen-Bouterse & Kowluru, 2008). As the disease progresses, obvious alterations in the vascular structure can be seen upon ophthalmoscope examination, such as microaneurysms. Other signs of the disease are the formation of cotton-wool spots.

Almost all these abnormalities are not associated with any clinical signs, with the exception of microaneurysms and cotton-wool spots, which are the earliest clinically observable lesions of retinopathy. The blockage of retinal capillaries will ultimately result in localized ischemia and hypoxia, which lead to increased production of angiogenic factors that induce neovascularization. The release of these growth factors is involved in the increase in permeability and contributes to the pathological vascular growth in a later stage.

1.3.2.2 - Retinal neural dysfunction

Microvascular alterations are undeniable associated with diabetic retinopathy and the vascular features of the disease are all well documented. Nevertheless, results of sensitive psychophysical examination methods, such as contrast sensitivity (Di Leo et al., 1992; Dosso et al., 1996) and color vision (Hardy et al., 1994) clearly show that diabetic retinopathy affects not only retinal vasculature, but also neurons and glial cells of retina. Furthermore, multifocal electroretinograms analyses, which can detect electrical potentials generated by retinal neurons, reveal retinal ganglion cell dysfunction early in diabetic patients (Ghirlanda et al., 1991; Han et al., 2004).

1.3.2.2.1 - Changes in retinal neuronal cells

Early studies have noted loss of neurons in the retinas of humans with diabetes (Wolter, 1961; Bloodworth, 1962). These studies identified pyknotic nuclei, now recognized as a typical characteristic of apoptosis, a mechanism of cell death, in sections of the retina. Recent evidences suggest that retinal ganglion cells, inner nuclear layer cells and photoreceptors die by apoptosis very early in the course of diabetes (Bek, 1994; Barber et al., 1998; Park et al., 2003; Martin et al., 2004), suggesting that retinal neurodegeneration occurs in diabetic retinopathy.

1.3.2.2.2 - Changes in retinal glial cells

Glial cells (Figure 3) play a central role in the homeostatic regulation of the retina. The spatial arrangement of retinal glia, intercalated between vasculature and neurons, points to their important functions. Macroglial cells are implicated in the maintenance of the BRB (Gardner et al., 1997), optimization of the extracellular ionic environment and also maintenance of low synaptic levels of neurotransmitters (Ohira & de Juan, 1990). They are important for the regulation of the glutamate-glutamine cycle. Glutamate is the major excitatory neurotransmitter in the retina, an amino acid that has excitotoxic effects to retinal neurons when present in high abundance (Romano et al., 1995). In diabetic animals, it was reported a decrease in the metabolism of glutamate concomitant with a decrease in the conversion of glutamate to glutamine taking place in the Müller cells of the retina (Lieth et al., 1998). Furthermore, elevated levels of glutamate were detected in the vitreous humor of humans with proliferative diabetic retinopathy and in animal models (Ambati et al., 1997; Kowluru, 2001). Alterations in the content of ionotropic glutamate receptor subunits have been also described in retinal neural cell cultures exposed to high glucose (Santiago et al., 2006). Therefore, elevated glucose levels may alter glutamate neurotransmission which affects calcium homeostasis in retinal neural cells and may induce cell death and visual field defects.

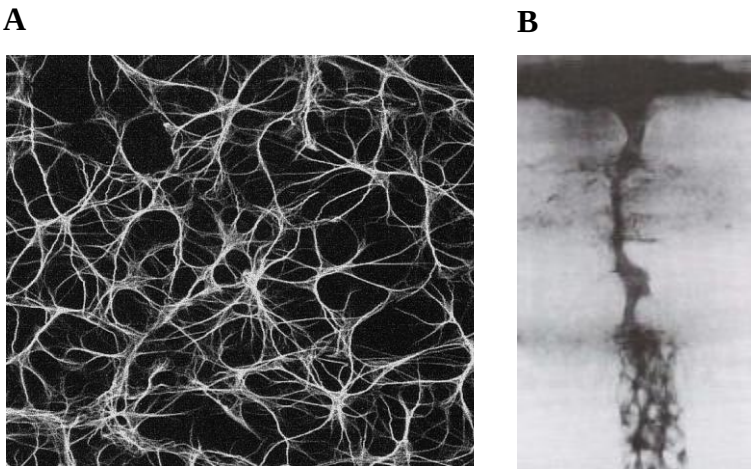

Figure 3. Retinal glial cells. **(A)** Astrocytes immunoreactivity to glial fibrillary acid protein (GFAP) was detected by immunofluorescence in flatmounted rat retina (Adapted from Barber et al., 2000); **(B)** Micrograph of a horseradish peroxidase (HRP) filled Müller cell of a rabbit retina. The dark band at the top is composed of Mller cell end-feet and the labeled axons of ganglion cells in the nerve fiber layer (Adapted from Forrester et al., 2002).

Moreover, several studies have reported glial reactivity in the retina of diabetic animal models, soon after the onset of experimental diabetes (Lieth et al., 1998; Rungger-Brandle et al., 2000). Glia reactivity is a general response to injury and inflammation (O'Callaghan, 1991; Norton et al., 1992). Increased expression of glial-fibrillary-acidic protein (GFAP) is a well-known marker of macroglial cell reactivity. The individual glial cell types react differentially to diabetes. In the retina, GFAP is normally expressed mainly in astrocytes, being minimally expressed by Müller cells (Fletcher et al., 2005). In a diabetic condition, Müller cells and astrocytes show opposite reactions. Astrocytes dramatically decrease GFAP expression, whereas Müller cell express more GFAP, in short-term diabetes. Overall, there is an increase in GFAP expression in the retinas of diabetic animals compared to control animals (Lieth et al., 1998; Mizutani et al., 1998; Rungger-Brandle et al., 2000). Müller cells also dye by apoptosis in diabetes in an advanced stage (Hammes et al., 1995).

Regarding microglial cells (Figure 4), which are normally quiescent, they become activated by diabetes, as described by Rungger-Brandle and colleagues (2000) and Gaucher and colleagues (2007). Microglial cells display a phenotype characteristic of activation, reflected by an increase in cell number and changes in cellular shape (Gaucher et al., 2007).

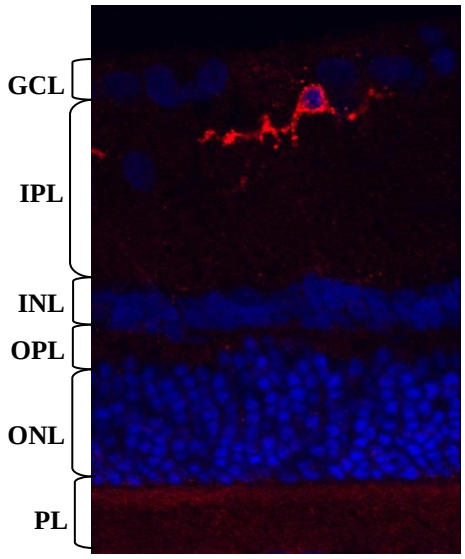

Figure 4. A ramified retinal microglia in a retinal cross section. Confocal image presented as a maximum projection of a retina section with 12µm stained with CD11b antibody (red) and nuclear DAPI staining (blue). Legend: GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; PL, photoreceptors layer.

1.3.3 - Pathogenesis of diabetic retinopathy

1.3.3.1 - Biochemical pathways

Hyperglycemia is considered the initiating event in the development of diabetic retinopathy. Prolonged exposure of cells to high glucose causes cumulative and irreversible long-term changes, although reversible changes also occur. Moreover, clinical trials have shown that improved glycemic control in diabetic patients is associated with decreased development and progression of retinopathy (DCCT Research Group, 1995; UKPDS, 1998). However, early intervention seems to be crucial to prevent the progression of retinopathy in diabetes (Robison et al., 1997; Robison et al., 1998; Kowluru, 2003).

Many biochemical abnormalities have been identified early in the retina during diabetes. Some of the major pathways implicated in the development of retinopathy include the increase of the flux in the polyol pathway, the accumulation of advanced glycation end products (AGEs), oxidative stress and protein kinase C (PKC) activation. Activation of these pathways is directly linked to hyperglycemia and the accumulation of various glycolytic intermediates.

1.3.3.1.1 - Polyol pathway

Aldose reductase is the first enzyme in the polyol pathway, and normally has the function of cell detoxification by reducing toxic aldehydes to inactive alcohols. In a normal condition, glucose is preferentially metabolized by the glycolytic pathway and by the pentose pathway, being a small percentage of the total glucose metabolized by the polyol pathway. However, in with glucose concentrations, cells increase the flux through the polyol pathway with an increased activity of aldose reductase (Gabbay, 1973). This increases the conversion of glucose to the polyalcohol sorbitol with a concomitant decrease in nicotinamide adenine dinucleotide phosphate (NAD(P)H), which functions as a hydrogen donor. Sorbitol is then oxidized to fructose by the enzyme sorbitol dehydrogenase, being nicotinamide adenine dinucleotide (NAD^+) reduced to NADH. These reactions initiate several mechanisms of cellular damage.

Sorbitol is hydrophilic and slowly metabolized, and therefore it is not easily diffusible through cell membranes thus accumulating inside the cells. This accumulation can result in osmotic cell stress (Gabbay, 1973). On the other hand, fructose produced by the polyol pathway might be phosphorylated to frutose-3-phosphate, which is broken down to 3-deoxyglucosone. Both compounds can be glycosylating agents for the formation of AGEs (Szwergold et al., 1990). Additionally, the increased use of NAD(P)H by the enzyme aldose reductase is critical, because NAD(P)H is also the essential cofactor for regenerating an intracellular antioxidant, reduced glutathione (GSH). By reducing the amount of intracellular glutathione the capability to respond to an intracellular oxidative injury is decreased (Barnett et al., 1986). Finally, it has been proposed that excessive NADH may become a substrate for NADH oxidase, and this would be a mechanism for generating intracellular oxidant species and thus increasing oxidative stress (Lassegue & Clempus, 2003; Lorenzi, 2007).

Retinal ganglion cells, Müller cells, vascular pericytes and endothelial cells are the ones known to be endowed with aldose reductase (Asnaghi et al., 2003; Dagher et al., 2004). Interestingly, these are the cells that also manifest the best-known changes or damage in diabetes (Lorenzi & Gerhardinger, 2001; Asnaghi et al., 2003).

1.3.3.1.2 - Hexosamine pathway activation

Hexosamine pathway has also been suggested to play a role in the pathogenesis of diabetic retinopathy. Under normal physiological conditions, only a small part of the intracellular glucose follows through hexosamine pathway. However, during hyperglycemia, increased flux of glucose into the glycolysis process will induce overexpression of frutose-6-phosphate. Hyperglycemia increases hexosamine pathway flux by providing more fructose-6-phosphate. Thus, frutose-6-phosphate will get diverted into another signalling pathway, the hexosamine pathway. In this pathway frutose-6-phosphate is converted to glucosamine-6 phosphate, by an enzyme called glutamine fructose-6-phosphate amidotransferase (GFAT), which is then rapidly metabolized to uridine diphosphate N-acetylglucosamine (UDP-GlcNAc) (Brownlee, 2005; Madsen-Bouterse & Kowluru, 2008). UDP-GlcNAc is a substrate for the

addition, via an O-linkage, of a single N-acetylglucosamine to serine or threonine residues of nuclear and cytoplasmic proteins. This form of protein glycosylation modifies proteins involved in transcription, signaling, and metabolism (Copeland et al., 2008; Durand et al., 2008). Also, increased activity of the hexosamine pathway is a putative mediator of glucose-induced insulin resistance but the mechanisms are still not very clear. The activation of hexosamine pathway has been reported in endothelial cells, pericytes and retinal neurons, and has been correlated wtih alterations in those cell types, including apoptosis (Nakamura et al., 2001; Du et al., 2003).

1.3.3.1.3 - Advanced glycation end products

Glucose and glycolytic intermediates can form, via nonenzymatic reactions, reversible Schiff base adducts and Amadori products with the amino groups of proteins, lipids, and nucleic acids. Gradually, after a cascade of reactions (condensation, dehydration or rearrangement) these early glycation products are converted to irreversible and cross-linked advanced glycation end-products (AGEs) (Brownlee et al., 1984; Wautier & Guillausseau, 2001). Dicarbonyl compounds, such as 1-, 3- and 4-deoxyglucosones, glyoxal, and methylglyoxal, are highly reactive glycolytic intermediates that appear upregulated during hyperglycemia, and they will in turn react with proteins and propagate intramolecular or intermolecular AGE formation (Thornalley, 1998).

AGEs can induce cell damage by three general mechanisms. The first mechanism, involves the modification of intracellular proteins, most importantly, proteins implicated in the regulation of gene transcription that appear with altered function (Giardino et al., 1994; Shinohara et al., 1998). The second mechanism, includes the AGE precursors that can diffuse out of the cells and modify extracellular matrix components (McLellan et al., 1994), thereby causing abnormal interactions with other matrix components and with the receptors for matrix proteins, the integrins (Charonis et al., 1990). The third mechanism is related to the modification of the circulating blood proteins by the AGE precursors that diffuse out of the cell. These modified proteins can then bind to AGE receptors (RAGEs), most likely at endothelial cells, pericytes or macrophages and cause the activation of oxidative stress cascades involving transcriptional nuclear factor-κB

(NF-κB), therefore inducing pathological changes in gene expression (Schmidt et al., 1994).

The increase of AGEs in diabetes is due to increased blood glucose levels (Schleicher et al., 1997). The accumulation of AGEs can be detected in the vitreous humor in patients with diabetes (Stitt, 2003) and in the retina in experimental diabetes (Bendayan, 1998; Nakamura et al., 2003). More precisely, increased AGEs and/or Amadori products have been localized within the retinal capillary wall of human diabetic retinas (Murata et al., 1997; Hammes et al., 1999). The accumulation of AGEs in the retinal vessels cause apoptosis in pericytes and upregulation of vascular endothelial growth factor (VEGF) in the endothelial cells, resulting in the activation of pathological angiogenesis seen in diabetic retinopathy (Stitt, 2003). Moreover, recent findings suggest that retinal microvascular endothelium exposed to AGEs, *in vitro* and *in vivo*, by inducing the activation of NF-κB, can promote intercellular cell adhesion molecule-1 (ICAM-1) expression, which causes significant adherence of isolated leukocytes (Stitt, 2003). In addition, during late stages, it was reported a marked AGE accumulation within the neural retina of diabetic rats (Hammes et al., 1999) which may have important effects on neural dysfunction too.

1.3.3.1.4 - Protein kinase C activation

Glucose is transported into the cells by glucose transporters (GLUT). In endothelial cells GLUT-1 is the predominant isoform. Once inside the cell, glucose can enter the glycolytic pathway and then be converted into glyceraldehyde-3-phosphate which in turn can be converted to dihydroxyacetone phosphate. Its reduction will result in the formation of glycerol-3-phosphate that leads to subsequent synthesis of diacylglycerol (DAG) (Koya & King, 1998). DAG appears as a physiologic activator of the PKC, which acts as a signalling component for a variety of growth factors, hormones, cytokines, and neurotransmitters. Under conditions of chronic hyperglycemia, the content of intracellular DAG increases which leads to an augment of PKC activity in vascular endothelial cells of the retina (Xia et al., 1994; Ganz & Seftel, 2000). Among the different eleven PKC isoforms (Rosse et al., 2010), the beta isoform (PKC-β) is the

predominant isozyme activated in vascular tissue during hyperglycemia (Shiba et al., 1993; Aiello, 2002).

