Protective effects of the dipeptidyl peptidase IV inhibitor sitagliptin in the kidney in a T2DM animal model – Focus on endoplasmic reticulum stress

Catarina Isabel Rodrigues Marques

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Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica da Professora Doutora Rosa Fernandes (Universidade de Coimbra) e do Professor Doutor Rui de Carvalho (Universidade de Coimbra)

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In the middle of difficulty lies opportunity.

Albert Einstein

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Poster presentation

"Protective effects of the dipeptidyl peptidase IV inhibitor sitagliptin in the kidney in a type 2 diabetes animal model – Focus on endoplasmic reticulum stress."

Catarina Marques, Andreia Gonçalves, Edite Teixeira-Lemos, Frederico Teixeira, Carlos Fontes Ribeiro, Flávio Reis, Rosa Fernandes.

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Abbreviations

ADP	Adenosine diphosphate
ANG-II	Angiotensin-II
AP-1	Activator protein-1
ASK1	Apoptosis signal-regulating kinase
ATF	Activating transcription factor
ATF4	Activating transcription factor 4
ATF6	Activating transcription factor 6
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
bZIP	Basic region-leucine zipper
cAMP	Cyclic adenosine monophosphate
cAMP-GEFII	cAMP-regulated guanine nucleotide ex-change factor II
CHOP	Transcription factor C/EBP homologous protein
CREB	Cyclic-AMP-responsive-element-binding protein
CRP	C-reactive protein
DAPI	4',6-diamidino-2-phenylindole
DM	Diabetes mellitus
DN	Diabetic nephropathy
DNA	Deoxyribonucleic acid
DOC	Sodium deoxycholate
DPP-IV	Dipeptidyl peptidase IV
DPP-IX	Dipeptidyl peptidase IX
DPP-VIII	Dipeptidyl peptidase VIII
DR5	Death receptor 5
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
eIF2 α	Eukaryotic initiation factor 2α
eNOS	Endothelial nitric oxide synthase
ER	Endoplasmic reticulum

ERAD	ER-associated protein degradation
ERO1	ER oxidoreductin 1
ERSE	ER stress response element
ESRD	End-stage renal disease
FAD	Flavin adenine dinucleotide
FFAs	Free fatty acids
GADD153	Growth arrest and DNA damage-inducible protein 153
GADD34	Growth arrest and DNA damage-inducible protein 34
GBM	Glomerular basement membrane
GFR	Glomerular filtration rate
GIP	Glucose-dependent insulinotropic polypeptide
GIPR	Glucose-dependent insulinotropic polypeptide receptor
GLP-1	Glucagon-like peptide-1
GLP-1R	Glucagon-like peptide-1 receptor
GLUT-1	Glucose transporter-1
GRP78/BiP	Glucose-regulated protein 78
GSH	Reduced glutathione
HbA1c	Glycosylated hemoglobin
HRP	Horseradish peroxidase
IAD	Iodoacetamide
ICAM-1	Intercellular adhesion molecule-1
IDF	International Diabetes Federation
IFN-γ	Interferon gamma
IL-1	Interleukine-1
IL-18	Interleukin-18
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IRE1α	Inositol requiring 1α
IRS	Integrated stress response
IRS-1	Insulin receptor substrate-1
IκB	NF-κB inhibitors
JNK	c-Jun N-terminal kinase
MCP-1	Monocyte chemoattractant protein 1

mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
NADPH	Nicotinic acid adenine dinucleotide phosphate
NF-κB	Nuclear factor κB
NO	Nitric oxide
ORFs	Open reading frames
P-Akt	Phosphorylated Akt
PBS	Phosphate-buffered saline
PDI	Protein disulfide isomerase
PERK	PKR-like ER kinase
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PMSF	Phenylmethylsulfonyl fluoride
PP1	Type 1 protein serine/threonine phosphatase
PVDF	Polyvinylidene difluoride
RAS	Renin-angiotensin system
RNA	Ribonucleic acid
ROS	Reactive oxygen species
S1P	Site 1 protease
S2P	Site 2 protease
SDS	Sodium dodecyl sulfate
SNAP	S-nitroso-Nacetyl-D, L-penicillamine
SOD	Superoxide dismutase
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TBS	Tris-buffered saline
TGF- β	Transforming growth factor-β
TGF- β1	Transforming growth factor-β1
TNF	Tumor necrosis factor
TRAF2	TNF receptor-associated factor 2
TRB3	Tribbles homolog 3
UPR	Unfolded protein response
VCAM-1	Vascular cell adhesion molecule-1

VEGF	Vascular endothelial growth factor
WHO	World Health Organization
XBP1	X-box binding protein 1
ZDF	*Zucker Diabetic Fatty*

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Resumo

A nefropatia diabética é uma das maiores complicações diabéticas microvasculares e é a principal causa de doença renal em estado terminal. Cerca de 80% de pessoas não tratadas com diabetes tipo 1 e microalbuminúria desenvolvem nefropatia, e uma percentagem significativa de pacientes com diabetes tipo 2 (20-40%) são susceptíveis ao desenvolvimento da doença. Esta patologia é caracterizada por uma acumulação excessiva de matriz extracelular, com espessamento da membrana basal glomerular e hipertrofia de vários tipos de células glomerulares e tubulares, que acaba por progredir para glomerulosclerose e fibrose túbulo-intersticial. Existem evidências que sugerem um envolvimento do stresse do retículo endoplasmático no desenvolvimento de nefropatia diabética, mas os mecanismos moleculares subjacentes permanecem por clarificar.

Tal como acontece com todas as complicações da diabetes, a hiperglicemia activa uma série de alterações no rim, que levam á disfunção glomerular e tubular e acelera a apoptose celular. Portanto, terapias anti-diabéticas obtendo um bom controlo glicémico diminuem o risco para o desenvolvimento da nefropatia diabética. A sitagliptina faz parte de uma nova classe de anti-hiperglicémicos orais, os inibidores da DPP-IV, os quais melhoram o controlo glicémico através de um aumento da secreção de insulina nos pacientes com diabetes do tipo 2 potenciando um sistema fisiológico, o efeito incretina.

Até ao início deste trabalho a informação existente sobre os potenciais efeitos benéficos da sitagliptina na atenuação/reversão de algumas das lesões no rim induzidas pela diabetes era escassa. Nesse contexto, o objectivo deste estudo foi avaliar a capacidade desse fármaco na atenuação das complicações microvasculares no rim, num modelo animal de diabetes do tipo 2, os ratos ZDF (*fa/fa*). Às 26 semanas de idade, estes animais diabéticos apresentaram alterações nos estados inflamatório e apoptótico. A imunoreactividade das citocinas pró-inflamatórias IL-1β e TNF aumentou em células localizadas ao redor do glomérulo, provavelmente células tubulares e/ou células inflamatórias intersticiais. A hiperglicemia crónica induziu também um aumento significativo na razão BAX/Bcl-2, acompanhado por um aumento dos níveis da proteína pro-apoptótica Bid, mostrando que a diabetes promove um carácter pro-apoptótico no rim. Estes animais diabéticos apresentaram ainda um aumento significativo nos níveis proteicos da P-Akt no rim, associada a hipertrofia de células mesangiais e expansão da matriz extracelular. Dados preliminares mostraram que a diabetes parece aumentar a

capacidade de translocação da CHOP e ATF4 para o núcleo, dois factores de transcrição relacionados com o stresse do retículo endoplasmático; no entanto, não houve alterações significativas nos níveis totais das proteínas CHOP e GRP78/BiP no rim de animais diabético.

No grupo de animais diabéticos, a administração da sitagliptina promoveu uma melhoria no controlo glicémico, após 6 semanas de tratamento. A sitagliptina aumentou significativamente os níveis proteicos de GLP-1 no rim, acompanhado por uma diminuição na imunoreactividade de GLP-1R, provavelmente devido a um feedback negativo. Relativamente às citocinas pro-inflamatórias, a sitagliptina diminuiu acentuadamente a imunoreactividade de IL-1β e TNF no rim de animais diabético, assim como preveniu o aumento da razão BAX/Bcl-2 e dos níveis proteicos de Bid. A sitagliptina também diminuiu os níveis de P-Akt no rim dos animais diabéticos. Curiosamente, a administração de sitagliptina promoveu um aumento significativo nos níveis da proteína CHOP, no entanto a translocação de CHOP e ATF4 para o núcleo parece diminuir.

Em suma, podemos concluir que o tratamento de sitagliptina tem efeitos benéficos no rim, possivelmente por mecanismos que envolvem uma melhoria das lesões renais, com consequente redução da apoptose e inflamação. Adicionalmente, resultados preliminares obtidos neste estudo sugerem que a sitagliptina poderá alterar a resposta do stresse do retículo endoplasmático. No entanto, estudos futuros serão necessários para uma melhor compreensão dos mecanismos moleculares e celulares envolvidos nas acções directas mediadas pela sitagliptina no rim, os quais poderão contribuir para o desenvolvimento de novas estratégias terapêuticas mais eficazes e direccionadas para a prevenção ou redução do efeito nocivo da hiperglicemia crónica nas células do rim.