Activation of PKC-β elicits numerous cellular changes leading to microvascular abnormalities in diabetes. The PKC activation increases the expression of matrix proteins, such as collagen and fibronectin and also increases the expression of vasoactive mediators, such as endothelin (Studer et al., 1993; Glogowski et al., 1999) Chu & Ali, 2008). The excess of these compounds will result in vascular occlusion and microthrombosis seen in the pre-clinic diabetic retinopathy stage and also in alterations of retinal blood flow (Ishii et al., 1996). In another way, PKC also mediates the increase of leukocyte adhesion to endothelial cells via upregulation of ICAM-1 (Chu & Ali, 2008). This will in turn contribute to the increase in retinal permeability. PKC-β also mediates the effects of VEGF, a key factor in neovascularization (Xia et al., 1996).

Moreover, due to the cross-talk between signaling pathways, chronic exposure of pericytes to AGEs leads to an amplification of DAG levels, with a subsequent increase in the PKC activity. Enhanced of oxidative stress can also activate PKC (Kowluru, 2001), and the augmented activation of the polyol pathway can have the same effect (Keogh et al., 1997), probably by increasing the amount of reactive oxygen species (ROS).

1.3.3.1.5 - Oxidative stress

Oxidative stress is defined as a cytopathic consequence of the generation of excessive ROS beyond the capacity of a cell to defend against them (Kowluru & Chan, 2007). Under normal physiological conditions, free radicals are produced continuously in all cells as part of normal function. About 95% of oxygen (O_2) consumed by tissues is utilized in metabolic processes, but approximately 0.1% - 5% of the oxygen that enters in the mitochondrial electron transport chain is transformed into ROS. The body is equipped with a competent detoxification system that consists of endogenous enzymatic free radical scavengers. However, under a pathological condition, the body´s natural defense mechanisms do not efficiently scavenge ROS that are being produced leading to an excessive bioavailability of ROS (Madsen-Bouterse & Kowluru, 2008).

Consequences of chronic oxidative stress include damage to DNA, proteins, lipids and also induction of expression of so-called *stress-response genes* further stimulating additional ROS generation from endogenous sources, disrupting cellular homeostasis.

Retina is extremely susceptible to oxidative stress, because it has a high content of polyunsaturated fatty acids and has the highest oxygen uptake and glucose oxidation relative to any other tissue in the body (Anderson et al., 1984). It has been suggested that ROS generated by high glucose are considered as a causal link between elevated glucose and the other metabolic abnormalities in the development of diabetic retinopathy (Brownlee, 2001). Thus, all pathways are in touch with ROS. In the diabetic retina, oxidative stress results from a variety of abnormalities, including decreased activities of antioxidant enzymes, namely GSH and manganese superoxide dismutase (MnSOD), formation of AGEs and interactions of those AGEs with membrane receptors, auto-oxidation of glucose and activation of PKC (Du et al., 2003). In turn, ROS can activate aldose reductase and PKC, induce the formation of AGEs and activate NF-κB (Nishikawa et al., 2000).

Superoxide ($O^{\cdot-}_2$) levels are elevated in the retina of diabetic rats and in endothelial and Müller cells exposed to high glucose (Du et al., 2003; Kowluru & Abbas, 2003; Cui et al., 2006), and hydrogen peroxide (H_2O_2) content is increased in the retinas of diabetic rats (Ellis et al., 2000). Under normal conditions, MnSOD reduce superoxide to hydrogen peroxide which is subsequently neutralized when it is enzymatically converted to water by GSH peroxidase or catalase (Droge, 2002). In contrast, hydrogen peroxide may be converted to another highly reactive ROS. In the presence of metals, hydrogen peroxide results in the production of the reactive hydroxyl radical that induce carbonyl formation on proteins (Davies et al., 1987; Shringarpure et al., 2001). Proteins constitute one of the major targets of ROS, and oxidation of proteins can lead to a loss of protein function as well as conversion of proteins to forms that are more susceptible to degradation.

Superoxide can be produced by NAD(P)H oxidase (enzymatic mechanism), the primary enzyme responsible for enzymatic reduction of O_2 to superoxide. Oxygen molecule is capable of accepting an additional electron to create the superoxide ion, a more reactive form of oxygen (Raha & Robinson, 2000; Droge, 2002). In other way, superoxide can also be produced as a consequence of mitochondrial respiration (non-

enzymatic mechanism). Hyperglycemia seems to induce superoxide anion overproduction by the mitochondrial electron-transport chain (Figure 5). Mitochondrial electron transport chain is considered the main source of hyperglycemia-induced superoxide (Du et al., 2003; Brownlee, 2005). The complex III of the electron respiratory chain is described to be the main mitochondrial target of hyperglycemia-induced injury (Rosca et al., 2002; Rosca et al., 2005). Its activity is decreased in the retina of diabetic mice (Kanwar et al., 2007). Therefore, the electrons tend to accumulate at coenzyme Q that then donates them to molecular oxygen creating a high amount of superoxide.

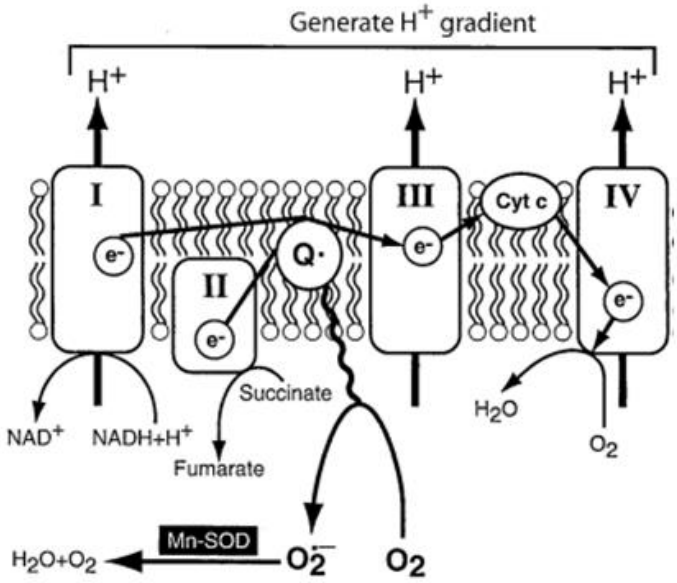

Figure 5. Hyperglycemia-induced production of superoxide by the mitochondrial electron transport chain. Electrons tend to accumulate at $Q^{\cdot}$, when a critical threshold in voltage gradient is reached. Q- donates them to molecular oxygen creating superoxide. In diabetes, the isoform of SOD in the mitochondria is suppressed leading to superoxide accumulation in the cells. Legend: I, mitochondrial complex I; II, mitochondrial complex II; III, mitochondrial complex III; IV, mitochondrial complex IV; $Q^{\cdot}$, coenzyme Q; O_2, oxygen; $O^{\cdot-}_2$, superoxide; Mn-SOD, manganese superoxide dismutase; H_2O, water (Adapted from Brownlee, 2005).

In diabetic retina, mitochondria become dysfunctional (Kowluru et al., 2006a; Kowluru et al., 2006b) and this might lead to enhanced sensitivity of retinal cells to oxidative stress, because they cannot scavenge ROS effectively. Mitochondrial dysfunction also includes damage to mitochondrial DNA (Maassen et al., 2004), which

appears increased in the retina in diabetes (Kanwar et al., 2007). The increase in the permeability of mitochondrial membrane represents another dysfunction caused by ROS. Swelling of the mitochondria is observed in the retina of diabetic mice (Kanwar et al., 2007) and that can causes the release of cytochrome C from mitochondria to the cytoplasm. The translocation of Bax (pro-apoptotic protein) from the cytosol to the mitochondria can lead to the activation of the apoptotic pathway resulting in increased apoptosis (Kowluru & Abbas, 2003; Kowluru, 2005). Bax immunostaining is seen in retinal capillary cells and ganglion cells of the retina (Podesta et al., 2000; Du et al., 2003; Kowluru, 2005). Caspase-3, which is activated in the course of the cascade of events triggered by cytochrome C release, appears to be activated in the retina in diabetes, prior to histopathological detection (Kowluru & Koppolu, 2002). In addition, NF-κB activation occurs in endothelial cells and pericytes incubated with high glucose, and also in the retina of diabetic animals (Romeo et al., 2002; Kowluru et al., 2003). Antioxidant therapy that inhibits the development of retinopathy in diabetic rats also inhibits caspase-3 and NF-κB activation in the retina. These results strongly support the link between oxidative stress and accelerated capillary cell death. Loss of capillary cells, such as endothelial cells and pericytes contribute to loss of intercellular junctions, increase vessel permeability and alterations in retinal blood flow.

Several studies have demonstrated that oxidative stress contributes not only to the development of diabetic retinopathy but also to the resistance of retinopathy to reverse after reinstitution of good glycemic control, a phenomenon designed as hyperglycemic memory (Kowluru, 2003). This resistance certainly derives from increased accumulation of damaged molecules and altered metabolism.

1.3.3.1.6 - Nitrative stress

Nitric oxide (NO) production is increased in the retina under diabetic condition, apparently via up-regulation of inducible nitric oxide synthase (iNOS) (do Carmo et al., 1998; Kowluru, 2001; Du et al., 2002; Kowluru, 2003; Leal et al., 2007). iNOS is one of the three isoforms of nitric oxide synthase and the only form inducible. It was demonstrated that iNOS is expressed in retinal endothelial cells and pericytes and that iNOS protein levels are elevated in the retina of diabetic animals (Chakravarthy et al.,

1995; Du et al., 2002; Leal et al., 2007). Furthermore, increased ROS can activate PKC and NF-κB and these can in turn induce the expression of iNOS and increase NO levels (Kowluru, 2001; Miralles et al., 2000). NO plays a role in the regulation of retinal vascular function (Tilton et al., 1993), and it has been postulated to contribute to the pathophysiology of retinopathy. In conditions of oxidative stress, such as those occurring in the retina during diabetes, NO can react spontaneously with superoxide to form peroxynitrite ($ONOO^-$), a highly reactive intermediate that initiates a variety of pathological processes. Peroxynitrite can contribute to vascular injury by inducing DNA damage, depletion of intracellular GSH levels, lipid peroxidation and inactivation of enzymes by oxidation of proteins sulfhydryls (Behar-Cohen et al., 1996; Szabo et al., 1996; Pieper et al., 1999). Peroxynitrite modifies tyrosine in proteins to form nitrotyrosine residues, and nitration of proteins can inactivate mitochondrial and cytosolic proteins and damage cellular constituents, resulting in nitrative stress (Kowluru et al., 2000). Nitrotyrosine levels are elevated early in the retina in diabetes, and remain elevated when the pathology is developing in the retina of animal models (Kowluru et al., 2000; Kowluru et al., 2001; Du et al., 2002; Leal et al., 2007). Also, nitrotyrosine levels are increased in retinal vasculature and in the ganglion cell layer of diabetic rats (Du et al., 2002; Leal et al., 2007), suggesting that peroxynitrite may also contribute to neuronal injury in diabetic retinopathy.

Overall, diabetes results in an imbalance between the production of free radical species, and the defense mechanism against them in the retina. The term, free radical species, encompasses several highly reactive molecules that include reactive oxygen species and reactive nitrogen species, superoxide and $ONOO^-$ being prominent members of those groups. The formation of oxidized and nitrosylated proteins are early events in the development of diabetic retinopathy and cannot be easily corrected after reestablishment of good glycemic control in rats.

1.3.3.1.7 - Growth factors

Finally, growth factors also seem to be involved in retinal vascular dysfunction and contribute to the pathophysiology of diabetic retinopathy.

Hyperglycemia enhances biochemical pathways associated with the production of growth factors, such as VEGF, insulin-like growth factor I (IGF-I) and pigment epithelium-derived growth factor (PEDF).

VEGF is specifically mitogenic for vascular endothelial cells and also increases vascular permeability. Therefore, VEGF is considered to play a key role in BRB breakdown (Caldwell et al., 2003). The increase in the VEGF expression has been demonstrated in diabetic rats retinas (Aiello et al., 1994; Hammes et al., 1998; Cukiernik et al., 2004). Increased VEGF levels were also reported in nonvascular cells in the retina of diabetic patients even in the absence of retinopathy (Amin et al., 1997). Activation of PKC-β and AGEs signalling pathway can enhance VEGF production, leading to alterations in BRB permeability, and during the later stages of the disease to neovascularization. In addition, VEGF expression is enhanced by hypoxia (Aiello et al., 1995), a major stimulus for retinal neovascularization. VEGF expression co-localizes with areas of retinal vascular occlusion, an early feature of diabetic retinopathy, which are associated with the induction of hypoxia.

Retinopathy progression has also been associated with elevated IGF-I levels (Lee et al., 1994; Chantelau, 1998), raising the hypothesis that IGF-I has a role in the pathogenesis of diabetic retinopathy. In fact, IGF-1 induces retinal neovascularization similar to VEGF (Danis & Bingaman, 1997; Smith et al., 1999; Rosenthal et al., 2004)

Finally, PEDF is produced by the retinal pigment epithelium and it was found to inhibit neovascularization (Dawson et al., 1999; Hutchings et al., 2002). It has been shown that reduced levels of PEDF may contribute to diabetic retinopathy. Animal studies have shown that systemic injection of PEDF reduces the development of retinal neovascularization in a mouse model of retinopathy (Stellmach et al., 2001). PEDF levels are decreased in the vitreous or aqueous humor of diabetic patients with proliferative diabetic retinopathy (Spranger et al., 2001). These observations support a possible involvement of PEDF in new blood vessel formation in the retina at proliferative diabetic retinopathy stage.

1.3.3.2 - Inflammation in diabetic retinopathy

Several findings strongly suggest that diabetic retinopathy is a chronic inflammatory disease. In fact, it has been proposed that inflammation plays a central role in the development and progression of retinopathy. Several genes involved in inflammatory processes are upregulated early in the diabetic rat retinas (Joussen et al., 2002b). Also, diabetic retinopathy exhibits features occurring in chronic inflammation, such as increased vascular permeability, edema, inflammatory cell infiltration, microglial activation, cytokine and chemokine expression, tissue destruction and neovasculatization.

Enhanced oxidative and nitrative stress and PKC activity induced by hyperglycemia can lead to the activation of the transcription factor NF-κB in endothelial cells and pericytes. Although NF-κB can activate either prosurvival or proapoptotic pathways, in the retina, under diabetic conditions, it seems to induce adverse effects. NF-κB appears activated in the retinal vasculature, early in the course of development of retinopathy, and remains activated when both capillary cell death and histopathology are developing (Mizutani et al., 1996; Kern et al., 2000; Kowluru et al., 2003; Kowluru & Odenbach, 2004c). Activation of NF-κB can modulate the expression of several genes, including proapoptotic genes and upregulate the production of various pro-inflammatory mediators such as cytokines, chemokines and adhesion molecules, including ICAM-1 (Baldwin, 1996; Kowluru et al., 2003; Chang & LoCicero, 2004). Increased levels of cytokines, such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α), have been found in vitreous fluid of diabetic patients (Yuuki et al., 2001; Funatsu et al., 2002) and in the retinas of diabetic rats (Carmo et al., 1999; Krady et al., 2005; Joussen et al., 2002b; Kowluru & Odenbach, 2004b; Kowluru & Odenbach, 2004c). IL-1β and TNF-α are pro-inflammatory cytokines produced by a variety of cell types including monocytes, macrophages and fibroblasts, and they can activate different types of cells. In the diabetic retina, endothelial, glial and microglial cells constitute the major potencial source of IL-1β (Kowluru & Odenbach, 2004b; Kowluru & Odenbach, 2004c; Krady et al., 2005) and TNF-α production (Tezel et al., 2001; Joussen et al., 2002b). Conversely, stimulation of retinal cells with IL-1β and TNF-α initiate a series of signal transduction events that lead to further NF-κB activation (Joussen et al., 2002b; Chang & LoCicero, 2004; Kowluru & Odenbach, 2004c).

1.3.3.2.1 - Inflammation and apoptosis

The increase in the production of pro-inflammatory cytokines has been correlated with an increase in cell death by apoptosis mediated by the activation of NF-κB and caspase-3 in retinal neurons and capillary cells (Kotsolis et al., 2010 ; Kowluru & Koppolu, 2002; Kowluru et al., 2003; Kowluru & Odenbach, 2004c). The levels of Bax, a NF-κB-dependent proapoptotic gene, is increased in the mitochondria obtained from the retina of diabetic rats and from retinal endothelial cells and pericytes incubated with high glucose (Pimentel et al., 2002). This supports a role of NF-κB in the apoptosis of retinal endothelial cells and pericytes.

1.3.3.2.2 - Inflammation and leukostasis

IL-1β and TNF-α can alter the expression of vascular cell adhesion molecules to recruit lymphocytes and macrophages to injury sites (Cook-Mills & Deem, 2005; Krady et al., 2005). Increased NF-κB mediated transcription leads to increased expression of ICAM-1 in endothelial cells, resulting in excessive leukostasis in retinal vessels (Morigi et al., 1998; Joussen et al., 2001; Zheng et al., 2004)

1.3.3.2.3 - Inflammation and angiogenesis

IL-1β and TNF-α were suggested to be involved in the angiogenic process seen in the late stage of diabetic retinopathy. These cytokines induce the expression of cyclooxygenase-2 (COX-2), an intermediate reported to contribute to the development of diabetic retinopathy by modulating VEGF-mediated vascular permeability and angiogenesis (Joussen et al., 2002b; Wilkinson-Berka, 2004).

1.3.3.2.4 - Inflammation overpowers inflammation

Since NF-κB activation also increases the expression of iNOS, this will result in an excessive production of NO. Elevated NO levels result in the augment of nitrative stress, which enhance the production of more ROS, with subsequent induction of oxidative stress creating a continuous positive feedback loop on the inflammatory process.

Under diabetic conditions increased IL-1β and TNF-α notably increase the glucose-induced damage in retinal cells by exacerbating the activation of NF-κB and its signalling pathways. The normal inflammatory response is designed to limit tissue injury. However, diabetes may disrupt the ability of tissues to respond appropriately and recover.

1.3.4 - Treatments for diabetic retinopathy

Hyperglycemia is considered the primary pathogenic factor for the development of diabetic retinopathy. It has been demonstrated that early tight glycemic control can prevent or delay the progression of diabetic retinopathy. Clinical trials have shown that improved glycemic control in diabetic patients is associated with decreased development and progression of retinopathy either in type 1 (DCCT Research Group, 1995) or type 2 diabetes (UKPDS, 1998). Additionally, controlled blood pressure and normalization of lipids seem to delay the onset and the progression of the disease.

Once visual impairment in diabetic retinopathy is detected, the treatment options are limited. Laser photocoagulation therapy, introduced in the 1960s, has long been the recommended treatment for patients with advanced proliferative diabetic retinopathy or clinically significant diabetic macular edema. While this treatment is usually effective because it can cause regression of the new vessels, the procedure destroys retinal neural tissue and can decrease peripheral vision, impair night vision and change color perception. Therefore, the development of new non-invasive therapies to prevent and treat diabetic retinopathy is required. With the progression of the research in this field, a

group of potential therapeutic strategies have been studied focusing on the main elements of the biochemical pathways activated by hyperglycemia in retinopathy.

Aldose reductase, a key enzyme in the development of diabetic retinopathy can be inhibited by sorbinil that prevents endothelial cell and pericyte apoptosis and acellular capillary formation in diabetic rats (Asnaghi et al., 2003; Dagher et al., 2004). Although sorbinil appears to have some efficacy in animal models, human trials with this inhibitor have been inconclusive (SRTRG, 1990). Aldose reductase inhibition with fidarestat also prevents the increase of oxidative stress and vascular permeability in rats (Kato et al., 2003; Obrosova et al., 2003). Thus, aldose reductase inhibitors have proven their efficacy in animal models, but their efficacy was not proved in humans.

Inhibition of AGEs by a small nucleophilic hydrazine compound, aminoguanidine (Pimagedine), reduces the development of retinopathy and retinal metabolic abnormalities, including oxidative stress and PKC activation in diabetic rats (Hammes et al., 1991; Kern et al., 2000; Kowluru et al., 2000). Aminoguanidine is a potent inhibitor of AGE-mediated crosslinking and has been also shown to prevent retinal microvasculature dysfunction in experimental animals (Agardh et al., 2000; Kern et al., 2000). However, such optimism has been tempered by the realization that aminoguanidine is a relatively non-AGE specific inhibitor with antioxidant and iNOS inhibitory properties (Vasan et al., 2001). Also, some studies have suggested that aminoguanidine may protect against experimental diabetic retinopathy independently of AGEs (Kern & Engerman, 2001; Du et al., 2002). Other successful approach has been to screen for compounds with post-Amadori product scavenging potential, which aminoguanidine does not have. Pyridoxamin appeared to be highly effective in reducing retinal AGE accumulation and also in preventing diabetes-associated capillary dropout (Stitt et al., 2002). Therefore, AGE-receptor inhibition is a potential therapeutic option in diabetic retinopathy but remains less developed than other strategies.

Another promising therapy is the use of oral inhibitors of PKCβ that have shown hopeful results in inhibiting diabetes-induced retinal dysmetabolisms and functional alterations. It was documented that intravitreal injection of VEGF induces BRB breakdown and an increase in PKC-β activity (Xia et al., 1996; Xu et al., 2004). The effects of VEGF injection can be blocked by treatment with the PKC-β inhibitor, ruboxistaurin (LY333531), which has minimal interference on normal metabolism. The

compound can effectively inhibit certain pathological changes associated with retinopathy, including retinal permeability and new vessel formation (Frank, 2004). In addition, PKC inhibition by LY333531 reduces leukocyte adhesion in diabetic retina (Nonaka et al., 2000). However, a clinical trial with ruboxistaurin revealed that this compound had no impact in the progression of retinopathy in patients, but has demonstrated a small beneficial effect on vision loss (Aiello et al., 2006).

Oxidative stress is considered to play a major role in the development of diabetic retinopathy and this makes it one of the favourite targets for therapeutic strategies. To reduce oxidative stress, normally antioxidants or compounds that increase the generation or activity of antioxidants are used.

Lipoic acid is a potent regenerator of other antioxidants such as vitamins C and E, and reduces glutathione to a healthy cellular redox state. It also functions as a direct scavenger of ROS. Lipoic acid attenuates the apoptosis of rat retinal capillary cells and decreases the levels of nitrotyrosine (Kowluru & Odenbach, 2004a). Antioxidant supplementation prevents diabetes-induced increase in nitrotyrosine residues and activation of NF-κB, and decreases VEGF levels and oxidized proteins in diabetic rat retinas (Kowluru et al., 2003; Kowluru & Odenbach, 2004b; Lin et al., 2006). It also induces beneficial effects on capillary cell apoptosis and on the number of acellular capillaries in the diabetic retina. By contrast, clinical studies have demonstrated some beneficial effects against oxidative stress but data are inconsistent and have focused mostly on outcomes related to complications other than retinopathy (Vincent et al., 2004; Guerrero-Romero & Rodriguez-Moran, 2005).

Benfotiamine, another antioxidant drug, inhibits the increase in acellular capillaries in the retina of diabetic rats via blocking the major pathways involved in hyperglycemia-induced retinal dysmetabolism, including AGEs formation, PKC, and hexosamine pathways (Hammes et al., 2003). In addition, the administration of vitamins C and E prevents the decrease in the activity of several antioxidant enzymes in the retinas of diabetic animals (Kowluru et al., 1997; Kowluru et al., 2001). Vitamins C and E demonstrate better potency in multi-antioxidant combination therapies that include Trolox, N-acetyl cysteine, β-carotene, and selenium. This multi-antioxidant combination therapy decreases lesions in the retinal microvasculature and inhibits the increase in PKC and NO levels. Additionally, administration of vitamin E reverses

some alterations in the retinal vessels and normalizes retinal blood flow (Bursell et al., 1999; Bursell & King, 1999).

Recent studies using genetic manipulation techniques have shown that overexpression of mitochondrial SOD in mice can prevent diabetes-induced decrease in retinal oxidative stress and mitochondrial dysfunction (Kowluru & Odenbach, 2004b; Kowluru & Odenbach, 2004c).

Antioxidants have proved to have beneficial effects on the development of retinopathy in experimental diabetes. However, the results from clinical trials are ambiguous. Intake of antioxidants, based on diet recall by diabetic patients, is ineffective in treating diabetic retinopathy (Millen et al., 2003). In contrast, pycnogenol, a compound with both free radical scavenging and anti-inflammatory properties, is reported to have beneficial effects on the progression of retinopathy in diabetic patients (Spadea & Balestrazzi, 2001; Steigerwalt et al., 2009). Pycnogenol has been tested for treatment and prevention of retinopathy and it has been shown a favourable outcome in the majority of the patients. However, it is not yet currently used in the management of this disease.

VEGF is upregulated in the eyes of diabetic patients and diabetic rats (Hammes et al., 1998; Qaum et al., 2001), and it is considered to have a predominant role in diabetic maculopathy and retinal neovascularization, which are the major causes of vision loss and blindness in diabetic patients. Therefore, the development of agents that directly target VEGF and its receptors is an area of active clinical research. New therapeutic strategies involve the use of pharmacological approaches to block VEGF overexpression or prevent its adverse effects on vascular permeability and growth. Blocking VEGF synthesis and the vitreal injection of anti-VEGF antibodies prevent retinal neovascularization in animal models. Pegaptanib sodium (Macugen), ranibizumab (Lucentis) and bevacizumab (Avastin) are the currently available anti-VEGF agents. Pegaptanib is an aptamer against $VEGF_{165}$ isoform. Intravitreal administration of pegaptanib has been shown to significantly inhibit leukostasis, retinal neovascularization and VEGF-mediated vascular leakage in rodents (Usui et al., 2004). Moreover, clinical studies reported that patients who were assigned to pegaptanib had better visual acuity outcomes (Cunningham et al., 2005) and showed regression of neovascularization (Adamis et al., 2006). Even more, this compound appeared to be

associated with fewer adverse effects than ranibizumab and bevacizumab (Simo & Hernandez, 2008).

Ranibizumab is derived from an anti-VEGF antibody and in contrast to pegaptanib, this anti-VEGF agent binds and inhibit the biological activity of all isoforms of human VEGF. It was proved to reduce the incidence of new vessel formation and to decrease leakage from established vessels in an animal model (Krzystolik et al., 2002). Ranibizumab has mainly been tested in patients with age-related macular degeneration (Rosenfeld et al., 2006), where two years of intravitreal administration prevented vision loss and improved mean visual acuity. However, in retinal macular edema associated with diabetes this compound has also showed positive results (Kotsolis et al. 2010). Bevacizumab, like ranibizumab, blocks all circulating VEGF isoforms, thus impairing both physiological and pathological neovascularization and so inducing a higher frequency of adverse effects. Bevacizumab is a successfully compound used in tumour therapy as a systemic drug, and recent clinical studies have demonstrated the usefulness of an intravitreal injection of bevacizumab in the reduction of vascular permeability and choroidal neovascularization (Kook et al., 2008; Kohno et al., 2010).

The inhibition of VEGF receptor tyrosine kinase activity also seems to decrease neovascularization (Chu & Ali, 2008). A similar effect has been demonstrated by corticosteroids, a class of substances with anti-inflammatory properties (Brooks et al., 2004) that act by inhibiting the expression of the VEGF gene (Nauck et al., 1998). Intravitreal injection of triamcinolone acetonide was found to reduce neovascularization and macular edema in patients with proliferative diabetic retinopathy (Bandello et al., 2006). Glucocorticoids, like dexamethasone, can also induce the repression of the inflammatory response by down-regulating several genes associated with pro-inflammatory cytokines and NF-κB, and by reducing recruited leukocytes and ICAM-1 levels (Barnes, 1998); (Tamura et al., 2005). Moreover, corticosteroids appear also linked with barrier properties. Hydrocortisone is reported to increase barrier properties through its effects on endothelial cell tight junction and thus it is claimed to have protective effects against retinal edema in diabetes (Antonetti et al., 2002). Also, cyclooxygenase seems to play a role in VEGF induction. Cyclooxygenase-2 is induced under inflammatory conditions and is elevated in the retina during diabetes (Carmo et al., 2000). Thus, selective inhibition of cyclooxygenase activity with celecoxib, itself an

inhibitor of VEGF mRNA expression, reduces vascular leakage in the retinas of diabetic rats (Ayalasomayajula & Kompella, 2003).

In other way, high-dose of aspirin and meloxican, a selective cyclooxygenase-2 inhibitor, both non-steroidal anti-inflammatory drugs, reduces diabetes-induced leukocyte adhesion and suppress BRB breakdown in diabetic rats (Joussen et al., 2002b). These two compounds also decrease the levels of retinal TNF-α and ICAM-1 (Joussen et al., 2002b).

Diabetic retinopathy involves a chronic, low-grade inflammatory component. Gene expression of IL-1β and TNF-α appeared increased in the retinas of diabetic rats (Joussen et al., 2001; Krady et al., 2005). Minocycline, a semi-synthetic tetracycline derivative, is able to repress the production of inflammatory cytokine in diabetic retinas and significantly reduces apoptosis (Krady et al., 2005). Also, it prevents the activation of caspase-1 in the retina (Mohr, 2004). Caspase-1 activation is essential for the production of IL-1β. Thus, minocycline is also a strong candidate for further consideration as a therapeutic drug in diabetic retinopathy.

In summary, several new compounds are starting to bring some benefits to diabetic patients with retinopathy in controlled clinical trials. However, since several mechanisms underlie the initiation and progress of the disease, probably it will be necessary to use a group of drugs with divergent modes of action to combat this multifactorial complication.

1.3.5 - Calcium dobesilate as a therapeutic agent in diabetic retinopathy

Calcium dobesilate (calcium 2,5-dihydroxybenzenesulfonate, CaD) is an angioprotective agent orally used. This synthetic benzene sulfonate derivative (Figure 6) was discovered more than 45 years ago and it is a registered compound used in more than 20 countries. Frequently known as Doxium, it is often recommended for venous disorders, and also prescribed for diabetic retinopathy and other microvascular disorders such as hemorrhoidal disease, varicose veins and erythematotelangiectatic rosacea.

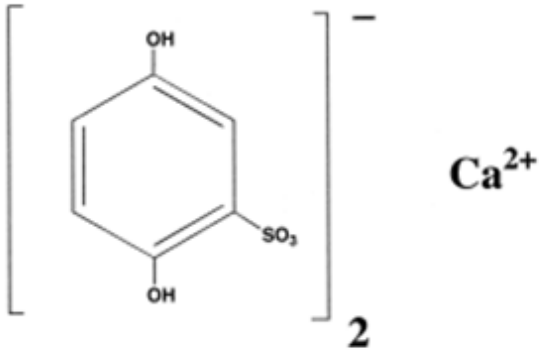

Figure 6. Chemical structure of calcium dobesilate (Adapted from Brunet et al., 1998).