Palavras-chave: Nefropatia diabética, Sitagliptina, Dipeptidil peptidase-IV, Incretinas, Stresse do retículo endoplasmático, Inflamação, Apoptose.

Abstract

Diabetic nephropathy is one of the major microvascular diabetic complications and is the leading cause of end-stage renal disease. About 80% of untreated people with type 1 diabetes and microalbuminuria progress to overt nephropathy, and a significant percentage of patients with type 2 diabetes (20-40%) are susceptible to the development of disease. This pathology is characterized by excessive accumulation of extracellular matrix, with thickening of glomerular basement membrane and hypertrophy of various cell types of the glomerulus and tubules, which ultimately progress to glomerulosclerosis and tubulointerstitial fibrosis. There are evidences that suggest an involvement of ER stress in the development of diabetic nephropathy, but the underlying molecular mechanisms remain unclear.

As in all complications of diabetes, hyperglycaemia promotes a series of changes in the kidney, that lead to glomerular and tubular dysfunction and accelerating cell apoptosis. Therefore, anti-diabetic therapies achieving a good glycaemic control reduce the risk for the development of diabetic nephropathy. Sitagliptin is part of a new class of oral anti-hyperglycaemic agents, the DPP-IV inhibitors, which improves glycaemic control by increasing insulin secretion in patients with type 2 diabetes enhancing a physiological system, the incretin effect.

At the beginning of this work the existing information on the potential beneficial effects of sitagliptin in the attenuation/reversal of some of the kidney lesions induced by diabetes was scarce. In this context, the aim of this study was to assess the ability of this compound in the attenuation of microvascular complications in the kidney, in an animal model of type 2 diabetes, ZDF (*fa/fa*) rats. At 26 weeks of age, these animals showed changes in inflammatory and apoptotic states. The immunoreactivity of the proinflammatory cytokines IL-1β and TNF was increased in cells around the glomeruli, probably tubular cells and/or interstitial inflammatory cells. Chronic hyperglycaemia also induced a significant increase in BAX/Bcl-2 ratio, accompanied by an increase in the levels of pro-apoptotic protein Bid, showing that diabetes increases the pro-apoptotic state in the kidney. These diabetic animals also showed a significant increase in P-Akt protein levels in the kidney, associated with mesangial cells hypertrophy and extracellular matrix expansion. Preliminary data show that diabetes seems to increase the capacity in translocation of CHOP and ATF4, transcription factors associated with the ER stress, to

the nucleus; however, there were no significant changes in the total levels of CHOP and GRP78/BiP proteins in the diabetic kidney.

In the diabetic animal group, administration of sitagliptin promoted an improvement in glycaemic control after 6 weeks of treatment. Sitagliptin significantly increased the GLP-1 protein levels in the kidney, accompanied by a decrease in the immunoreactivity for GLP-1R, probably due to a feedback mechanism. Concerning the proinflammatory cytokines, sitagliptin markedly decreased the immunoreactivity of IL-1β and TNF in the diabetic kidney, as well as prevented the increase of BAX/Bcl-2 ratio and Bid protein levels. Sitagliptin also decreased the P-Akt protein levels in the kidney of diabetic animals. Interestingly, the administration of sitagliptin promoted a significant increase in CHOP protein levels, however, CHOP and ATF4 translocation to the nucleus seems to decrease.

Overall, we can conclude that sitagliptin treatment has beneficial effects on the kidney, possibly by mechanism involving an improvement in renal lesions with consequent reduction in apoptosis and inflammation. Additionally, the preliminary results obtained in this study suggest that sitagliptin could modulate the ER stress response. However, further studies are needed to better understand the molecular and cellular mechanisms involved in the direct actions mediated sitagliptin in the kidney, which may contribute to the development of new therapeutic strategies more effective and focused on preventing or reducing the injurious effects of chronic hyperglycaemia on cells of the kidney.

Key words: Diabetic nephropathy, Sitagliptin, Dipeptidyl peptidase-IV, Incretins, ER stress, Inflammation, Apoptosis.

CHAPTER 1

INTRODUCTION AND AIMS

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1. Diabetes Mellitus

1.1. General definition and epidemiology

Diabetes mellitus (DM) has been defined as a complex and heterogeneous metabolic disorder, which is mainly characterized by chronic hyperglycaemia, with abnormal metabolism of carbohydrates, lipids and proteins, resulting from defects in insulin secretion and/or insulin action (Alberti *et al.*, 1998). The effects of DM include long-term dysfunction and failure of various organs and tissues, with progressive development of specific complications.

Several pathogenic processes are involved in the development of diabetes. These include processes which destroy the β-cells of the pancreas with consequent insulin deficiency, and others that result in resistance to insulin action (Rossetti *et al.*, 1990; Zimmet *et al*., 2001).

DM prevalence rates have risen markedly in recent years. According to the International Diabetes Federation (IDF), DM is one of the most common non-communicable diseases, with high rates of morbidity and mortality, recording about 4.8 million deaths in 2012. At present, diabetes affects about 371 million people (Figure 1) and it is estimated to increase to 552 million by 2030, thus representing 8.3% of the adult population. However, these numbers are underestimated due to the high rate of undiagnosed diabetes in patients, which represents half of people with the disease (IDF, 2012).

Recent estimates for 2011 from the Portuguese Society of Diabetology have indicated that 12.7% of portuguese adult population is diabetic, from which 5.4% were undiagnosed (OND, 2012).

The prevalence of diabetes is reaching pandemic proportions on a global scale, becoming a threat to public health throughout the world, highlighting the importance of implementing effective preventive and therapeutic strategies (Zimmet *et al.*, 2001; Hossain *et al.*, 2007).
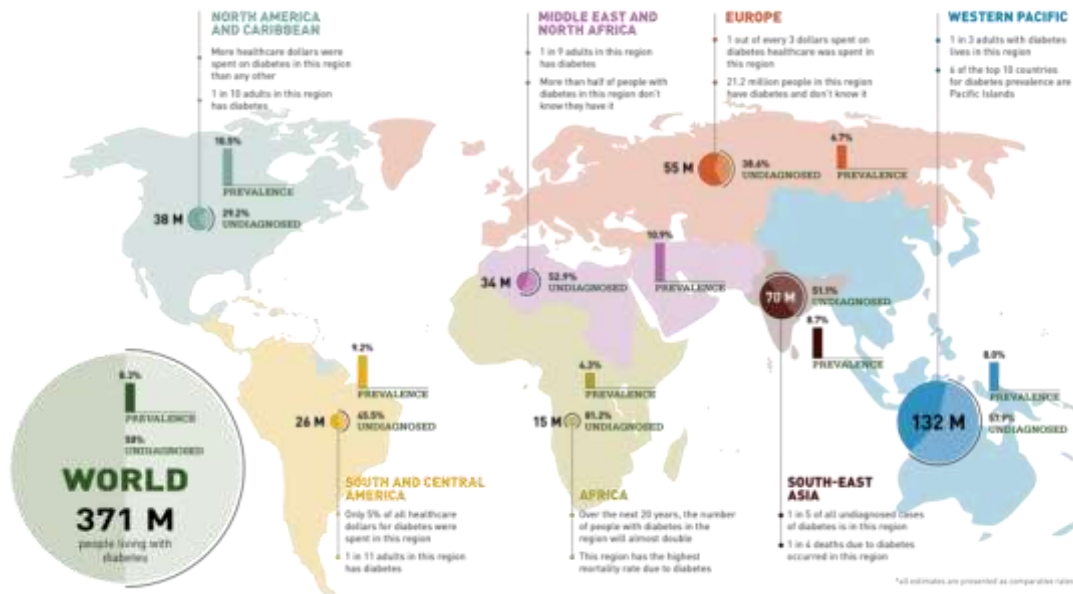
Figure 1: Global prevalence (%) of diabetes mellitus in adult population (20-79 years) in 2012, shown by geographic region (Image taken from IDF Diabetes Atlas, 5th Edition (IDF, 2012)).

DM is a multifactorial disease in which multiple genetic and environmental factors contribute to its onset and progression. The concomitant increase in life expectancy, obesity, sedentary lifestyle, hypertension and hyperlipidemia might raise the chance of citizens from both developed and developing countries of having glucose intolerance. This suggests that diabetes is certainly one of the most challenging health problems in the 21st century (Zimmet *et al.*, 2001; Tuomi, 2005).

1.2. Characterization of diabetes mellitus

According to the WHO and IDF there are three main types of diabetes, as follows: type 1 diabetes mellitus (T1DM), type 2 diabetes mellitus (T2DM) and gestational diabetes. However, most prevalent forms of diabetes are T2DM, known as insulin-resistant, representing about 90% of the cases, and T1DM, also called insulin-dependent, responsible for 5-10% of the cases (Zimmet *et al.*, 2001).

DM is associated with a decreased of pancreatic β-cell, due to cell death by apoptosis and defective β-cell regeneration, which are mediated by activation of metabolic pathways, in response to chronic hyperglycaemia condition (Cnop *et al.*, 2005).