The compound has been used in the management of diabetic retinopathy for more than 40 years. Controversy exists regarding the benefits of CaD in the treatment of diabetic retinopathy. Although several clinical studies show a slowdown of the progression of the disease after long-term treatment (Benarroch et al., 1985; Leite et al., 1990; Ribeiro et al., 2006), others have shown no beneficial effect of CaD (Daubresse et al., 1977; Haas et al., 1995). CaD primarily known effects involve its vasoactive properties. It was shown to reduce capillary permeability and fragility (Leal et al., 2010; Beyer et al., 1980; van Bijsterveld & Janssen, 1981; Leite et al., 1990) and to decrease blood hyperviscosity (Vojnikovic, 1991) which is often related with changes in plasma protein composition and increased aggregation of red blood cells. It has also been described to inhibit platelet aggregation (Michal & Gotti, 1988) and microcirculatory thrombus formation (Michal & Giessinger, 1985) in animals.

The antioxidant properties of CaD are known since long time. Brunet and colleagues (1998b) have demonstrated the effectiveness of CaD in scavenging hydroxyl radicals *in vitro*, at therapeutically relevant concentrations. However, higher concentrations of the compound were required to scavenge superoxide radicals or to protect the cells against the deleterious effects of intracellular reactive oxygen species. Moreover, this study suggested that CaD acts predominantly in the extracellular compartment, as expected from its hydrophilic properties. A study published by the same authors refers that in an *in vivo* model for quantification of microvascular permeability, CaD orally given can significantly reduce the increase in ROS as well as significantly inhibit Evans blue

extravasation in retina, suggesting that its antioxidant properties might underlie its angioprotective effects *in vivo* (Brunet et al., 1998). So, the pharmacological activity of CaD may be explained in part by its antioxidant properties. However, its vasoactive properties may be due to the synthesis of nitric oxide, which increases the endothelium-dependent relaxation in rabbit isolated aorta (Ruiz et al., 1997).

The effects of CaD in the retina are still not completely understood. Szabo and colleagues (2001) have shown that oral treatment of diabetic rats with CaD significantly protects against the oxygen-free radical production and the metabolic and histological disturbances induced by ischemia/reperfusion in retinal tissue. They have tested the compound in their model of free radical generation by reperfusion. This model has previously been shown to aggravate the biochemical changes resulting from oxidative stress in a diabetic rat retina (Szabo et al., 1991; Szabo et al., 1995). Thus the antioxidant properties of CaD were confirmed by the reduction in hydroxyl radical production and by the prevention of the loss of cellular GSH (Szabo et al., 2001). The involvement of CaD in the increase in the activity of the enzymes that are known to be implicated in the GSH metabolic cycle has been also reported by Losa and colleagues in 1999. In normal cells, GSH is mainly in the reduced form that is known to be a potent antioxidant, protecting membrane proteins and enzymes. Thus, reduction in retinal GSH is associated with increased oxidative stress in the retina and that seems to be prevented by CaD (Szabo et al., 2001). The antioxidant effect of CaD was also correlated with its protective effects against retinal hyperpermeability in diabetic rats. CaD significantly reduced retinal hyperpermeability in association with reductions in retinal AGEs content and retinal VEGF overexpression (Rota et al., 2004). Taking into account that: (1) increased levels of AGEs and VEGF were found in the aqueous humor of patients with diabetic retinopathy (Endo et al., 2001); and (2) AGEs are likely to be involved in the increase of VEGF through generation of oxidative stress and activation of the PKC pathway; the authors suggested that the reduction of AGEs by CaD might mediate the partial reversal of VEGF overexpression. Altogether these results suggest that CaD stabilizes BRB disruption in diabetic retinopathy via an *in situ* antioxidant action.

Furthermore, CaD was found to prevent several histological changes induced by diabetes (Padilla et al., 2005). CaD ameliorates the loss of pericytes in retinal capillaries. Pericyte degeneration is one of the earliest histological changes observed in

the development of diabetic retinopathy. They are extremely important to maintain the regulatory properties of the vessels. Thus the maintenance of their number might reflect the ability of CaD to reduce vascular permeability in diabetes. In the same study, the authors reported an increase in noradrenaline (NA)-induced vasoconstriction in aortic tissue of diabetic animals. Animals treated with CaD were found to have less vasoconstriction. They hypothesized that this effect of CaD might be due, in part, by its action on intracellular calcium and/ or β-adrenoreceptors. To complement this study, the authors also found that the treatment with CaD restored to normal the decrease of endothelium-dependent relaxation induced by diabetes, and suggested that this effect of CaD was due to its antioxidant properties.

The finding of a positive result against VEGF overexpression in diabetic rats was the basis to evaluate the potential effect of CaD on intraocular angiogenesis (Lameynardie et al, 2005). VEGF plays an important role in mediating intraocular angiogenesis manifested in the last stages of diabetic retinopathy with typical proliferative lesions. Lameynardie and colleagues evaluated the effect of CaD *in vitro* and *ex vivo* on choroidal explant angiogenesis in diabetic rats and found that CaD inhibits both microvessel formation and VEGF production *in vitro*, and reduce choroidal angiogenesis in diabetic rats. These results suggest that the antiangiogenic effect of CaD can be one of the therapeutic benefits of CaD in diabetic retinopathy.

More recently, another clinical trial reinforced the positive effects of CaD in diabetic patients (Ribeiro et al., 2006). In patients expressing early phases of diabetic retinopathy and with two years of treatment, CaD decreased BRB permeability, showing significant beneficial effects in the control of hemorrhages and in the evolution of microaneurysms. Conversely, a different clinical trial, with a follow up of five years involving patients with mild-to-moderate non-proliferative diabetic retinopathy, concluded that CaD was not able to reduce the risk of clinically significant macular edema (Haritoglou et al., 2009). However, this study shows that a particular subgroup of patients, namely patients with accumulation of risk factors, such as high blood pressure and blood glucose can benefit with CaD treatment. Also, this study highlights the hypothesis that CaD might be effective in the early stages of diabetic retinopathy, losing effectiveness in advanced stages.

Despite the use of CaD in the last decades in the treatment of diabetic retinopathy, very low attention has been given to the molecular and cellular mechanisms underlying its protective effects. Recently, it emerged the first study aimed to create a better understanding on the protective effects of CaD against the increase of BRB permeability induced by diabetes (Leal et al, 2010). In this study, authors clearly showed that CaD inhibits changes in tight junctions proteins, ICAM-1 and leukocyte adhesion to retinal vessels, which are correlated with the increase in BRB permeability in diabetes. Tight junctions provide the direct cell-to-cell attachment responsible for the crucial diffusion barrier properties in endothelial cells that are critical for the normal functioning of the retina. Thus, CaD seems to stabilize BRB permeability by preventing alterations in tight junction proteins induced by diabetes. Moreover, CaD prevented the increase in ICAM-1 levels, decreasing, at least in part, the number of leukocyte adhering to retinal vessels. These results suggest a role for CaD against the inflammatory process, considered a key player in BRB breakdown.

Adverse effects caused by CaD did not occur frequently and in some cases appear unrelated to the pharmacological properties of the compound. Some cases of agranulocytosis and fever related to the use of CaD were reported (Galindo Bonilla et al., 1996; Ibanez et al., 2000; Allain et al., 2004). Agranulocytosis is a rare but serious acute condition involving a severe lack of granulocytes, most commonly neutrophils (Andersohn et al., 2007). However the risk of an adverse effect with CaD is very low.

In summary, CaD is a registered compound used as an alternative therapy for the treatment of diabetic retinopathy. It is considered to improve visual acuity, at least in some studies. However, its efficacy in the treatment of diabetic retinopathy is still a matter of controversy. An overall review of published studies involving CaD depicts a non-specific compound acting moderately, but significantly, on the various and complex alterations that contribute to the progression of diabetic retinopathy. The beneficial effects of this compound are believed to be due to its antioxidant properties reflected through the ability in scavenging hydroxyl radicals and in preventing the decrease of cellular GSH. Its efficacy appeared also to be linked to the decrease in the levels of AGEs, and consequently to the partial decrease in VEGF overexpression. Its good effectiveness is shown as the ability to prevent changes in tight junction proteins as well as changes in ICAM-1, structurally connected with leukocyte adhesion to retinal

endothelial cells. Altogether these findings suggest that many possibilities are still open and other molecular and cellular mechanisms are expected to be involved in the protective effects of CaD in diabetic retinopathy.

1.4 - Objectives of the study

An increased burden of evidence suggests that diabetic retinopathy is as an inflammatory disease. Several findings strongly propose that diabetes induce an overall increase in oxidative stress in the retina which subsequently activates p38 MAPK and NF-κB pathways resulting in the up-regulating TNF-α and IL-1β expression. Both cytokines are players of an inflammatory response, and appear to be crucial in the progression of diabetic retinopathy, contributing, in addition to VEGF, to the vascular permeability. Therefore oxidative stress is strongly in touch with inflammation.

Despite the use of CaD in the treatment of diabetic retinopathy in the last decades, its mechanisms of action are still not completely elucidated. In clinical trials, the protective effect of CaD is shown by a reduction in capillary permeability and fragility. Apparently, the primary known antioxidant properties of this compound are involved in the good outcome against diabetic retinopathy. However, how this antioxidant effect impact in other pathways triggeres in the retina is not yet documented. Thus, the present study was designed to explore the effect of the treatment with calcium dobesilate on the inflammatory process induced by diabetes in the retina. Special attention was given to glial reactivity, and TNF-α and IL-1β expression. Additionally, it was also analyzed its potential preventive effect on the formation of nitrotyrosine residues and oxidized carbonyl groups.

Chapter 2 Materials & Methods

2.1 - Materials

Calcium dobesilate was obtained from OM Pharma (Meyrin, Geneva, Switzerland). STZ (streptozotocin), DAPI (4',6-diamidino-2-phenylindole), goat serum, TRIZOL Reagent, ethidium bromide, DNPH (2,4-dinitrophenylhydrazine) and BSA (albumin bovine serum) (Fluka) were purchased from Sigma-Aldrich Corporation (Madrid, Spain). Glucose strips were obtained from Bayer (Carnaxide, Portugal). Sodium thiopental was acquired from Emivete (Veterinary products, Portugal). BCA (bicinchoninic acid) Protein Assay kit was purchased from Pierce (Thermo Scientific, Rockford, Illinois, USA). Protease inhibitor cocktail tablets *complete–mini* was acquired from Roche (Basel, Switzerland). PVDF (polyvinylidene fluoride) membranes and anti-DNP (Chemicon) and anti-nitrotyrosin (Upstate) antibodies were obtained from Millipore Iberica (Madrid, Spain). Primary antibody anti-GFAP was purchased from Oncogene Science (Cambridge, USA). Antibody anti-vimentin (Lab Vision Corporation) and anti-TNF-α (Endogen) were acquired from Thermo Scientific (Rockford, Illinois, USA). Caspase-3 was purchased from Cell Signaling (Danvers, MA, USA). The antibody anti-IL-1β was obtained from R&D Systems (Northeast, Minneapolis, USA). SDS (sodium dodecyl sulfate), polyacrilamide and nitrocellulose membranes were acquired from Bio-Rad Laboratories (Amadora, Portugal). ECF (enhanced chemifluorescence) substrate, primary anti-actin antibody and anti-mouse, anti-rabbit and anti-goat alkaline phosphatase-conjugated secondary antibodies were purchased from GE Healthcare (Chalfont St. Giles, UK). Secondary antibodies Alexa 488 goat anti-rabbit and Alexa 568 goat anti-mouse were obtained from Molecular Probes (Invitrogen, Barcelona, Spain). SuperScript First-Strand Synthesis System kit, Platinum Taq DNA polymerase, TNF-α, IL-1β and actin primers, and agarose were obtained from Invitrogen (Barcelona, Spain). TFA (trifluoroacetic acid) was acquired from VWR (Leicestershire, UK). TCA (trichloroacetic acid) was obtained from Merck (Lisbon, Potugal). All other reagents were acquired from Sigma-Aldrich Corporation (Madrid, Spain).

2.2 - Animal model

Male *Wistar* rats (Charles River Laboratories, Barcelona, Spain), 8 weeks, weighing approximately 280g (two *per cage*), were fed standard laboratory chow and allowed free access to water in an air-conditioned room (22 ± 1ºC) with a 12 h:12 h light-dark cycle. All procedures involving animals were in agreement with the European Community guidelines (86/609/EEC) for the protection of animals used for experimental and other scientific purposes. All efforts were made to minimize the suffering and the number of animals used.

Body weight was monitored before the induction of experimental diabetes. Diabetes was induced in randomly assigned *Wistar* rats with a single intraperitoneal streptozotocin (STZ) injection (60 mg/kg, in sodium citrate buffer 0.1 M, pH 4.5). Tail blood samples were obtained and glucose concentration was measured with a glucometer Ascencia Elite (Bayer Diagnostics, Munich, Germany), 48 h after the injection and before the sacrifice. Animals were considered diabetic when glycemia was higher than 250 mg/dl. The development of diabetes was also confirmed by measuring glycohemoglobin with a glucometer (Elite, Bayer, Portugal) before euthanizing the animals. The experiments were performed 2 months after diabetes induction.

2.3 - Treatment protocol

Animals were randomly assigned to receive calcium dobesilate (100 mg/kg/day, in sterile 0.9% NaCl solution) or sterile 0.9% NaCl saline solution orally given through gentle esophageal canulation. Calcium dobesilate was administered during the last 14 days of 2 months diabetes duration. Calcium dobesilate was also administered to non-diabetic age-matched rats, for the same period. The animals were divided into four groups, according to the following scheme:

Control Group (*n* = 6/7): Non-diabetic rats (received saline solution);

CaD Group (*n* = 6/7): Non-diabetic rats treated with calcium dobesilate;

Diabetic Group (*n* = 6/7): Streptozotocin-induced diabetic rats (received saline solution);

Diabetic+CaD Group (*n* = 6/7): Streptozotocin-induced diabetic rats treated calcium dobesilate.

The last administration of calcium dobesilate (or saline) was performed one day before the sacrifice. On the day of sacrifice, rats were sequentially anesthetized with sodium thiopental (Emivete, Veterinary products, Portugal) before careful removal of the eyes. The retinas were dissected under a microscope and used for biochemical measurements. Body weight was measured before the initiation of oral treatment protocols and after the sacrifice.

2.4 - Immunohistochemistry in whole mounted retinas

The retinas were isolated, washed in ice-cold phosphate-buffered saline (PBS – 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4) and immersed in 2% paraformaldehyde for 2 x 5 min at room temperature. After washing 2 x 5 min in PBS with 0.3% Triton X-100 for permeabilization, the retinas were immersed in blocking solution (10% goat serum) in PBS with 0.3% Triton X-100, for 30 min, and incubated for 3 days at 4ºC with anti-GFAP antibody (1:200) in PBS with 0.3% Triton X-100. After incubation, the retinas were washed in PBS with 0.3% Triton X-100 for 24 h, and incubated with an Alexa Fluor-568-(red) conjugated secondary antibody (goat anti-mouse IgG, 1:250) in PBS with 0.3% Triton X-100. Following, the retinas were washed, again in PBS with 0.3% Triton X-100 for more 24 h and flat mounted on glass slides using Glycerogel (DakoCytomation, Glostrup, Denmark) and coverslipped for visualization under a confocal scanning laser microscope (LSM510, Carl Zeiss, Gottingen, Germany).

2.5 - Immunohistochemistry in retinal sections

2.5.1 - Preparation of cryosections

The eyes, immediately enucleated after the sacrifice, were washed in ice-cold PBS and fixed in 4% (w/v) paraformaldehyde in PBS for 1 h. The cornea was removed and the posterior segments were fixed in 4% (w/v) paraformaldehyde in PBS for an additional period of 5 h. The tissue samples were transferred to 20% sucrose buffer overnight at 4°C for cryoprotection and then were embedded in OCT (optimal cutting temperature gel, Shandon Cryomatrix, Shandon, Pittsburg, USA). The blocks were stored in a deep freezer (-80ºC) until use.

Transverse sections with 12 μm were obtained on a cryostat (Leica CM3050S, Nussloch, Germany) at -20ºC. The cryosections were then collected on gelatin-coated glass slides and allowed to air dry for 1 hour. Retina sections were then stored at -20ºC for later use.

2.5.2 - Immunohistochemistry

For staining, frozen sections were placed 45 min at room temperature. After thawing, the sections were fixed in cold acetone (-20ºC) during 10 min and subsequently hydrated 3 times in PBS, during 5 min each time, to remove OCT. Sections were permeabilized with 0.25% Triton X-100 in PBS, for 30 min and blocked with 10% goat serum in PBS with 1% albumin bovine serum (BSA), for 30 min. Then they were incubated with primary antibodies against GFAP (1:500 PBS with 1% BSA), vimentin (1:750 in PBS with 1% BSA), or nitrotyrosine residues (1:200 PBS with 1% BSA) at 4°C overnight in a humid atmosphere, to avoid tissue dehydratation. After washing in PBS, 3 times for 5 min, conjugated secondary antibodies [Alexa Fuor-568-(red) goat anti-mouse IgG (1:500 in PBS with 1% BSA), for sections stained for GFAP and vimentin, or Alexa Fluor-488-(green) goat anti-rabbit IgG (1:250 in PBS with 1% BSA), for sections stained for nitrotyrosine residues], plus DAPI (1:5000), to stain cell nuclei, were added for 1 h in the dark, at room temperature. After washing the sections, 3 times for 5 min in PBS, coverslips were mounted over retina sections using

Glycerogel (Dako). Nitrotyrosine stained sections were observed with a confocal microscope (LSM 510, Carl Zeiss) and GFAP and vimentin stained section were visualized with an inverted fluorescence microscope (Leica DM IRE2).

2.6 - Tissue extracts

After dissection, retinas were washed with ice-cold PBS. Samples for Western blotting were then lysed and homogenized, on ice, with RIPA buffer [150 mM NaCl, 50 mM Tris, 5 mM EGTA, 1% Triton X-100, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate (SDS)] supplemented with *complete-mini* protease inhibitor cocktail tablets and phosphatase inhibitors (1mM dithiothreitol, 50mM NaF and 1mM Na_3VO_4). The resulting homogenate was sonicated (4 pulses, 2 seconds each) and stored at -80ºC.

2.7 - Protein quantification

All tissue lysates were centrifuged at 13,200 rpm for 10 min at 4ºC. The supernatant was transferred to clean tubes and protein concentration was then measured by the colorimetric bicinchoninic acid (BCA) (Pierce, Rockford, USA) assay. This method combines the well-known biuret reaction (reduction of Cu^{2+} to Cu^{+} by protein in an alkaline medium) with the highly sensitive and selective colorimetric detection of Cu^{+} cation by the bicinchoninic acid, giving a purple color to the final reaction product. This complex exhibits the maximum absorbance at 562 nm directly proportional to the protein concentration.

Samples were diluted in deionized water and incubated with BCA reagent for 30 min at 37ºC, in a 96-well plate. The content of each well was mixed and the absorbance of each sample was read at 570 nm in a microplate reader (Synergy HT; Biotek, Winooski, USA). The protein standard curve was prepared using BSA (2.0mg/ml) in deionized water, and the absorbance of BSA standard samples was used to construct the curve and to determine protein concentration in the retina.

2.8 - Western blotting

After the determination of protein concentration, retina samples were denaturated with 6x concentrated sample buffer (0.5 M Tris-HCl, 30% glycerol, 10% SDS, 0.6 M DTT, 0,02% bromophenol blue, pH 6.8) at 95ºC, for 5 min. Samples were loaded (same amount per lane) and separated by electrophoresis on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), for 60min at 160 V in Tris-Bicine buffer (25 mM Tris, 25mM Bicine, 1% SDS, pH 8.3). When blotting for vimentin 10μg of protein were loaded and 15 μg of protein were loaded when blotting for GFAP, in a 4-10% polyacrylamide gel; 40 μg of protein were loaded when blotting for caspase-3, in a 4-12% polyacrylamide gel; 80 μg of protein were loaded when blotting for TNF-α and IL-1β, in a 4-12% polyacrylamide gel. Proteins were transferred electrophoretically to PVDF membranes (Amersham Biosciences, Uppsala, Sweden) in CAPS-methanol buffer (10mM CAPS, pH 11, 10% methanol), for 90 min at 750 mA (4ºC). The membranes were then blocked for 1 h at room temperature, in Tris-buffered saline with Tween 20 (TBS-T) (20 mM Tris-HCl, 137 mM NaCl, 0.1% Tween 20 (v/v), pH 7.6) containing 5% (w/v) low-fat milk when blotting for GFAP, vimentin and caspase-3; and in TBS-T with 4% BSA when blotting for TNF-α and IL-1β. After that, the membranes were incubated for 2 h at room temperature with the primary antibodies: anti-GFAP (1:5000), anti-vimentin (1:1000), anti-caspase-3 (1:1000). The primary antibodies anti-IL-1β (1:1000) and anti-TNF-α (1:500) were incubated overnight at 4ºC. The antibodies were diluted in TBS-T solution with 1% low-fat milk when blotting for GFAP, vimentin and caspase-3; and diluted in TBS-T solution with 4% BSA when blotting for TNF-α and IL-1β. After washing for 1 h (3 x 20 min) in TBS-T, the membranes were incubated for 1 h at room temperature with an alkaline phosphatase-linked secondary antibodies (1:20,000) [goat anti-rabbit IgG in TBS-T with 1% low-fat milk for membranes incubated with anti-caspase-3 and goat anti-rabbit IgG in TBS-T with 4% BSA for membranes incubated with anti-TNF-α; rabbit anti-goat IgG in with 4% BSA for membranes incubated with anti-IL-1β; goat anti-mouse IgG in TBS-T with 1% low-fat milk for the remaining membranes]. The membranes were then washed again with TBS-T (3 x 20 min). The proteins were detected using ECF substrate, and fluorescence was detected on an imaging system (Typhoon FLA 9000, GE Healthcare, Chalfont St. Giles, UK). In the end, digital quantification of the densitometry of the bands was performed

using Image Quant 5.0 software (Molecular Dynamics, Amersham Biosciences, Uppsala, Sweden). To check if the amount of loaded protein was similar between the different lanes the membranes were re-incubated with a primary antibody anti-actin in TBS-T with 1% low-fat milk (1: 20,000) followed by re-incubation with alkaline phosphatase-linked secondary antibody: goat anti-mouse IgG in TBS-T with 1% low-fat milk (1: 20,000).

2.9 - Semi-quantitative analysis of TNF-α and IL-1β mRNA

2.9.1 - Isolation of total RNA from tissue

Total RNA was isolated from the retina of *Wistar* rats using TRIZOL Reagent (Sigma-Aldrich, Madrid, Spain) according to manufacturer's instructions. Tissue was homogenized in guanidinium isothiocyanate and phenol using a glass-Teflon. Chloroform was added and after centrifugation (15,300 rpm, 15 min, 4ºC), RNA was isolated in the aqueous phase and precipitated with isopropanol. The pellet was then washed with 75% ethanol in diethylpyrocarbonate (DEPC)-treated water, briefly dried, and redissolved in 16 μl of (DEPC)-treated water. The total amount of RNA was quantified by optical density (OD) measurements at 250 nm in a NanoDrop ND 1000 Spectrophotometer (Thermo Scientific, Massachusetts, USA).

2.9.2 - Reverse transcription polymerase chain reaction (RT-PCR) analyses

Pro-inflammatory cytokines IL-1β and TNF-α mRNA levels were determined by reverse transcriptase-PCR (RT-PCR). Reverse transcription into cDNA was carried out by using the SuperScript First-Strand Synthesis System kit for RT-PCR (Invitrogen, Barcelona, Spain) following the manufacter'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, 0.5 mM dNTP mix, 5 mM $MgCl_2$, 10 mM DTT, 40 units of RNase inhibitor, 1x random hexamers, and 1 μl of SuperScript II Reverse Transcriptase.

Afterwards, PCR was performed in a 25 μl reaction volume containing 2 μl (IL-1β, TNF-α and β-actin) of cDNA, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 1× Taq DNA

polymerase buffer, 1 unit of Platinum Taq DNA polymerase (Invitrogen, Barcelona, Spain), 0.3 μM of both forward and reverse primers, and a variable volume of water. Forward and reverse primers (Invitrogen, Barcelona, Spain) used in PCR reactions were as follows: IL-1β, 5´-AGCAGCTTTCGACAGTGAGGAGA-3´ and 5´-TCCTGGAAGCTCCACGGGCA-3´; TNF-α, 5´-TCCAGGCGGTGTCTGTGCCT-3´ and 5´-TGGTTTGCTACGACGTGGGCT-3´; β-actin, 5´-CTGGAGAAGAGCTATGAGCTG-3´ and 5´-AATCTCCTTCTGCATCCTGTC-3´. The PCR cycling profile for IL-1β and TNF-α was the following: 30s at 94 ºC for denaturation, annealing at 61 ºC for 30s, extension at 72 ºC for 15s, for 35 cycles, and a 10 min final extension period at 72 ºC. β-actin PCR cycling profile was similar but with an annealing temperature of 55 ºC for 30s. The mRNA levels of β-actin were used as a loading control, and negative controls were performed to ensure that PCR products result from RNA transcription. PCR products were separated by gel electrophoresis (1.5% agarose) and were stained with ethidium bromide. The identification of the bands was carried out using the Universal Hood II (Bio-Rad Laboratories, Milan, Italy). Then, mRNA expression was evaluated by analyzing the band-intensity using Image Quant 5.0 software (Molecular Dynamics, Amersham Biosciences, Uppsala, Sweden).

2.10 - Detection of Oxidized Carbonyl in Proteins

The protein concentration of retinal lysates was adjusted to 1.5 mg/ml with lysis buffer (the same used for Western blotting samples), and 50 μl of protein solution (75 μg) was incubated with 50 μl of 10 mM 2,4-dinitrophenylhydrazine (DNPH) in 10% of trifluoroacetic acid (TFA) (prepared one day before the experiments) for 15 min, at room temperature. The reaction was stopped with trichloroacetic acid (TCA) during 10 min, at room temperature. After centrifugation at 14,000 rpm for 5 min, the supernatant was discarded and the pellet was washed twice, followed by centrifugation at 14,000 rpm for 5 min with ethylacetate:ethanol (1:1) to remove free DNPH. The pellet was solubilized in 100 μl 1x Laemmli sample buffer (15.6 mM Tris-HCl pH 6.8, 2.5% glycerol, 0.5% SDS, 1.25% 2-β-mercaptoetanol, 0.01% bromophenol blue) with a brief sonication (4 pulses, 2 seconds each) and stored at -20ºC, for later use.

2.10.1 - Dot blot

The DNP binding sites of the oxidized proteins were specifically detected using an anti-DNP antibody (1:5,000, in TBS-T solution with 5% low-fat milk) by dot blot. For the dot blot, 2 µl of the protein carbonyl-DNPH derivatives (20x diluted) were loaded on a nitrocellulose membrane at each dot. Membranes were then placed within an incubator during 30 min at 50ºC, to fix proteins to the membrane. After blocking the membrane with TBS-T containing 5% (w/v) low-fat milk, during 1 h at room temperature, followed for anti-DNP antibody incubation, for 1h at room temperature, membranes were washed, 6 times for 5 min, in TBS-T. Then membranes were incubated for 1 h more, at room temperature, with an alkaline phosphatase-linked secondary antibody goat anti-rabbit IgG (1:5 000), in TBS-T containing 5% (w/v) low-fat milk, followed by washing for more 6 times, 5 min each time, in TBS-T. Protein immunoreactive dots were then visualized using ECF substrate, and fluorescence was detected on an imaging system (Typhoon FLA 9000, GE Healthcare, Chalfont St. Giles, UK). In the end, digital quantification of the densitometry of the bands was performed using Image Quant 5.0 software (Molecular Dynamics, Amersham Biosciences, Uppsala, Sweden).

2. 11 - Statistical analysis

The results are presented as mean ± standard error of the mean (SEM). The data were analyzed using one-way analysis of variance (ANOVA), followed by Bonferroni´s post-hoc test. The statistical analysis was performed in Prism 4.0 Software (GraphPad Software). *p* values less than 0.05 were taken as significant.

Chapter 3 Results

3.1 - Animals

Before diabetes induction, the body weight of animals assigned for control and diabetic groups was similar (274.9 ± 3.4 g for control animals and 276.9 ± 3.0 g for diabetic animals). The average weight and blood glucose levels for both diabetic and aged-matched control rats at the time of death are given in Table I. Diabetic rats showed a marked impairment in weight gain comparing with age-matched controls. Furthermore, diabetic animals had a marked hyperglycemia and glycohemoglobin levels in comparison with age-matched controls. CaD treatment did not alter the body weight, hyperglycemia or glycohemoglobin of both control and diabetic rats.

Table I: Average weight, blood glucose and HbA_{1c} levels of diabetic and aged-matched control rats.

	Weight (g)	Glycemia (mg/dL)	HbA_{1c} (%)
Control	394.1±10.7	89.2±2.5	3.6±0.0
CaD	390.9±5.2	85.0±2.7	3.6±0.0
Diabetic	259.5±6.3***	470.5±20.2***	8.3±0.2***
Diabetic+CaD	266.2±4.6***	427.3±20.3***	8.4±0.1***

Statistical significance was determined by one-way ANOVA, followed by Bonferroni´s post-hoc test. Data are presented as percentage of control and represent the mean ± SEM of 24-25 animals ***$p<0.001$, significantly different from control.

3.2 - Effect of calcium dobesilate on glial cells reactivity

Previous data have reported changes in GFAP levels, a common marker for reactive gliosis, in the retina during diabetes (Lieth et al., 1998; Rungger-Brandle et al., 2000). In this study, in order to evaluate the effect of CaD in glial reactivity during diabetes, the levels of GFAP was evaluated by both immunoblot and immunofluorescence analysis in the retina of diabetic animals (2 months duration). By western blotting analysis (Figure 7), there was a significant increase in GFAP protein levels in the retina of diabetic animals (147.8 ± 4.4% of control, Figure 7A). Moreover, in diabetic rats

treated with CaD for 14 days, the GFAP levels were also significantly increased comparing with control rats (174.8 ± 5.6% of control, Figure 7A), but not significantly increased compared with the values obtained in diabetic animals. Furthermore, analyzing the non-diabetic group of rats receiving CaD, it is evident a statistical increase comparing to age-matched controls (138.6 ± 12.8% of control, Figure 7A). This suggests that CaD alone was able to stimulate glial cells reactivity by inducing an increase in the content of GFAP.