1.2.1. Type 1 diabetes mellitus (T1DM)

T1DM usually occurs in young subjects, and is an autoimmune disease characterized by progressive destruction of pancreatic β-cells and multiple genetic predispositions and environmental factors seems to be associated with the suppression of insulin synthesis. T1DM patients are, therefore, dependent on exogenous insulin to maintain adequate insulin levels, which leads to better control of blood glucose levels (Cnop *et al.;* 2005; Ichinose *et al.*, 2007).

1.2.2. Type 2 diabetes mellitus (T2DM)

T2DM is one of the most prevalent and serious metabolic diseases in the world. It occurs predominantly in adults (people older than 40 years); however, there has been a rise in diagnosed cases of T2DM among people with younger ages, suggesting an association with the increase of childhood obesity (Hossain *et al.*, 2007). Insulin resistance and pancreatic β-cell dysfunction are the hallmarks of this disease.

T2DM has higher prevalence than T1DM, due to sedentary lifestyle, lack of physical activity, increasing obesity and unhealthy diet (Houssain *et al.*, 2007; Villary *et al.*, 2007).

In the early stage of T2DM, there is a state of insulin resistance, leading to qualitative and quantitative changes in its secretion. Insulin resistance is defined as the inability of insulin (endogenous and exogenous) to promote glucose cell uptake, resulting in elevated blood glucose (hyperglycaemia) (Villary *et al.*, 2007). As a consequence of hyperglycaemia, there is a compensatory increase of insulin secretion by pancreatic β-cells (hyperinsulinaemia), in order to maintain normoglycaemia, a process termed β-cell compensation. This process declines β-cell secretory capacity, leading to eventual failure and progressive loss of these cells (Butler *et al.*, 2003; Virally *et al.*, 2007).

1.3. Diabetic complications

Diabetics can live for years without showing any symptoms, during which high glucose levels will silently damage several organs. The frequency, severity and progression of diabetic complications are related with several factors, such as levels of

hyperglycaemia, metabolic changes associated with the duration of disease, exposure to risk factors and also with genetic environment.

Patients with a diagnosis of diabetes can have many macrovascular (atherosclerosis, stroke, coronary heart disease, acute myocardial infarction, peripheral vascular disease) and microvascular (retinopathy, nephropathy or peripheral neuropathy) complications (Figure 2) (Fowler, 2008; Calcutt *et al.*, 2009).

These complications have been directly associated with glucose toxicity and activation of several intracellular metabolic pathways, including increased protein glycosylation, abnormal activation of signaling cascades, such as protein kinase C (PKC), increased activation of hexosamine and polyol pathways, and elevated production of reactive oxygen species (ROS) (Giacco and Brownlee, 2010). Moreover, accumulating evidence suggests that inflammation and endoplasmic reticulum (ER) stress also plays an important role in the pathogenesis of diabetes (Saraheimo *et al.*, 2003; Eizirik *et al.*, 2008).

DM and its complications have become a major public health problem, highlighting important research advances and the development of novel methods to both prevent and treat this disease.
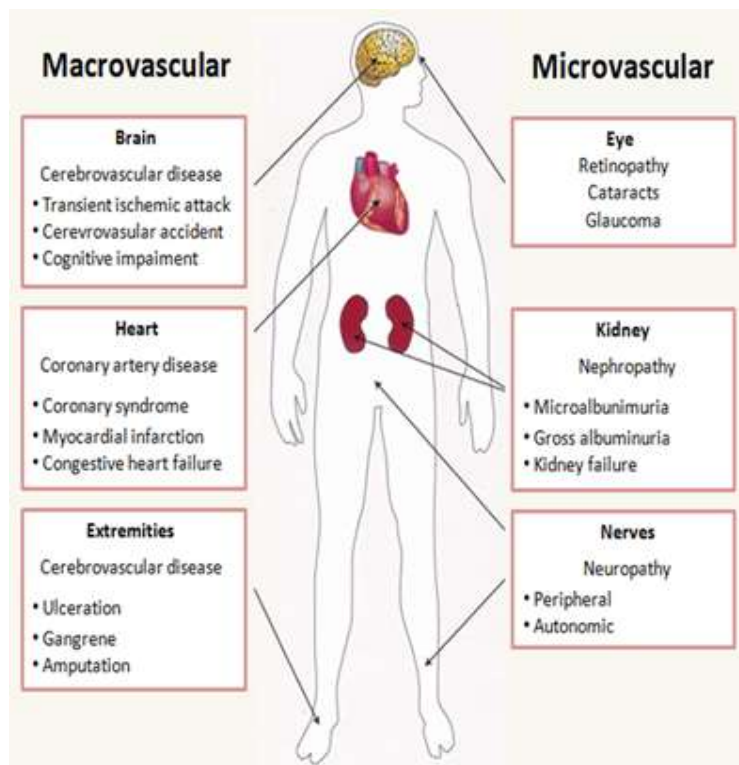
Figure 2. Schematic diagram of macro and micro complications of diabetes (Adapted from Cefalu and Canon, 2007).

2. Diabetes and the kidney

2.1. Kidney anatomy and function

The kidney is one of the most complex and dynamic organs of human body and plays an essential role in maintaining body homeostasis through excretion of waste products and excess water in the form of urine, regulating the chemical composition of the blood by controlling the fluid, electrolyte and acid-base balance of the circulation system, and producing hormones that help to maintain blood pressure, keep healthy bones and prevent anemia (Brenner, 2000).

The kidney has a high content of epithelial cells with extensive intracellular membrane trafficking machinery. The basic functional unit of the kidney is the nephron (Figure 3), and each kidney contains approximately 0.5-1 million nephrons, which are responsible for blood filtration and urine production (Roestenberg, 2006; Dankers *et al.*, 2011). The nephron consists of a glomerulus, surrounded by parietal epithelial cells of Bowman's capsule, a proximal tubule, a loop of Henle and a distal tubule that is connected to a collecting duct. Nephrons remove excess water and soluble matter from the body by first filtering the blood, and then reabsorbing some necessary fluid and molecules back into the blood while secreting other unneeded molecules (Dankers *et al.*, 2011).
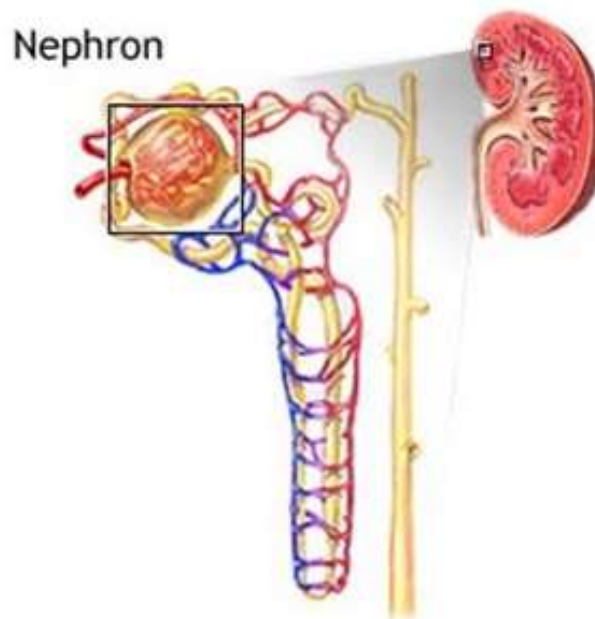
Figure 3. The basic functional unit of the kidney - Nephron
(Adapted from http://health.rush.edu/HealthInformation/BodyGuide/reftext/html/urin_sys_fin.html).

Glomerulus consists of a capillary tuft enclosed in Bowman's capsule, and the capillaries are enclosed by specialized visceral epithelial cells, named podocytes (Figure 4) (Roestenberg, 2006). The endothelial cells of the glomerulus and the podocytes are separated by a glomerular basement membrane (GBM), which is composed of extracellular matrix (ECM) proteins, such as collagen IV, and negatively charged glycoproteins, such as heparin sulfate proteoglycans (Dankers *et al.*, 2011). Podocytes form a crucial component of the glomerular filtration barrier. The glomerular filtration barrier is characterized by extensive interdigitating foot processes, that are separated by narrow spaces with a constant width of 40 nm, called slit diaphragm, which are pores composed by specific proteins and are freely permeable to water and small solutes but selectively impermeable to plasma proteins (Tryggvason, 1999; Wartiovaara *et al.*, 2004). Furthermore, it has been reported that nephrin, a protein of slit diaphragm of glomerular podocytes, is crucial for maintaining the integrity of the slit diaphragm structure and an intact filtration barrier (Ruotsalainen *et al.*, 1999). Functionally, podocytes are responsible for the GBM turnover, maintenance of the filtration barrier, support of the capillary tuft and regulation of the glomerular filtration (Leewis *et al.,* 2010).
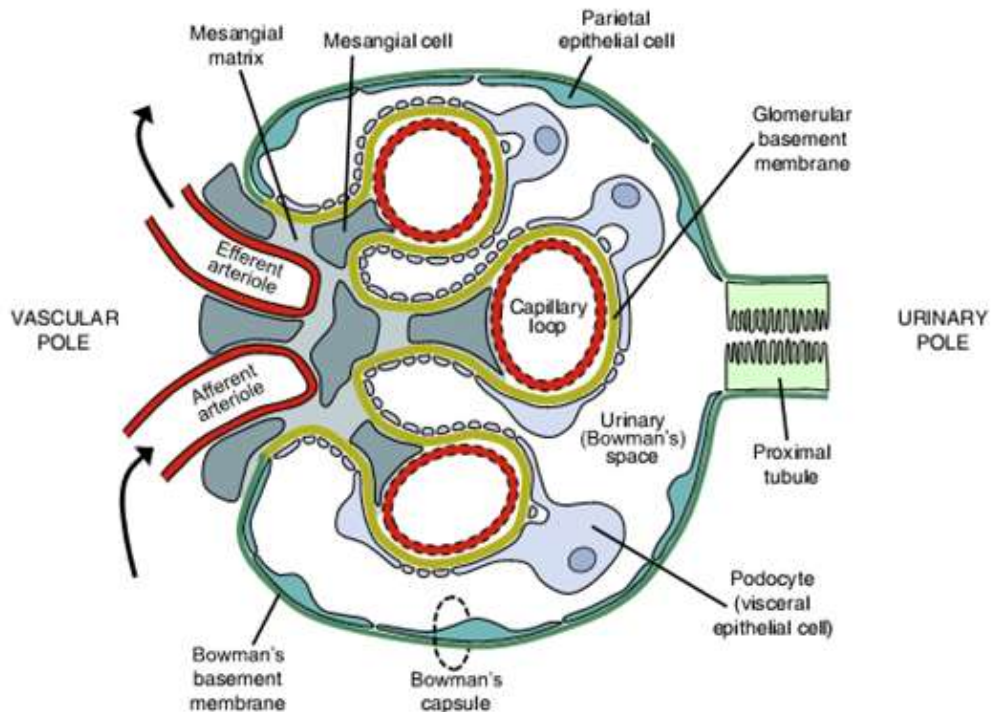
Figure 4. Schematic representation of a glomerulus (Image taken from Leeuwis *et al.*, 2010)