Vimentin, like GFAP, is an intermediate filament protein, normally expressed by Müller cells. Müller cell reactivity has been associated with increased expression of vimentin or GFAP (or both) (Verardo et al., 2008). Thus, in order to evaluate the effect of CaD and diabetes on retinal glial cells, vimentin immunoreactivity was also assessed. However, neither diabetes nor CaD induced any changes in the protein levels of vimentin (Figure 7B).

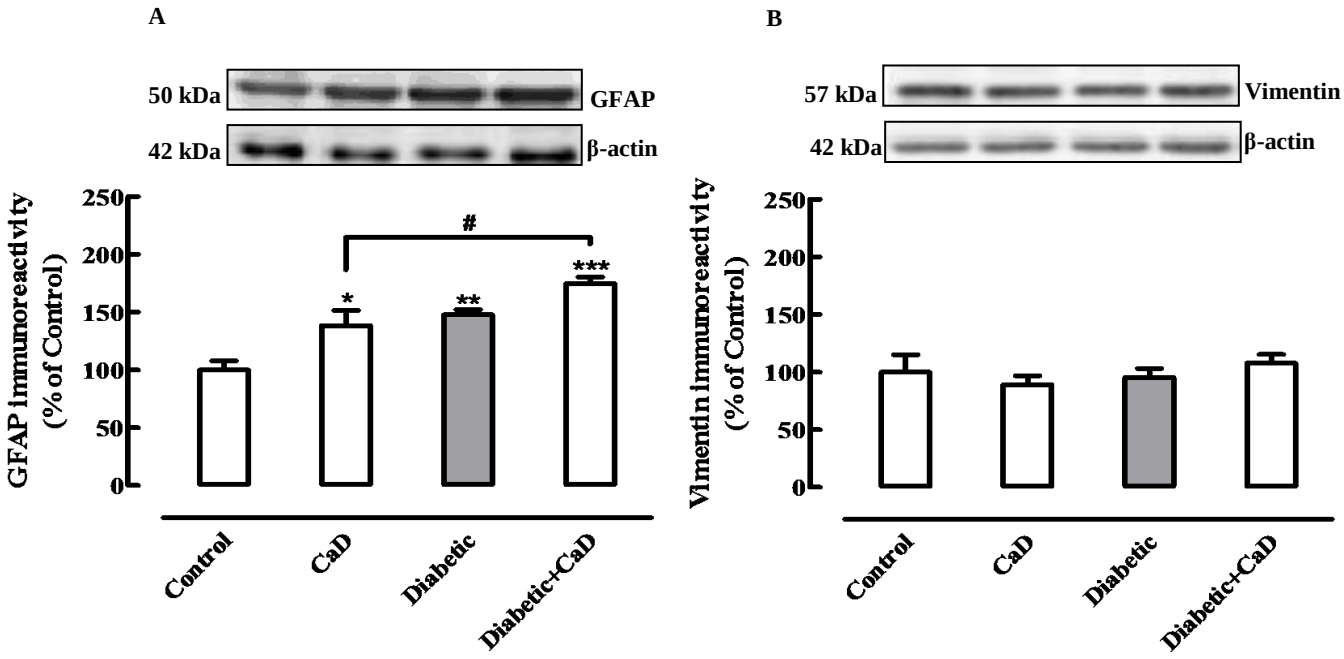

Figure 7. Effects of diabetes and calcium dobesilate on GFAP and vimentin protein levels. The protein levels of GFAP and vimentin were assessed by immunoblotting in total protein extracts of the retina. A representative Western blotting is presented above the graphs, with the respective loading controls (β-actin), to confirm that identical amounts of protein were loaded into the gel. Data are presented as percentage of control and represent the mean ± SEM of 5-6 animals. *$p<0.05$, **$p<0.01$, ***$p<0.001$, significantly different from control; #$p<0.05$, significantly different from CaD, as determined by ANOVA followed by Bonferroni's post test. Legend: CaD – Calcium dobesilate.

To further confirm the results obtained by immunoblotting, GFAP distribution was also assessed by immunohistochemistry in flatmounted retinas (Figure 8) and in retinal sections (Figure 9). We used confocal stacking images of flatmounted retinas, that help us to focus at the different layers of the retina, namely at the layer of astrocytes and at the most inner layer of the retina where the end-feet of Müller cells are located. In flatmounted retinas, GFAP immunoreactivity in control rats and in CaD group, was restricted to astrocytes (Figure 8A/B), but GFAP staining appeared to be more intense in the CaD group (Figure 8B). No GFAP staining was detected at the level of Müller cell end-feet. In the diabetic retinas (Figure 8C/D), GFAP immunoreactivity was reduced in the astrocytes (Figure 8C) comparing to controls (Figure 8A). In contrast, Müller cells end-feets appeared immunoreactive for GFAP (Figure 8D). In the retina of diabetic animals treated with CaD (Figure8D/E), GFAP immunoreactivity was increased in the astrocytes (Figure 8E) compared with the untreated diabetic rats (Figure 8D). Moreover, GFAP immunoreactivity in the end-feet of Müller cells of diabetic rats treated with CaD presented a modest increase (Figure 8F) when compared to diabetic non-treated animals (Figure 8D). The staining always appeared more intense in the diabetic group treated with CaD comparing with control and with the untreated diabetic rats, which is in agreement with the results obtained by immunoblotting.

In retinal sections, GFAP immunoreactivity of control animals was confined to the most inner retinal layer. GFAP immunoreactivity increased in the diabetic group, and in a similar way, it also increased in non-diabetic animals treated with CaD, essentially in ganglion cell layer and inner retinal layers. The immunoreactivity was even more intense in the retinas of diabetic animals treated with CaD (Figure 9).

Furthermore, by immunohistochemistry studies in retinal sections, no significant differences were observed in vimentin immunoreactivity between the different groups (Figure 9).

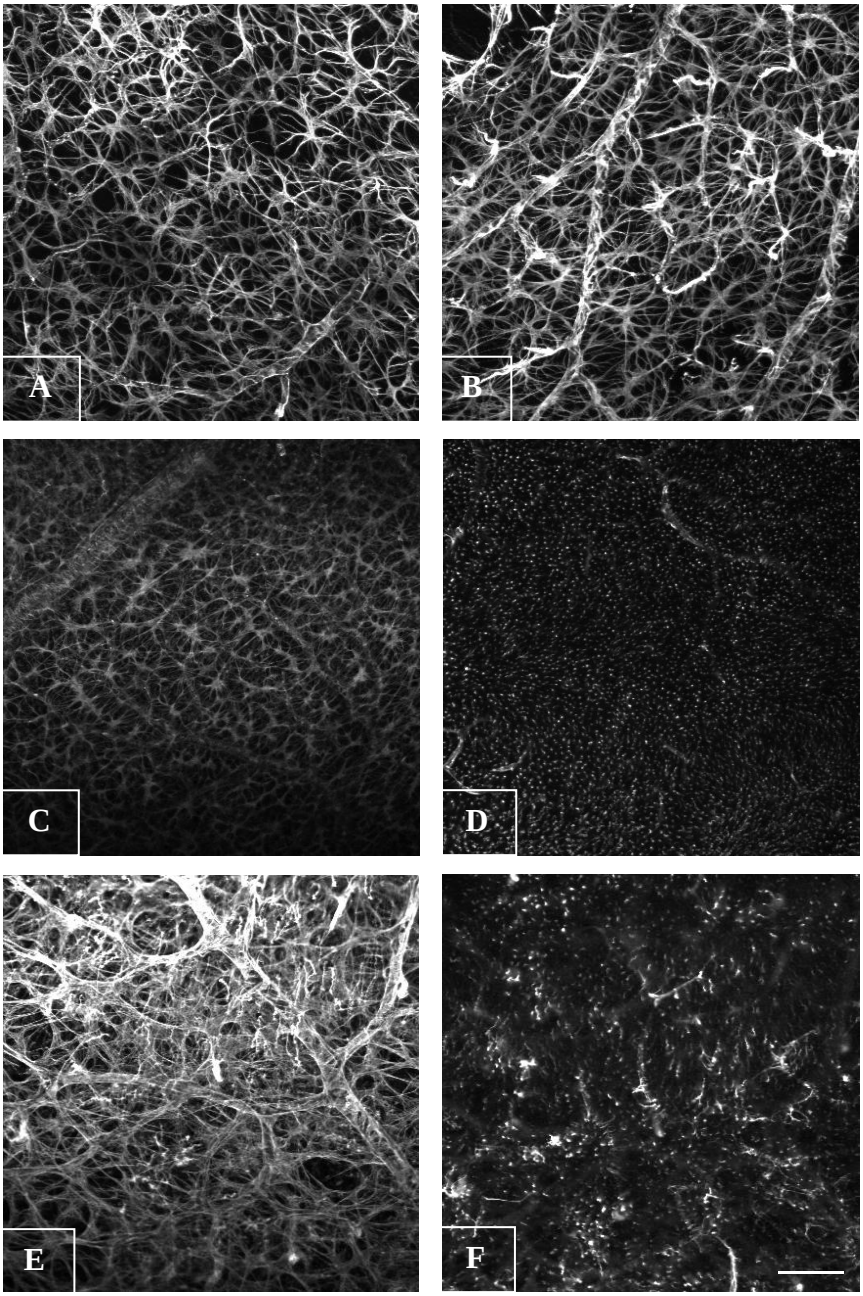

Figure 8. GFAP expression in astrocytes and Müller cells of the retina. GFAP expression was detected by immunofluorescence in flatmounted retinas. Stacking images were taken by confocal microscopy.The images shown represent the astrocyte layer or Müller cell end-feet layer. **(A)** Control, astrocytes layer; **(B)** CaD-treated, astrocytes layer; **(C)** Diabetic, astrocytes layer; **(D)** Diabetic, Müller cells end-feet layer; **(E)** Diabetic CaD-treated, astrocytes layer; **(F)** Diabetic CaD-treated, Müller cells end-feet layer. 200x magnification (bar 100 μm).

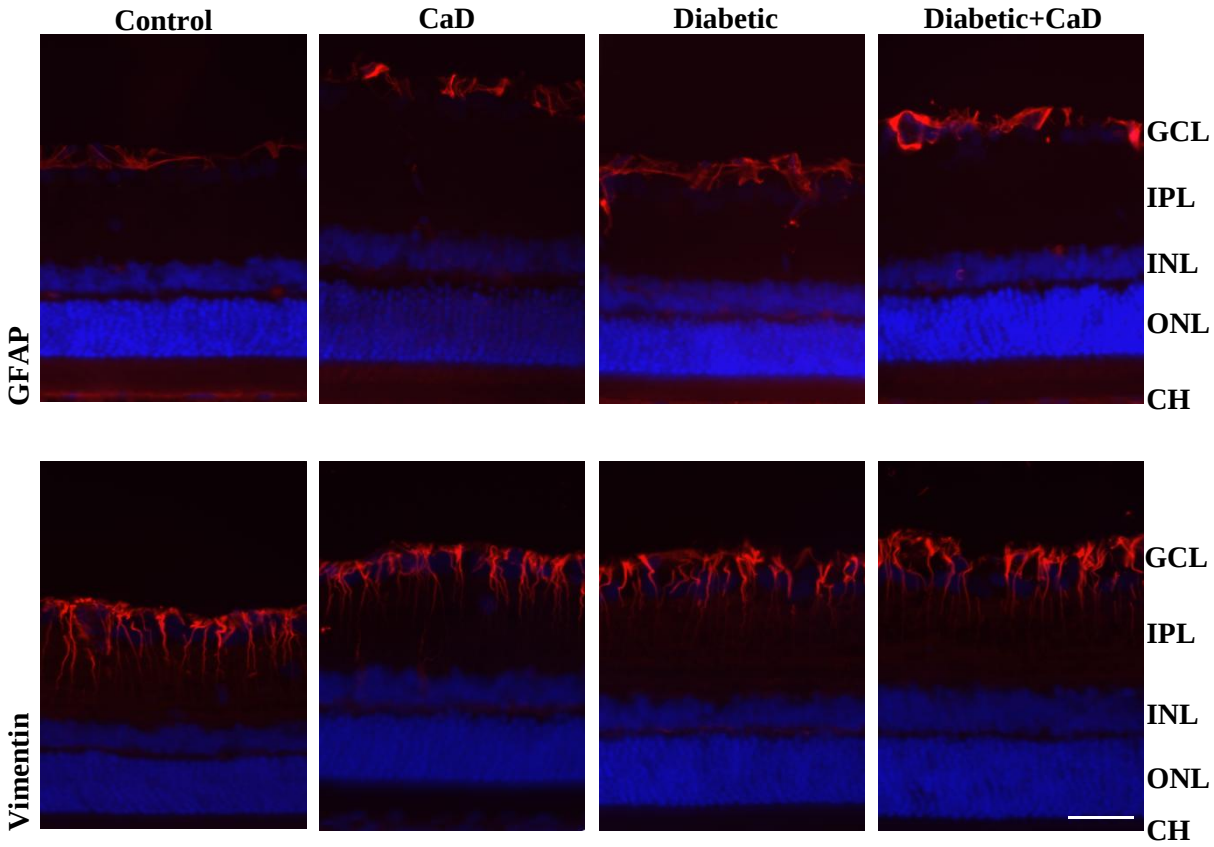

Figure 9. Effect of calcium dobesilate and diabetes on GFAP and vimentin immunoreactivity in the retina. Representative images of retinal slices showing GFAP and vimentin immunoreactivity (red), which allows the detection of astrocytes and/or Müller cells, and nuclear DAPI staining (blue). 400x magnification (bar 50 μm). Legend: CaD, Calcium dobesilate; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; ONL, outer nuclear layer; CH, choroid.

3.3 - Effect of calcium dobesilate on mRNA expression and protein levels of inflammatory markers, TNF-α and IL-1 β

The increase in the production of pro-inflammatory cytokines, namely TNF-α and IL-1β, has been correlated with the increase in retinal vascular permeability and angiogenesis in diabetic retinas (Joussen et al., 2001; Wilkinson-Berka, 2004). In order to evaluate a potential protective effect of CaD on the inflammatory process, TNF-α and IL-1β mRNA (Figure 10) and protein (Figure 11) levels were analyzed. After 2 months of diabetes there was a significant increase in TNF-α (140.3 ± 8.0% of control, Figure 10A) and IL-1β (188.6 ± 8.4% of control, Figure 10B) mRNA expression levels. The expression levels of TNF-α and IL-1β in controls and CaD-treated rats were similar

(Figure 10A/B). CaD treatment completely prevented the increase in TNF-α (99.2 ± 1.2% of control, Figure 10A) and IL-1β (103.0 ± 8.65% of control, Figure 10B) mRNA levels induced by diabetes.