Another cell type found in glomerulus is the mesangial cell. These cells are in direct contact with the capillaries and are essential for maintaining the structural and functional dynamic stability of glomerular tufts, providing structural support for capillary loops and modulating glomerular filtration (El-Nahas, 2003). Mesangial cells produce ECM and are separated from podocytes by the GBM. These cells regulate wall tension and intracapillary flow, as well as produce many growth factors and other proteins that are important for the normal physiological function of the podocytes and glomerular endothelial cells (Schlöndorff and Barnas, 2009).

2.2. Diabetic nephropathy

2.2.1. Prevalence

Diabetic nephropathy (DN) is one of the major microvascular complications of both types 1 and 2 diabetes, which can result in end-stage renal disease (ESRD), requiring dialysis or transplantation (Dronavalli *et al.*, 2008; Kanwar *et al.*, 2008). This stage of advanced chronic renal failure contributes to the decrease of quality of life and significantly shortened life expectancy of the patients, conferring high morbidity and mortality rates of diabetic patients, as well as contributes to rising medical costs. In addition, chronic kidney disease also contributes to the development of cardiovascular disease which leads to an increase in mortality rates (Go *et al.*, 2004; Zeeuw *et al.*, 2006). About 20-40% of the diabetic patients develop microalbuminuria, a sign of nephropathy (Dronavalli *et al.,* 2008), mostly within 15-20 years after diagnosis (Parving *et al.*, 1988; Ismail *et al.*, 1999).

The incidence rate of ESRD has been increasing in worldwide (Xue *et al.*, 2001; Wakai *et al.*, 2004), and the clinical history of DN differs according to the type of diabetes. About 80% of untreated people with T1DM and microalbuminuria progress to overt nephropathy, whereas only 20-40% of those with T2DM are susceptible to the development of disease (Dronavalli *et al.,* 2008). Although the highly probability of T1DM patients to develop DN, the T2DM patients constitute the majority of diabetic people with this pathology, because of the much greater prevalence of this type of diabetes (Ritz *et al.,* 1999).

2.2.2. Pathophysiology of the diabetic nephropathy

DN is characterized by a progressive renal disease with functional and structural abnormalities, and hyperglycaemia is the driving force for the development of this pathology, inducing metabolic and hemodynamic factors that are mediators of this injury. Moreover, several genes have been associated with susceptibility for development of DN (Tanaka and Babazono, 2005).

DN has several distinct phases of development and multiple mechanisms can contribute to the development of the disease and its outcomes, through damage of various cells, including glomerular podocytes, mesangial and endothelial cells, and tubular epithelia. The early stage of DN is characterized by glomerular hyperfiltration (increased urine production) represented by high values of glomerular filtration rate (GFR) (Chawla *et al.*, 2010), leading to the development of microalbuminuria (30-300 mg/day or 20 µg/min) (Dronavalli *et al.*, 2008), and is also associated with development of other complications, such as cardiovascular disease (Go *et al.*, 2004; Zeeuw *et al.*, 2006). The morphological changes of the kidney for these functional abnormalities include glomerular and tubular epithelial hypertrophy, thickening of GBM and expansion of mesangial ECM (Figure 5) (Adler, 1994; Chawla *et al.*, 2010). Decreased levels of nephrin are also connected to the early changes of DN, and may contribute to the loss of glomerular filtration function (Aaltonen *et al.*, 2001). Advanced DN is characterized by glomerulosclerosis, demise of glomerular capillaries, tubulointerstitial degeneration and fibrosis, associated with precipitous decline of GFR, developing macroalbuminuria (>300 mg/day or >200 µg/min) (Dronavalli *et al.*, 2008), leading to chronic renal insufficiency that declines to ESRD.

Several factors have been shown to influence the renal cells loss and progression of DN, such as hyperglycaemia-induced metabolic and hemodynamic factors (Vinod, 2012), as well as hyperlipidemia (Kasiske *et al.*, 1988) and ER stress (Cunard and Sharma, 2011). In diabetic patients, instead of dumping glucose in the urine to correct hyperglycaemia, the kidney chooses to hold on to the glucose. The ability of the kidney to reabsorb glucose is augmented by an absolute increase in the renal glucose influx capacity, possibly by GLUT-1, a surface receptor of resident renal cells, which is overexpressed in mesangial cells under high glucose concentrations (Heilig *et al.*, 1997), associated with overexpression of transforming growth factor-β1 (TGF-β1) (Inoki *et al.*, 1999).

The hemodynamic factors implicated in the pathogenesis of DN include increased systemic and intraglomerular pressure, as well as hyperfiltration and hyperperfusion injuries, through activation of various vasoactive hormone pathways, including the renin-angiotensin system (RAS), specifically angiotensin-II (ANG-II), and endothelins (Ichinose *et al.*, 2007; Dronavalli *et al.*, 2008). In fact, ANG-II stimulates resident renal cells to produce TGF-β1 that induces mesangial expansion and GBM thickening, through augmentation of ECM protein deposition, such as collagen types I, IV, V and VI, and fibronectin (Schena and Gesualdo, 2005), and is also been implicated in production of monocyte chemoattractant protein 1 (MCP-1) and ROS (Chawla *et al.*, 2010). Moreover, Akt signaling has an emerging role in pathogenesis of DN, namely in mesangial hypertrophy and ECM composition (Zdychova and Komers, 2004; Alique *et al.*, 2011; Kattla *et al.*, 2008). Several studies have described increased Akt activity in diabetic kidney (Feliers *et al.*, 2001). Additionally, diverse factors including vascular endothelial growth factor (VEGF), prostanoids and diverse cytokines have also been implicated in hyperfiltration injury and increased vascular permeability (Wolf, 2004).
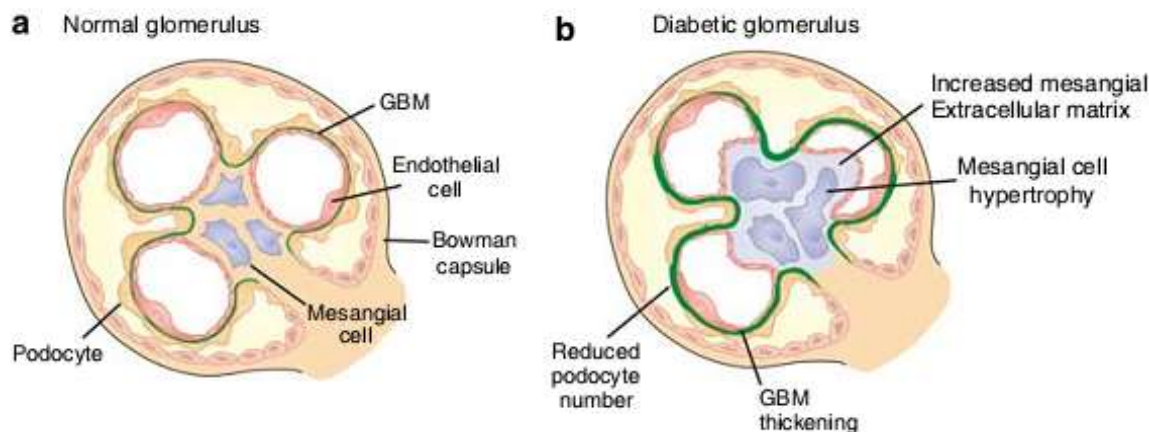
Figure 5. Characteristic glomerular changes in DN. (a) Normal glomerulus. (b) Diabetic kidney: thickening of GBM and mesangial expansion, due to increased mesangial matrix and increased mesangial cell size due to hypertrophy, as well as a reduction in podocytes number. (Image taken from Jefferson *et al.*, 2008)

Moreover, it is well known that hyperglycaemia increases oxidative stress, inflammation and activate several metabolic pathways, such as non-enzymatic glycosylation, activation of PKC, polyol pathway and hexosamine biosynthetic pathway, which contribute to the development and pathogenesis of DN.

2.2.3. Oxidative stress

Another contributing mechanism for renal injury is the oxidative stress that results from enhanced reactive oxygen species (ROS) generation and decreased antioxidant defense system. Chronic hyperglycaemia has been indicated as one of the major causes that lead to oxidative stress, due to activation of several pathways, uncoupled endothelial nitric oxide synthase (eNOS) activity, oxidative metabolism of glucose in the mitochondria and glucose auto-oxidation (Evans *et al.*, 2002; Susztak *et al.*, 2006).

ROS are chemical compounds that result from the activation or reduction of molecular oxygen or derivatives of the products of this reduction, such as the superoxide anion ($O_2^{\bullet-}$) and hydroxyl radical ($OH^{\bullet}$) (Irani, 2000; Evans *et al.*, 2002). Excessive levels of ROS lead to the damage of proteins, lipids and deoxyribonucleic acid (DNA), and also play a significant role in the activation of stress-sensitive signaling pathways that regulate gene expression resulting in cellular damage (Melov *et al.*, 1999; Dröge, 2002; Evans *et al.*, 2002). Therefore, in order to maintain proper cellular function and homeostasis, has to be fulfilled a balance between ROS production and consumption. However, hyperglycaemia has been shown to induce a decrease in antioxidant capacity, through a decline in important cellular antioxidant defense mechanisms, which significantly increases the susceptibility to oxidative stress (Wolff *et al.*, 1991). These antioxidant defense mechanisms involve an enzymatic strategy, including the enzymes superoxide dismutase (SOD), catalase and glutathione peroxidase, and non-enzymatic components, such as vitamins A, C and E, and also the α-lipoic acid (Maritim *et al.*, 2002). In addition, reduced glutathione (GSH) is an important scavenger of ROS, and, due to the fact that nicotinic acid adenine dinucleotide phosphate (NADPH) is consumed by glucose reduction to sorbitol, and since NADPH is required for regenerating GSH, this could induce intracellular oxidative stress (Giacco and Brownlee, 2010). Recent evidences suggest that NADPH oxidase is the primary source of vascular and renal ROS production (Shi *et al.*, 2008). Furthermore, the increased oxidative stress in diabetes has been associated with mitochondrial superoxide anion overproduction, which is the central and major mediator for the activation of several metabolic pathways, as before referred (Du *et al.*, 2000; Nishikawa *et al.*, 2000). Increased superoxide production is also correlated with endothelial dysfunction, through inactivation of the eNOS (Guzik *et al.*, 2002). Oxidative stress is not only associated with complication of diabetes, but also with

insulin resistance and pancreatic β-cell dysfunction (Evans *et al.*, 2002), and has been linked to lipid-induced ER stress (Borradaile *et al.*, 2006).

Oxidative stress is strongly correlated with the development of DN. Enhanced ROS production have been linked to vasoconstriction, endothelial dysfunction, modification of ECM proteins and increased renal sodium reabsorption (Elmarakby and Sullivan, 2010). The importance of oxidative stress in DN is underscored by the finding that inhibition of oxidative stress ameliorates the manifestations associated with diabetes-induced kidney injury. In fact, inhibition of hyperglycaemia-induced superoxide overproduction using a transgenic approach (overexpression of SOD) prevents long-term DN (Craven *et al.*, 2001; DeRubertis *et al.*, 2004). Additionally, it has been demonstrated that glucose-induced ROS production initiates podocyte apoptosis and podocyte depletion *in vitro* and *in vivo*, leading to DN (Susztak *et al.*, 2006).

There is also been described a link between oxidative stress and inflammation in diabetic kidney. Oxidative stress can increase cytokines production via the activation of nuclear factor κB (NF-κB) (by degradation of NF-κB inhibitors (IκB)) and activator protein 1 (AP1), leading to the transcription of genes encoding cytokines and growth factors, such as interleukins (IL-1, -6 and -18) and transforming growth factor-β (TGF-β) (Elmarakby and Sullivan, 2010).

2.2.4. Inflammation

DM is not only a metabolic disorder, several studies have shown that there is a correlation between inflammatory and metabolic processes involved in diabetes, developing and exacerbating the level of microvascular complications, such as DN. Therefore, an association between DN and the presence of a chronic low-grade inflammation was established (Saraheimo *et al.*, 2003; Dalla Vestra *et al.*, 2005). Moreover, a state of inflammation is also commonly associated with obesity and insulin resistance in T2DM (Shoelson *et al.*, 2006). Elevated inflammatory mediators cause insulin resistance since they reduce the insulin receptor substrate 1 (IRS-1) tyrosine phosphorylation and the activation of phosphoinositide 3-kinase (PI3K) and Akt3k, decreasing insulin signaling (Jager *et al.*, 2007).

Accumulating evidence indicates that immunologic and inflammatory mechanisms play a significant role in the development and progression of DN. This inflammatory response is mediated by diverse inflammatory cells, including

macrophages, monocytes and leukocytes (Chow *et al.*, 2004; Galkina and Ley, 2006), as well as other molecules, such as chemokines (MCP-1) (Chow *et al.*, 2006), adhesion molecules intercellular adhesion molecule 1 (ICAM-1) (Chow *et al.*, 2005) and inflammatory cytokines, mainly tumor necrosis factor (TNF), C-reactive protein (CRP), TGF-β, interleukin-6 (IL-6), interleukin-1β (IL-1β) and interleukin-18 (IL-18) (Navarro and Mora, 2006). There is also an association between oxidative stress and inflammation, as oxidative stress can stimulate cytokines production (Elmarakby and Sullivan, 2010), and, on the other hand, it is known that inflammatory mediators can trigger ROS production.

The NF-κB is a transcription that regulates the gene expression of several molecules involved in inflammation, such as ICAM-1, MCP-1, TGF-β, IL-1β and TNF (Guijarro and Egido, 2001). In fact, it has been reported that the inhibition of NF-κB activity significantly decreases the expression of proinflammatory cytokines in diabetic kidney, such as ICAM-1 and MCP-1, as well as TGF-β1 (Soetikno *et al.*, 2011). NF-κB is suggested to play an important role in mesangial cell activation leading to renal injury and NF-κB expression is increased in diabetic kidney (Massy *et al.*, 1999). Furthermore, MCP-1 is responsible for macrophage recruitment, leading to development of renal fibrosis and, indirectly, ECM formation (Tesch, 2008).

Although the proinflammatory cytokine TNF is mainly synthesized by monocytes and macrophages, various renal cells, including tubular, glomerular, mesangial and endothelial cells, are also able to produce this cytokine (Jevnikar *et al.*, 1991; Nakamura *et al.*, 1993; Hasegawa *et al.*, 1995). TNF induces the expression of other cytokines, chemokines and adhesion molecules (Ortiz *et al.*, 1995), and it was also described that this proinflammatory mediator induces oxidative stress in diverse cells, including mesangial cells, by promoting the local generation of ROS (Radeke *et al.*, 1990). Moreover, this cytokine stimulates sodium uptake by proximal tubule cells, contributing to sodium retention and renal hypertrophy (Melnikov *et al.*, 2002). Overall, TNF is cytotoxic to glomerular, mesangial and epithelial cells, and induce direct renal damage, contributing to the development of DN.

It has been described that interleukine-1 (IL-1) stimulates the proliferation of mesangial cells and matrix synthesis and enhances vascular endothelial permeability (Melcion *et al.*, 1982; Royall *et al.*, 1989), as well as induces the expression of ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1) by glomerular endothelial cells (Navarro-González and Mora-Fernández, 2008). IL-6 also stimulates proliferation of

mesangial cells and enhances endothelial permeability, as well as increases fibronectin expression and affects ECM dynamics at both mesangial and podocyte levels (Vestra *et al.*, 2005; Navarro and Mora, 2006).

Other potent inflammatory cytokine implicated in inflammatory process in diabetic kidney cells is IL-18. This cytokine induces the production of interferon gamma (IFN-γ) (Okamura *et al.*, 1995), that in turn modulates functional chemokine receptor expression in human mesangial cells (Schwarz *et al.*, 2002), and also stimulates other cytokines production, such as IL-1 and TNF, regulates ICAM-1 and induces apoptosis of endothelial cells (Navarro-González and Mora-Fernández, 2008).

Overall, the increased expression of cytokines, chemokines and adhesion molecules, and inflammatory cells recruitment, may be a critical factor for the development of microvascular complications associated with diabetes.