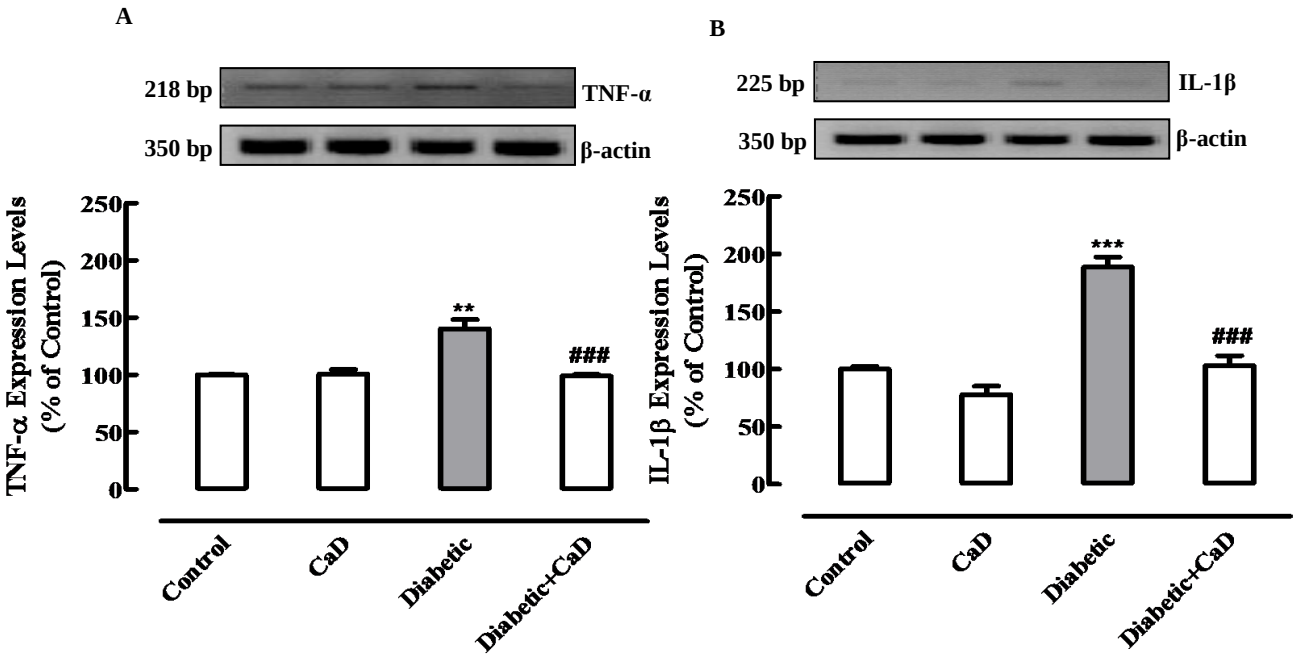

Figure 10. Calcium dobesilate prevents the increase in TNF-α and IL-1β mRNA levels in the retina, induced by diabetes. The mRNA levels of TNF-α **(A)** and IL-1β **(B)** were assessed by RT-PCR. Representative bands are presented above the graphs, with the respective loading controls (β-actin). Data are presented as percentage of control and represent the mean ± SEM of 3-4 animals. **p<0.01, ***p<0.001, significantly different from control; ###p<0.001, significantly different from diabetic rats, as determined by ANOVA followed by Bonferroni's post test. Legend: CaD – Calcium dobesilate.

In diabetic rat retinas, TNF-α protein levels were significantly increased (162.1 ± 15.8 % of control, Figure 11A). CaD treatment prevented the increase in TNF-α induced by diabetes (Figure 11A). CaD per se did not change TNF-α protein levels in the retina.

After 2 months of diabetes, the content of IL-1β in the retina increased, but this increase did not reach statistical significance. In the retinas of diabetic animals treated with CaD, the levels of IL-1β were similar to those found in control and CaD-treated animals (Figure 11B).

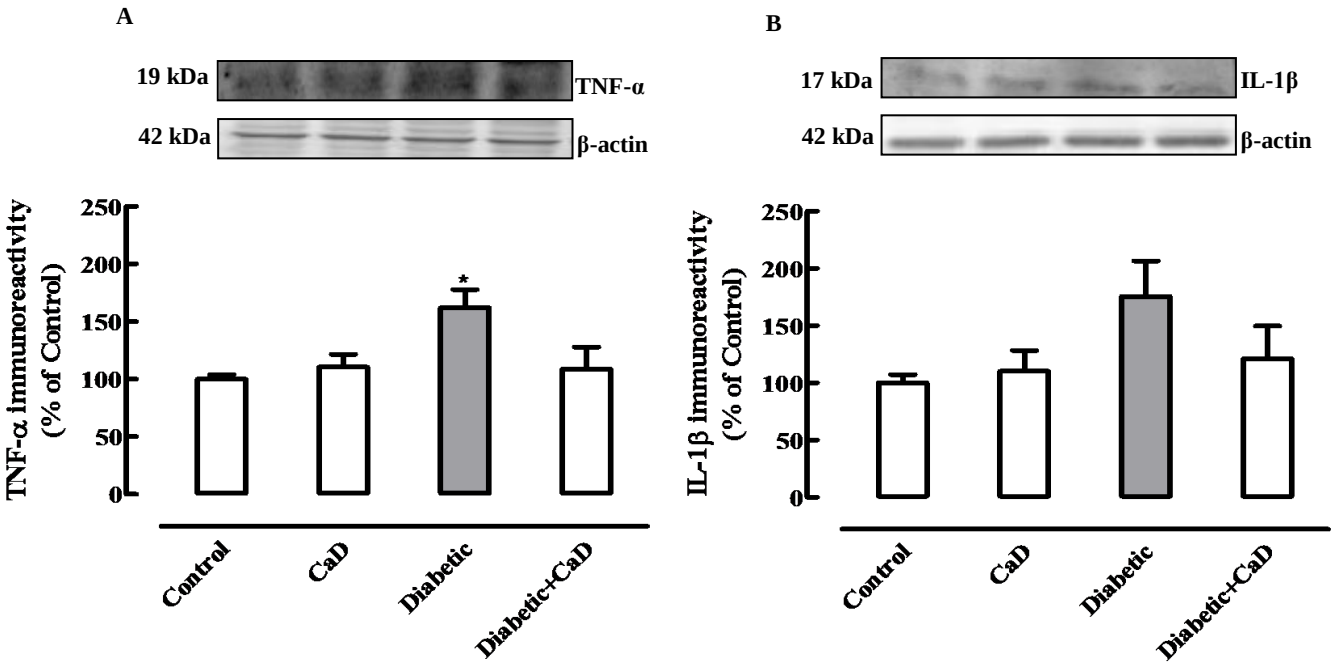

Figure 11. Calcium dobesilate attenuates the increase in pro-inflammatory cytokine levels in the retina induced by diabetes. The protein levels of TNF-α **(A)** and IL-1β **(B)** were assessed by immunoblotting in total extracts from retina. A representative Western blotting is presented above the graphs, with the respective loading controls (β-actin). Data are presented as percentage of control and represent the mean ± SEM of 3-4 animals. *$p<0.05$, significantly different from control, as determined by ANOVA followed by Bonferroni's post test. Legend: CaD – Calcium dobesilate.

3.4 - Effect of calcium dobesilate on caspase-3 activation

Several studies have correlated the increase in the production of pro-inflammatory cytokines with an increase in cell death by apoptosis mediated via activation of NF-κB and caspase-3 in retinal neurons and capillary cells (Abu El-Asrar, 2001; Kowluru & Koppolu, 2002; Kowluru et al., 2003; Kowluru & Odenbach, 2004c). Thus, to investigate if CaD was able to prevent the potential activation of caspases after 2 month of experimental diabetes, caspase-3 protein levels were assessed in total retinal extracts. However, by Western blotting, no bands corresponding to the p17 subunit (active form of caspase-3) were detected. Moreover, no significant differences were detected in the pro-caspase-3 protein levels (Figure 12).

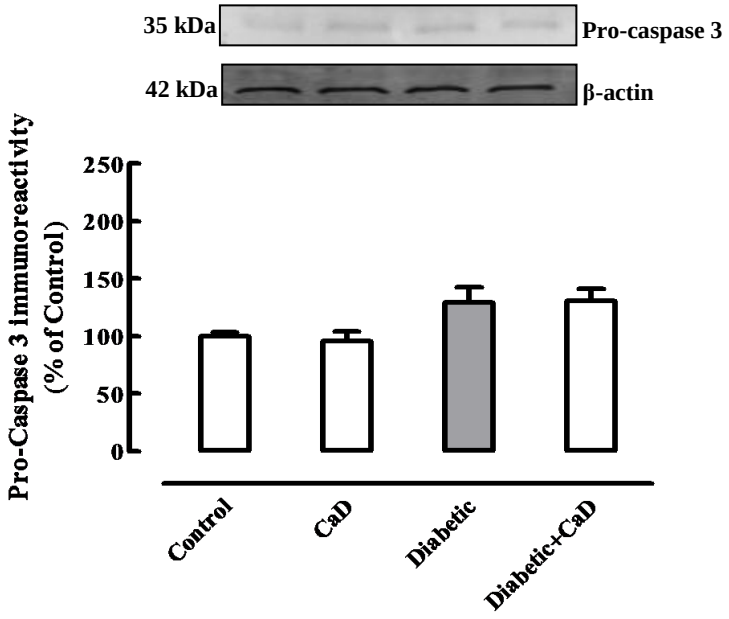

Figure 12. Diabetes (2 months duration) does not increase caspase activation in rat retinas. Pro- and active caspase levels were analyzed by Western blotting. The band corresponding to the activated caspase-3 (17 kDa) was not detected. Representative Western blots, including the loading control (β-actin), are presented above the graph. The results are expressed as mean ± SEM of at least 3 animals and are represented as percentage of control. Legend: CaD – Calcium dobesilate.

3.5 - Calcium dobesilate reduces diabetes-induced oxidative stress

A large body of evidence strongly suggests that diabetes induce an overall increase in oxidative stress in the retina, which in turn creates a continuous positive feedback loop on the inflammatory process (Joussen et al., 2002b; Kowluru et al., 2003; Chang & LoCicero 2004; Kowluru & Odenbach, 2004c; Zheng et al., 2004). It has also been claimed that CaD exerts its protective effects due to its antioxidant properties (Szabo et al., 2001; Rota et al., 2004; Leal et al, 2010). Taking this into account, the effects of CaD on the formation of oxidized carbonyl groups were evaluated. The presence of carbonyl groups in proteins has been used as a marker of ROS-mediated protein oxidation. Detection and quantification of protein carbonyl groups involve derivatization of the carbonyl group with DNPH (2,4-dinitrophenolhydrazine) and subsequent immunodetection.with anti-DNP antibody. Carbonyl residues were significantly increased in diabetic rat retinas (123.3 ± 6.7 % of control, Figure 13). CaD

treatment inhibited the increase in DNP immunoreactivity induced by diabetes (104.6 ± 7.5 % of control, Figure 13). The immunoreactivity of DNP in the retina of animals treated with CaD was similar to DNP immunoreactivity in controls.

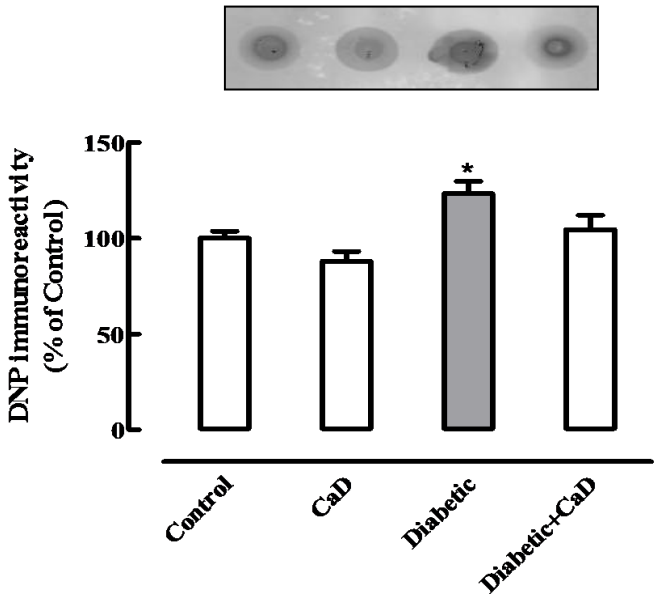

Figure 13. Calcium dobesilate prevents the increase in oxidized carbonyl groups formation induced by diabetes in the retina. The oxidized proteins were detected using an anti-DNP antibody by dot blot. A representative dot blot is shown above the graph. Data are presented as percentage of control and represent the mean ± SEM of 5-6 animals. *$p<0.05$, significantly different from control; ANOVA followed by Bonferroni's post test. Legend: CaD – Calcium dobesilate.

3.6 - Calcium dobesilate reduces diabetes-induced nitrosative stress

In conditions such as those occurring in the retina during diabetes, oxidative stress can induce the production of large amounts of peroxynitrite. Peroxynitrite modifies tyrosine in proteins forming nitrotyrosine residues, and nitration of proteins can inactivate mitochondrial and cytosolic proteins and damage cellular constituents, resulting in cellular dysfunction by nitrosative stress. Given the antioxidant properties of CaD, the effects of CaD treatment on tyrosine nitration were assessed by immunohistochemistry in retinal sections at 2 months of diabetes (Figure 14). Diabetes increased tyrosine nitration in the retina, particularly in the inner retinal layers (Figure

14C). The strongest immunoreactivity against nitrotyrosine residues was found within the ganglion cell layer. Treatment with CaD inhibited the increase in tyrosine residues nitration induced by diabetes (Figure 14D). CaD per se did not cause any change in nitrotyrosine immunoreactivity (Figure 14A/B).

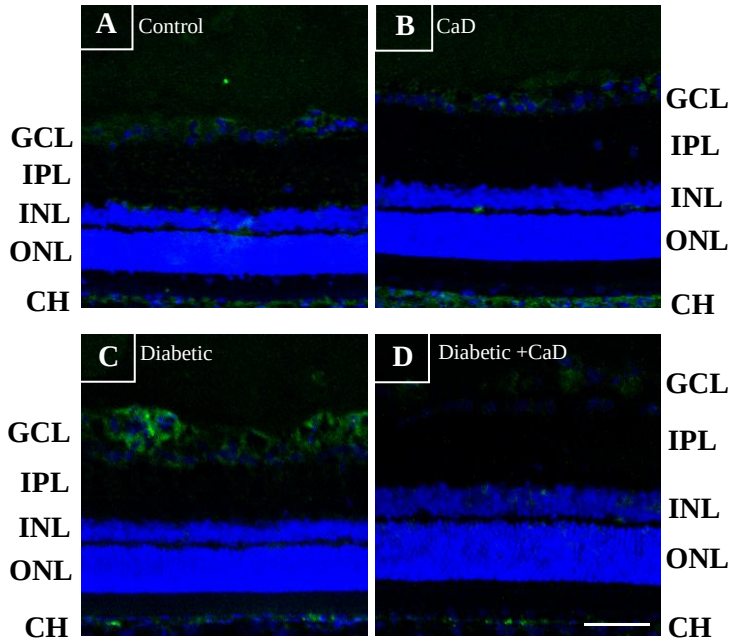

Figure 14. Calcium dobesilate inhibits the increase in the formation of nitrotyrosine residues induced by diabetes in the retina. Representative images of retinal sections showing nitrotyrosine immunoreactivity (green), which allows the detection of nitrated tyrosine residues, and nuclear DAPI staining (blue). Magnification: 200x, bar 100µm. Legend: **(A)** Control; **(B)** Non-diabetic treated with CaD; **(C)** Diabetic; **(D)** Diabetic treated with CaD; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; ONL, outer nuclear layer; CH, choroid.