3. Endoplasmic reticulum and diabetes

3.1. Endoplasmic reticulum stress

The endoplasmic reticulum (ER) is a highly dynamic central cellular organelle equipped with chaperones and folding enzymes, being responsible for the synthesis, folding and post-translational modifications of secretory and trans-membrane proteins, and lipid biosynthesis (Eizirik *et al.*, 2008). This organelle also functions as a dynamic calcium store and signaling, controlling a wide range of cellular processes such as organogenesis, transcriptional activity, stress responses and apoptosis (Zhang and Kaufman, 2008). Therefore, the efficient functioning of the ER is essential for most cellular activities and survival.

The unique oxidizing environment of the ER and the numerous protein chaperones present in the organelle are crucial for the proper folding of proteins and protein complexes. This organelle is exquisitely sensitive to alterations in homeostasis and environmental insults (Eizirik *et al.*, 2008). Thus, pathophysiological stress conditions, such as hypoxia, hyperglycaemia, elevated protein synthesis, ER-calcium depletion, nutrient excess or deprivation, altered protein glycosylation and glucose starvation, interfere with the correct folding of proteins (Asada *et al.*, 2011; Eizirik *et al.*, 2008; Cunard and Sharma, 2011; Chiang *et al.*, 2012). Accumulation of misfolded and unfolded proteins in the ER lumen induces their aggregation, causing an imbalance in protein-folding capacity and protein-folding load, and subsequent cellular toxicity (Eizirik *et al.*, 2008; Cunard and Sharma, 2011), a condition referred as ER stress.

The ER responds to these perturbations by activating an intracellular signaling pathway, called the unfolded protein response (UPR), to ensure the fidelity of protein folding and to prevent such an accumulation of unfolfed or misfolded proteins (Ezirik *et al*., 2008; Cunard and Sharma; 2011). Activation of the UPR leads to a transient translational attenuation, transcriptional induction of genes encoding ER-resident chaperones and ER-associated protein degradation (ERAD) (Schröder and Kaufman, 2005; Ron and Walter, 2007)

3.1.1. Unfolded protein response

The concept of an UPR pathway, which first appeared in the literature in 1988, had its origin in experiments performed by Kozutsumi *et al.* (1988), in which mammalian cells exposed to severe ER conditions, such as altered pH, low levels of glucose or oxygen, or alterations in the oxidizing state of the ER responded by up-regulating the ER chaperones, including glucose-regulated protein 78 (GRP78/BiP) (Kozutsumi *et al.*, 1988).

In resting, unstressed cells, GRP78/BiP binds to the ER luminal domains of the ER stress sensors inositosol requiring 1α (IRE1α), PKR-like ER kinase (PERK) and activating transcription factor 6 (ATF6) and maintains them in an inactivated state (Schröder and Kaufman, 2005). During ER stress, GRP78/BiP preferentially binds to unfolded and misfolded proteins and dissociates from the transmembrane sensors, facilitating their activation (Ni and Lee, 2007). After GRP78/BiP dissociation, it is not clear whether full activation of the ER stress response requires subsequent binding of unfolded proteins to the luminal domains of IRE1α, PERK and ATF6 (Ron and Walter, 2007).

The primary purpose of the UPR is to facilitate adaptation to the changing environment and reestablish normal ER function. The UPR is activated when misfolded and unfolded proteins accumulate in the ER lumen, and consist mainly of three simple adaptive mechanisms: 1) upregulation of the folding capacity of the ER through induction of ER-resident molecular chaperones, including GRP78/BiP (Ni and Lee, 2007; Ron and Walter, 2007), 2) down-regulation of the biosynthetic load of the ER through shut-off of protein synthesis on a transcriptional and translational level, and 3) increased clearance of unfolded proteins from the ER through upregulation of ERAD, thus preventing additional protein misfolding and overloading of the organelle (Ron and Walter, 2007; Eizirik *et al.*, 2008). ERAD mediates the retro-translocation of unfolded proteins from the ER lumen into the cytosol for degradation by the proteasome (Ron and Walter, 2007). However, if these three mechanisms do not restore ER homeostasis and ER stress persists, the UPR can activate a cell death pathway, by switching from pro-survival to pro-apoptotic signaling (Rao *et al.*, 2002; Szegezdi *et al.*, 2006), presumably to protect the organism from rogue cells that display misfolded proteins (Schröder and Kaufman, 2005; Ron and Walter, 2007).

GRP78/BiP has a conserved N-terminal ATPase domain and a C-terminal peptide binding domain (Ni and Lee, 2007). The region of GRP78/BiP that associates with ER stress sensors is its peptide-binding region that is the same region that binds to exposed hydrophobic residues in unfolded proteins, thus the accumulation of unfolded proteins would drive the equilibrium of GRP78/BiP binding away from these sensor proteins. Perturbations in the ER environment could lead to modification of GRP78/BiP by regulating their ATPase and nucleotide exchange activities, causing its release from ER stress sensors (Rutkowski and Kaufman, 2004). Furthermore, affinity panning and binding assays with random peptide libraries demonstrated that short hydrophobic peptides are preferentially bound by GRP78/BiP (Blond-Elguindin *et al.*, 1993).

In the ADP-bound form GRP78/BiP has high affinity for protein substrates. Substrates bound to GRP78/BiP are locked in their conformation and stimulate the exchange of ADP with ATP, releasing the substrate from GRP78/BiP, which then progresses on its folding pathway (Flynn *et al.*, 1989). Subsequent ATP-hydrolysis returns GRP78/BiP into the ADP high affinity state. Moreover, GRP78/BiP forms multiprotein complexes which lead to an ER chaperoning network processing the unfolded protein substrates (Meunier *et al.*, 2002), that regulates the conformation of its substrate binding domain by nucleotide exchange. Both reactions, nucleotide exchanged and ATP-hydrolysis, are regulated by co-chaperones, such as the DnaJ-like proteins that stimulate the ATPase activity of GRP78/BiP, and the GrpE-like protein BiP-associated protein and Sls1p that stimulate the nucleotide exchange reaction (Schroder and Kaufman, 2005).

3.1.2. PERK pathway

PERK is a Ser/Thr kinase, catalytic domain of which shares substantial homology to other kinases of the eukaryotic initiation factor 2α (eIF2α) family (Shi *et al.*, 1998; Cunard and Sharma, 2011), and, as IRE1, is a type I transmembrane kinase, sharing related luminal domains that are proposed to monitor ER stress via common regulatory mechanisms (Schröder and Kaufman, 2005).

PERK has an ER luminal stress-sensing domain that binds GRP78/BiP and a cytosolic kinase domain. When ER stress is sensed, GRP78/BiP dissociates from PERK that multimerizes in ER membranes, inducing its autophosphorylation and activating the kinase domain (Bertolotti *et al.*, 2000), generating active PERK. Once activated, PERK

phosphorylates (at Ser51) and inactivates eIF2α (Heather *et al.*, 1999; Heather *et al.*, 2000), thereby inhibiting 80S ribosome assembly (Zhang and Kaufman, 2008). This results in the inhibition of general protein translation, by shutting off mRNA translation and then reducing the protein load on the ER (Figure 6) (Harding *et al.*, 1999; Harding *et al.*, 2000). Phosphorylated eIF2α can also activate NF-κB, but still there are controversy regarding the precise mechanism involved in this process (Jiang *et al.*, 2003). However, certain mRNAs gain a selective advantage for translation under eIF2α phosphorylation, activating a signaling pathway called integrated stress response (ISR) (Heather *et al.*, 2003). In ISR, the phosphorylation of eIF2α is required for the translation of certain messenger RNAs that contain regulatory sequences, such as the short open reading frames (ORFs) in the 5'-untranslated region of the mRNA, including the mRNA encoding activating transcription factor 4 (ATF4) (Yaman *et al.*, 2003; Harding *et al.*, 2003). The ATF4 mRNA has two upstream ORFs before the initiation codon that mediates their translational regulation (Lu *et al.*, 2004).

ATF4 is a member of the ATF/CREB (activating transcription factor/cyclic AMP response element binding protein) family of basic region-leucine zipper (bZIP) transcription factors (Ameri and Harris, 2007). ATF4 can induce the expression of UPR target genes, which are involved in amino-acid biosynthesis and transport, protein secretion and resistance to oxidative stress (Harding *et al.*, 2003). It also induces the expression of growth arrest and DNA damage-inducible protein (GADD34) (Ma and Hendershot, 2003), which directly interacts with the catalytic subunit of type 1 protein serine/threonine phosphatase (PP1) and activates the ability of PP1 to dephosphorylate eIF2α, allowing recovery from the translation repression through a feedback mechanism (Novoa *et al.*, 2001).

However, not all the genes induced by ATF4 are anti-apoptotic. The transcription factor C/EBP homologous protein (CHOP) (Ma *et al.*, 2002) and tribbles homolog 3 (TRB3) (Ohoka *et al.*, 2005), which induction strongly depends on ATF4, are well known to promote apoptotic cell death.

3.1.3. ATF6 pathway

ATF6α, a 90 kDa bZIP (basic region and leucine zipper)-domain-containing transcription factor belonging to the CREB (cyclic-AMP-responsive-element-binding protein) and ATF family of transcription factors (Haze *et al.*, 1999), is a regulatory protein which promotes UPR-inducible gene expression and is activated by proteolysis in response to ER stress (Inagi, 2009). This protein is synthesized as inactive precursor, tethered to the ER membrane by a trans-membrane segment and has a stress-sensing portion that projects into the ER lumen (Ron and Walter, 2007).