Chapter 4 Discussion

It has been shown that CaD can have beneficial effects in the retina of diabetic patients, particularly at the level of blood-retinal barrier (Leal et al., 2010 ; van Bijsterveld & Janssen, 1981; Leite et al., 1990; Ribeiro et al., 2006). However, despite the use of CaD in the last decades in the treatment of diabetic retinopathy, a better understanding on the cellular and molecular actions of this compound was definitely lacking. Oxidative stress and inflammation are considered to play a central role in the development and progression of retinopathy (Joussen et al., 2002a; Romeo et al., 2002; Kowluru, 2003; Kowluru et al., 2003) and therefore they are favourite targets for therapeutic strategies. It is also known that oxidative stress can induce the production of inflammatory markers. Taking this into account, this study aimed to evaluate the impact of CaD on typical features of the inflammatory process. Since it has also been claimed that CaD has antioxidant properties, its potential preventive effect on oxidative and nitrosative stress markers was evaluated as well, namely on the formation of oxidized carbonyl groups and nitrotyrosine residues in the retinal tissue.

The results show that CaD is able to prevent the increase of TNF-α and IL-1β expression induced by diabetes in the retina, at least during the early phase of diabetes. These findings appeared in strong correlation with the inhibition of oxidative/nitrosative stress by CaD in the retina.

It is important to mention that CaD is not an anti-diabetic drug, and thus there were no effects neither on blood glucose levels nor in the glycohemoglobin status, like it was expected.

Previous data have reported an increase in the levels of GFAP, a common marker for reactive gliosis, in the retina during diabetes (Lieth et al., 1998; Mizutani et al., 1998; Rungger-Brandle et al., 2000). Glial reactivity is considered a general response to injury and inflammation (O'Callaghan, 1991; Norton et al., 1992). In the present study, in order to evaluate the effect of CaD in glial reactivity during diabetes, the levels of GFAP were evaluated. As expected, a significant increase in GFAP protein levels in the diabetic rat retinas was detected. However, and unexpectedly, the oral administration of CaD also increased GFAP levels, which suggests an astrocytic activation. Moreover, the results also show a partial additive effect of diabetes and CaD regarding GFAP immunoreactivity in diabetic animals treated with CaD. This was never referred before, and the meaning of this glial reactivity is unknown.

The central nervous system responds to diverse neurologic injuries with a vigorous activation of astrocytes (Pekny & Pekna, 2004). Glial activation leads to the upregulation of GFAP, commonly accompanied by proliferation and hypertrophy of glial cells (Eddleston & Mucke, 1993; Eng & Ghirnikar, 1994; Barber et al., 2000). Indeed, GFAP upregulation is a stereotypical response present in the retina during experimental diabetes and in age-related retinal degenerations (DiLoreto et al., 1995). Also, elevated levels of GFAP have been identified in the human retina (Mizutani et al., 1998). However, this process is still very controversial, and both harmful and beneficial activities have been attributed to astrocytes. Concepts about reactive glia have long been implicated in the inhibition of axon regeneration (Liuzzi & Lasek, 1987; Rudge & Silver, 1990), and reactive astrocytes are often regarded as detrimental to functional recovery after CNS injury. Nevertheless, the basic phenomena of reactive glia after CNS injury appear conserved throughout vertebrate evolution (Larner et al., 1995), suggesting that fundamental aspects of the process convey survival advantage.

Reactive gliosis is a range of potential molecular, cellular and functional changes in glial cells that occur in response to all forms of CNS injury and disease. The changes of reactive gliosis are regulated in a context-specific manner by inter- and intracellular signaling molecules. These changes undergone during reactive gliosis have the potential to alter glia activities both through gain and loss of functions that can impact both beneficially and detrimentally on surrounding neural and nonneural cells (Sofroniew, 2009). In this regard, many different types of intercellular signaling molecules are able to trigger or to regulate specific aspects of reactive glia. These include, increased levels of pro-inflammatory cytokines, increased levels of neurotransmitters, such as glutamate, elevated ATP, ROS, NO, hypoxia and regulators of cell proliferation, such as endothelin-1 (Sofroniew, 2009). In fact, all of these markers are present in the retina during diabetes. So, this led us to, normally, think that the entire process of reactive gliosis is a uniformly negative phenomenon that will unavoidably trigger glial cells to extend neurotoxicity and inflammation. This stereotyped viewpoint has led to the simplistic notion that total inhibition of reactive gliosis can be regarded as a good therapeutic strategy. However, it is possible that this exacerbated glial reaction could be a way to promote tissue recovery. A growing body of evidence has demonstrated that reactive astrocytes can protect cells and tissue by increasing the uptake of potentially excitotoxic glutamate (Rothstein et al., 1996; Suzuki et al., 2001; Aronica et al., 2003) by increasing cytosolic antioxidant proteins, such as GSH (Levison et al., 1996); (Chen

et al., 2001; Swanson et al., 2004); by neuroprotecting tissue via adenosine release (Lin et al., 2008) and through degradation of β-amyloid peptides (Koistinaho et al., 2004); by improving blood barrier repair (Bush et al., 1999; Abbott, 2000); by enhancing neuronal survival through the release of neurotrophic factors (Schwartz & Nishiyama, 1994; Marz et al., 1999) and by restricting inflammation thereby helping to limit tissue degeneration and preserve function after injury (Faulkner et al., 2004). Taking this into account, we speculate that reactive glial phenomenon has to be analyzed with prudence, concerning all the different perspectives. We suspect that the observed increase in GFAP levels in the diabetic-treated group could be due to a neuroprotective mechanism instead of an injury insult. Indeed, it was documented that CaD is able to prevent the loss of cellular GSH, but this observation was not related to glial reactivity (Szabo et al., 2001). Further investigation is needed to clarify the precise and real effect of CaD-induced reactive gliosis.

To further determine which cell types, astrocytes and/or Müller cells, were responsible for the increase in GFAP, immunohistochemistry experiments in flatmounted retinas and in retinal slices were performed. The results indicate that diabetes decreases the expression of GFAP in astrocytes and increases its expression in Müller cells, mostly at the end-feet of these cells. This is particularly evident in flatmounted retinas.

Normally, it is assumed that in the healthy retinal tissue GFAP is expressed only in astrocytes, being low expressed by Müller cells (Fletcher et al., 2005). In a diabetic condition, Müller cells and astrocytes show opposite reactions. Astrocytes dramatically decrease GFAP expression, whereas Müller cell express more GFAP, in short-term diabetes (Lieth et al., 1998; Barber et al., 2000; Asnaghi et al., 2003). The authors suggested that the observed reduction in GFAP levels by astrocytes during diabetes may be linked with altered metabolic capacity associated with an abnormal ability to response to injury. It was also pointed out that, these cells could be influenced by the surrounding environment in quite a different manner, because astrocytes contact directly with blood vessels and Müller cells extend across the entire neural retina.

In the case of CaD, the GFAP immunoreactivity increased in both astrocytes and Müller cells, causing an overall augment in the staining comparing with the control rats, as was shown by Western blotting. It is therefore clear that different mechanisms underlie the increase in GFAP immunoreactivity induced by diabetes or CaD. Taking into account the beneficial effects of CaD in the retina, we speculate that the

augmentation of GFAP not only in astroglial cells but also in Müller cells will predispose an appropriate regulation of glial cells to promote the recovery of retinal tissue.

Contrarily to what was observed in GFAP immnunoreactivity, the levels of vimentin were not affected neither by diabetes nor by CaD. Like GFAP, vimentin is a member of the intermediate filament family of proteins, but normally is expressed only by Müller cells. Müller cell reactivity is associated with an increased expression of vimentin or GFAP, or both (Verardo et al., 2008). Although it was expected an increase in vimentin reactivity, at least in the retina of diabetic animals, no clear evidence of significant changes in vimentin expression was obtained in this work, as assessed by different experiments. Probably, this could be noticed for longer periods of diabetes. It was however clear that Müller cells became reactive because the levels of GFAP increased.

Diabetic retinopathy exhibits the features occurring in chronic inflammation, such as increased vascular permeability (Wilkinson-Berka, 2004), edema, inflammatory cell infiltration (Miyamoto et al., 1999; Joussen et al., 2001; Tamura et al., 2005), cytokine and chemokine expression (Joussen et al., 2002a; Kowluru & Odenbach, 2004c), tissue destruction (Barber et al., 1998; Martin et al., 2004), neovascularization (Sone et al., 1997) and microglial activation (Gaucher et al., 2007). Enhanced oxidative and nitrative stress, PKC activity and DNA damage induced by hyperglycemia can lead to the activation of the transcription factor NF-κB in endothelial cell and pericytes in retinas of diabetic rats (Kowluru et al., 2003; Zheng et al., 2004). Although NF-κB can activate either prosurvival or proapoptotic pathways (Perkins, 2000), in the retina, under diabetic conditions, it seems to induce adverse effects. Activation of NF-κB can modulate the expression of several genes, including proapoptotic genes and upregulate the production of various pro-inflammatory mediators such as cytokines, chemokines and adhesion molecules, including ICAM-1 (Baldwin, 1996; Kowluru et al., 2003; Chang & LoCicero, 2004). Increased levels of cytokines, such as IL-1β and TNF-α, have been found in vitreous fluid of diabetic patients (Yuuki et al., 2001; Funatsu et al., 2002) and in the retinas of diabetic rats (Carmo et al., 1999; Joussen et al., 2002b; Kowluru & Odenbach, 2004c; Krady et al., 2005). In the diabetic retina, endothelial, glial and microglial cells constitute the major potencial source of IL-1β (Kowluru & Odenbach, 2004c; Krady et al., 2005) and TNF-α production (Tezel et al., 2001; Joussen et al., 2002a). Conversely stimulation of retinal cells with IL-1β and TNF-α initiate a series of

signal transduction events that lead to further NF-κB activation and augmentation of the inflammatory process (Joussen et al., 2002b; Chang & LoCicero, 2004; Kowluru & Odenbach, 2004c).

This work follows the line of our recent evidences. We have demonstrated that CaD treatment prevents some features of the inflammatory process, such as the increase in ICAM-1 levels, leukocyte adhesion to retinal vessels and also the activation of intracellular signaling pathways and transcription factors, like p38 MAPK and the redox-sensitive factor, NF-κB, at 1 month of experimental diabetes (Leal et al, 2010). Thus, given now attention to pro-inflammatory cytokines, we report here that CaD completely inhibited the increase in both TNF-α and IL-1β mRNA levels detected in the rats retinas after 2 months of diabetes, as well as the increase in TNF-α protein levels. Regarding IL-1β, diabetes did not induce a significant increase in its protein levels, but there was a clear tendency to an increase. Moreover CaD treatment appeared to inhibit that increase. We suggest that this effect could be correlated with the CaD proved ability to inhibit NF-κB activation, thus triggering inhibition of the pro-inflammatory cytokines transcription.

CaD ability to prevent the activation of caspases was also evaluated. Several studies have correlated the increase in the production of pro-inflammatory cytokines with an increase in apoptotic cell death in retinal neurons and capillary cells mediated by activation of NF-κB and caspase-3 (Abu El-Asrar et al., 2001; Kowluru & Koppolu, 2002; Kowluru et al., 2003; Kowluru & Odenbach, 2004c). However, neither caspase-3 activation nor significant changes in pro-caspase 3 levels were detected. In accordance with these results, Kowluru and Koppolu (2002) reported that no detected activation of caspase-3 was seen in the rat retina after 2 months of diabetes, in the same animal model. However, increased caspase-3 activity was detected in long-term studies (Kowluru et al., 2004), emphasizing that this issue needs additional investigation.

To further confirm the described antioxidant properties of CaD (Brunet et al., 1998; Szabo et al., 2001), the formation of oxidized carbonyls and nitrotyrosine residues was also evaluated. CaD treatment completely prevented oxidative and nitrosative injury in the retinal tissue after 2 months of diabetes, as assessed by the formation of oxidized carbonyl groups and nitrotyrosine residues, as we have previously demonstrated after 1 month of diabetes (Leal et al, 2010). Oxidative stress is considered to play a role in the inflammatory process (Kowluru & Odenbach, 2004b). Commonly, several findings strongly suggest that diabetes induces an overall increase in oxidative stress in the retina

which subsequently activates p38 MAPK and NF-κB pathways (Joussen et al., 2002a; Kowluru et al., 2003; El-Remessy et al., 2003) resulting in the upregulation of TNF-α and IL-1β expression. Both players of an inflammatory response, they are crucial in the progression of diabetic retinopathy, contributing, in addition to VEGF, to vascular permeability. Thus, the protective effects of CaD treatment can be explained by its antioxidant properties. CaD was effective in scavenging hydroxyl radicals *in vitro* (Brunet et al., 1998). Furthermore, CaD stabilized BRB in diabetic rats, via its antioxidant action (Rota et al., 2004), and reduced retinal edema thus protecting diabetic retina against the dysfunctions induced by ischemia/reperfusion (Szabo et al., 2001). In this study, administration of CaD to the diabetic rats for 14 days inhibited the elevation in cytokine expression, and the increase in oxidized carbonyls and nitrotyrosine formation, without ameliorating the severity of hyperglycemia. These results, in association with our published recent finding (Leal et al, 2010) suggest that the beneficial effects of CaD are inter-related with the inhibition of oxidative stress, which will in turn prevent NF-κB activation thereby inhibiting its downstream pathways in the retina. Indeed, this explanation was supported by similar finding with an antioxidant therapy (Kowluru, 2003; Kowluru & Odenbach, 2004b). CaD antioxidant properties might promote the attenuation of the inflammatory process that underlies retinal degeneration, and so be efficient in reducing capillary permeability and fragility, the primarily known beneficial effect of CaD in diabetic retinopathy.

We have reported previously the beneficial effects of CaD after 10 days of treatment in diabetic rats (1 month duration). In this work, we were particularly interested in evaluating the effects of CaD on the inflammatory process, which is more evident in a more advanced stage of the disease.This was the reason why a 2 months diabetes period was chosen. The period of treatment with CaD also increased from 10 days to 14 days. The results clearly show that CaD, after a longer period of diabetes, is still able to revert the alterations induced by diabetes, namely at the level of pro-inflammatory cytokines expression and oxidative/nitrosative stress. Previous data reported positive results in patients expressing early phases of diabetic retinopathy and with one (Leite et al., 1990) or two years of treatment with CaD (Ribeiro et al., 2006). Conversely, a recent clinical trial, with a follow up of five years involving patients with mild-to-moderate non-proliferative diabetic retinopathy, concluded that CaD was not able to reduce the risk of clinically significant macular edema (Haritoglou et al., 2009). However this study highlights the hypothesis that CaD might be effective in the early stages of diabetic

retinopathy, losing effectiveness in advanced stages. The present results, in an animal model, show that CaD still has protective effects after 6 weeks of diabetes duration (when treatment with CaD was initiated). However, for humans, early CaD treatment continues to be the best option to achieve a real efficacy against the development of diabetic retinopathy.

Chapter 5 Concluding Remarks

In summary, with this work, we complement our recent findings and contribute to give insight into the molecular and cellular mechanisms underlying the protective effect of CaD in the treatment of diabetic retinopathy. Experiments in a diabetic type 1 rat model, with 2 months diabetes duration, where CaD was orally and daily administered during the last 2 weeks of the 2 months period allowed to draw the following main conclusions:

1. CaD per se induces glial reactivity in the retina.

2. CaD is able to prevent the increase in TNF-α and IL-1β expression in the retinal tissue induced by diabetes.

3. CaD decreases oxidative and nitrosative stress induces by diabetes in the retina.

Taking this and previous finding into account, it appears that the protective effects of CaD are linked to its antioxidants effects.

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