After dissociating from GRP78/BiP, ATF6 is transported from the ER to the Golgi apparatus, packed into a COPII vesicle (Nadanaka *e al.*, 2004), and cleaved by Golgi-resident proteases, first by site 1 protease (S1P) and then in an intra-membrane region by site 2 protease (S2P) to release the functional fragment (bZIP-domain) of ATF6α into the cytosol (Shen *et al.*, 2002). This fragment (50 kDa cleaved ATF6α (Eizirik *et al.*, 2008) moves to the nucleus where it binds to the ER stress response element (ERSE) $CCAATN_9CCACG$ (Yoshida *et al.*, 1998) to activate the transcription of target genes (Figure 6), which encode ER chaperones, such as GRP78/BiP, GRP94, protein disulfide isomerase (PDI) and calreticulin (Okada *et al.*, 2002), and ERAD components (Yamamoto *et al.*, 2007). ATF6 also augments X-box binding protein 1 (XBP1) mRNA expression, providing more substrate for IRE1-induced generation of XBP1s (spliced variant) (Lee *et al.*, 2002), and the CHOP mRNA expression, although no reports have linked ATF6 to ER stress-induced apoptosis (Szegezdi *et al.*, 2006). ATF6 is also related to OASIS, CREBH, and CREB4, which have tissue-specific effects (Cunard and Sharma, 2011).

3.1.4. IRE1α pathway

The IRE1α is a membrane-bound serine/threonine kinase with endonuclease activity, owning a novel luminal domain and a cytoplasmic portion that contains a protein kinase domain (Cox *et al.*, 1993). After dissociation of GRP78/BiP from the ER luminal domain (Oikawa *et al.*, 2009), IRE1α oligomerizes and activates its RNase domain by autophosphorylation (Shamu and Walter, 1996). Once activated, IRE1α splices 26-bp intron from the mRNA encoding XBP1, generating a spliced variant that functions as a

potent transcriptional factor of genes involved in UPR-associated genes, ER homeostasis and expansion, and ERAD (Figure 6) (Yoshida *et al.*, 2001; Lee *et al.*, 2003).

IRE1α also activates apoptosis signal-regulating kinase (ASK1), c-Jun N-terminal kinase (JNK) and NF-κB, which are involved in apoptosis, autophagy and inflammation (Urano *et al.*, 2000; Kaneko *et al.,* 2003; Kim *et al.,* 2009).
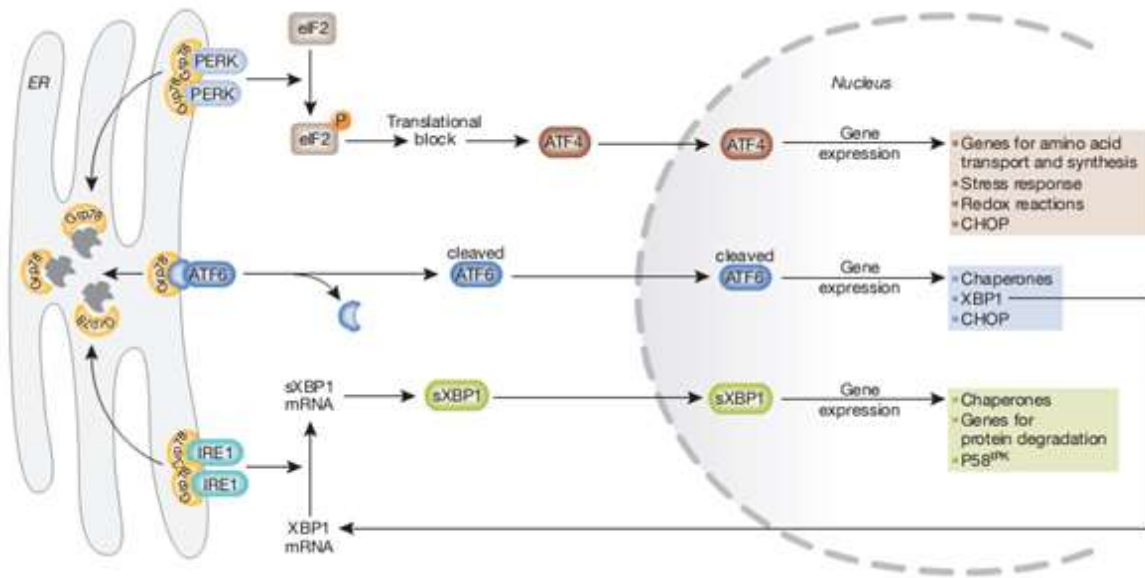
Figure 6. The unfolded protein response. In stress conditions, GRP78/BiP dissociates from the ER stress sensors, namely PERK, ATF6 and IRE1, allowing their sequential activation. Activated PERK blocks general protein synthesis by phosphorylating eIF2α. This phosphorylation enables translation of ATF4, a transcription factor that is translocated to the nucleus and induces the transcription of genes required to restore ER homesostasis. ATF6 is activated by limited proteolysis after its translocation from the ER to the Golgi apparatus. ATF6 is also a transcription factor that regulates the expression of ER chaperones and XBP1. To achieve its active form, XBP1 must undergo MRNA splicing, which is carried out by IRE1. Spliced XBP1 protein translocates to the nucleus and controls the transcription of chaperones, as well as genes involved in protein degradation. This concerted action aims to restore ER function by blocking further buil-up of client proteins, enhancing the folding capacity and initiating degradation of protein aggregates. (Image taken from Szegezdi *et al.*, 2006)

3.2. ER stress and apoptosis

Although the UPR initially serves as an adaptive response which acts to restore ER homeostasis, this physiological response induce an apoptotic response when the ER stress crosses a certain persistence and strength. ER stress-induce apoptosis is mediated by signaling through PERK, ATF6 and IRE1α, that initiate the activation of downstream molecules and activate several pro-apoptotic pathways, including caspase-mediated pathway, IRE1α-mediated pathway and CHOP-mediated pathway (Figure 7) (reviewed in Inagi, 2009).

3.2.1. IRE1α-mediated pathway

In response to ER stress, the cytoplasmic domain of activated IRE1α interacts with the adaptor protein TNF receptor-associated factor 2 (TRAF2), activating the JNK, via interaction with ASK1, which subsequently phosphorylates and activates JNK (Urano *et al.*, 2000; Nishitoh *et al.*, 2002), leading to the activation of JNK-mediated apoptotic pathway (Figure 7).

JNK is known to regulate the anti-apoptotic protein Bcl-2 by its phosphorylation and inactivation (Szegezdi *et al.*, 2006), which suppresses the anti-apoptotic activity of Bcl-2, inducing apoptosis (Bassik *et al.*, 2004). JNK also phosphorylates BH3-only Bcl-2 family members, which enhances their pro-apoptotic potential (Szegezdi *et al.*, 2006).

The IRE1-TRAF2 complex also activates procaspase 12, that also contribute to ER stress-mediated apoptosis (Yoneda *et al.*, 2001), and is involved in the activation of NF-κB, that can have a pro-apoptotic effect, depending on the cell type and context (Kaneko *et al.*, 2003).

3.2.2. Caspase-mediated pathway

Some components of the caspase cascade are reported to be involved in ER stress-induced apoptosis, such as caspases 2, 3, 4, 7, 9 and, in particular, caspase 12 (Cheung *et al.*, 2006; Dahmer, 2005; Di Sano *et al.*, 2005; Hitomi *et al.*, 2004) . Caspase 12, which resides on the outside of ER membrane, is activated during ER stress by calpain, which cleaves the ER-resident procaspase-12 to caspase-12, initiating caspase-dependent apoptosis (Figure 7) (Tan *et al.*, 2006). Caspase-12 triggers the activation of caspases 9, which in turns activate caspase-3, in a cytochrome c and Apaf-1-independent manner (Morishima *et al.*, 2002; Rao *et al.*, 2002), leading to cell death. ER stress also causes the translocation of cytosolic caspase-7 to ER surface, which is also required for caspase-12 activation (Rao *et al.*, 2002).

In addition, BAX-BAK pathway also activates caspase-12-mediated apoptosis (Inagi, 2009). BAX and BAK are present at the mitochondrial and ER membrane, and under ER stress conditions cause the release of Ca^{2+} from ER increasing the cytosolic Ca^{2+} concentration. This increased cytosolic Ca^{2+} concentration activates calpain present in the cytosol, which then leads to caspase-12 activation (Scorrano *et al.*, 2003; Oakes *et al.*, 2005).

3.2.3. CHOP-mediated pathway

CHOP, also known as growth arrest and DNA damage-inducible protein 153 (GADD153), is a bZIP transcription factor, induced through the ATF6 and PERK UPR pathways (Figure 7) (Szegezdi *et al.*, 2006), playing an important role in ER stress-mediated apoptosis (Nishitoh, 2012). However, the precise mechanism how CHOP mediates ER stress-induced apoptosis remains controversial (Malhotra and Kaufman, 2007; Nishitoh, 2012).

One of the mechanisms that is considered to contribute to CHOP-mediated apoptosis is the transcription of several genes involved in apoptosis, such as GADD34, ER oxidoreductin 1 (ERO1), death receptor 5 (DR5), TRB3, carbonic anhydrase VI (Yamaguchi and Wang, 2004; Ohoka *et al.*, 2005; Marciniak *et al.*, 2004; Malhotra and Kaufman, 2007), and the induction of the BH3-only pro-apoptotic proteins, such as Bim (Puthalakath *et al.*, 2007). The translocation of BAX from the cytosol to the mitochondria (Gotoh *et al.*, 2004), the down-regulation of anti-apoptotic Bcl-2 expression and perturbation of the cellular redox state by depletion of cellular glutathione (McCullough *et al.*, 2000), may also contribute to CHOP-mediated apoptosis.

ER stress-associated apoptosis induced by CHOP is dependent of the duration and degree of ER stress. Severe ER stress preferentially induces pro-apoptotic CHOP expression, while chronic exposure to a mild stress can lead to attenuation of CHOP expression, by degradation of CHOP mRNA and CHOP protein (Rutkowski *et al.*, 2006).
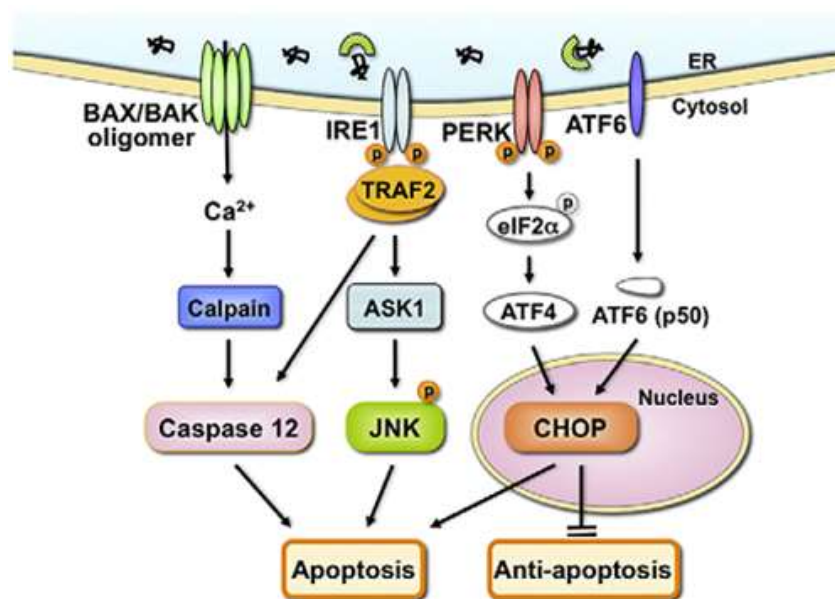

Figure 7. ER stress induced apoptosis. Three main pathways involved: CHOP-mediated pathway, Caspase-mediated pathway and IRE1 α -mediated pathway. (Adapted from Inagi, 2009)

3.3. ER stress and inflammation

Accumulating evidences suggests ER stress plays an important role in the pathogenesis of diabetes. ER may also link inflammation in T2DM. In fact, ER stress attenuation decreases the expression of proinflammatory cytokines in diabetic kidney (Qi *et al.*, 2010). The signaling pathways in the UPR and inflammation are connected through diverse mechanism, including the production of ROS and the activation of NF-κB and JNK.

ROS are important mediators of inflammation (Naik and Dixi, 2011), and generation and accumulation of intracellular ROS (state referred as oxidative stress) have been associated to the ER stress. The correct folding of proteins requires oxidizing conditions for the formation of intramolecular and intermolecular disulphide bonds. The oxidative protein folding is driven by a protein relay involving ERO1 and PDI (Tu and Weissman, 2004). PDI has been known to aid the formation of disulphide bonds, as it directly accepts electrons from protein-folding substrates, resulting in the oxidation of cysteine residues and the formation of disulphide bonds. Then, ERO1 operates in association with the flavin adenine dinucleotide (FAD) to transfer electrons from PDI to molecular oxygen (O_2), resulting in the production of ROS (Zhang and Kaufman, 2008). Given that the UPR increases the protein-folding load in the ER, an accumulation of ROS might initiate an inflammatory response.

Moreover, NF-κB, a key transcriptional regulator of genes involved in the inflammatory response, is activated by ER stress. In response to ER stress, the UPR can directly promote NF-κB activation through a PERK-eIF2α and IRE1-TRAF2 pathways, by translational attenuation and degradation of IκB, respectively (Zhang and Kaufman, 2008). The IRE1α-TRAF2 pathway can also lead to the activation of JNK, and activated JNK induces the expression of inflammatory genes by the phosphorylation of the transcription factor AP1 (Davis *et al.*, 2000).

Recent findings suggest that CHOP is a key molecule not only in apoptosis but also in inflammatory response. UPR-induced CHOP expression is implicated in the induction of caspase-11 that plays an important role in the processing of pro-IL-1β through caspase-1 activation (Endo *et al.*, 2006).

3.4. ER stress and diabetes

The ER stress response is implicated in a number of pathogenesis, including neurodegenerative diseases, atherosclerosis and metabolic diseases, such as diabetes (Malhotra and Kaufman, 2007). Accumulating evidence suggests that ER stress contribute to pancreatic β-cells loss and insulin resistance (Araki *et al.*, 2003; Kaneto *et al.*, 2006; Song *et al.*, 2008). Apoptosis in β-cells plays an important role in development of diabetes, both type 1 and 2 (Mathis *et al.*, 2001; Butler *et al.*, 2003), and these cells have a highly developed and active ER, showing a high expression of ER stress transducers proteins such as Ire1α, PERK and GRP78/BiP (Eizirik *et al.*, 2008; Malhotra and Kaufman, 2007). This may reflect the fact that β-cells are very susceptible to ER stress, and also highly engaged in protein secretion (Araki *et al.*, 2003).

In T1DM, β-cell loss is linked to nitric oxide (NO) production by inflammatory cytokines, such as IL-1β, TNF and IFN-γ (Darville and Eizirik, 1998; Thomas *et al.*, 2002). Oyodomari et al (2001) demonstrated that NO-induced apoptosis in β-cells is mediated by the ER stress pathway through induction of the ER stress-associated apoptosis factor CHOP. Treatment of mouse insulinoma cell line MIN-6 with NO donor SNAP (S-nitroso-Nacetyl-D, L-penicillamine) suggest that NO depletes ER Ca^{2+} stores (Oyadomari *et al.*, 2001). A severe Ca^{2+} ER depletion leads to disturbance of ER function and impairment in the quality of ER protein folding and assembly, causing ER stress and downstream CHOP-induced apoptosis (Araki *et al.*, 2003; Cardozo *et al.*, 2005; Eizirik *et al.*, 2008).

In T2DM, it is known that a high-fat diet and/or obesity contribute to the development of this pathology, as a consequence of lipotoxicity/glucotoxicity and insulin resistance. It has been shown that free fatty acids (FFAs) (lipotoxicity) as palmitate, and high glucose levels (glucotoxicity) induced the activation of ER stress response in β-cells, leading to β-cell apoptosis (Karaskov *et al.*, 2006; Cnop *et al.*, 2007; Elouil *et al.*, 2007; Lawrence *et al.*, 2007). Palmitate leads to phosphorylation of PERK and eIF2α, inhibition of protein synthesis and induction of ATF4 and CHOP, causing β-cell apoptosis (Cnop *et al.*, 2007). High glucose induces both the expression of CHOP and ER chaperones (Elouil *et al.*, 2007; Lawrence *et al.*, 2007). On the other hand, glucose is known to stimulate insulin biosynthesis (Itoh and Okamoto, 1980). Thus high glucose levels induce increased demand of insulin that result in increased proinsulin biosynthesis exceeding the folding

capacity of ER, leading to ER overload and ER stress. This, in turn, causes β-cell dysfunction and apoptosis (Kim *et al.*, 2012).

The importance of the UPR in diabetes is not restricted to β-cell function but is also involved in insulin resistance. ER stress has been proposed as one of the molecular mechanism linking obesity with peripheral insulin resistance, through alterations on insulin signaling (Ozcan *et al.*, 2004). ER stress suppresses insulin receptor signaling through activation of the protein kinase JNK. JNK phosphorylation of IRS-1 on Ser307 reduces insulin receptor-stimulated tyrosine phosphorylation, decreasing insulin signaling (Ozcan *et al.*, 2006; Hirosumi *et al.*, 2002). Indeed, Ozcan et al. (2004) demonstrated that, in obese mice, JNK activity increases dramatically.

Additionally, altered metabolic conditions associated with diabetes, such as ROS production, FFAs and hyperglycaemia, can lead to UPR response and contribute for the development of diabetic complications (King and Loeken, 2004; Kaneto *et al.*, 2005), including diabetic nephropathy (Lindenmeyer *et al.*, 2008).

3.4.1. ER stress and diabetic nephropathy

There is increasing evidence indicating that the ER stress has an important role in renal injury onset in diabetes (Liu *et al.*, 2008; Inagi *et al.*, 2005; Inagi *et al.*, 2010; Lim *et al.*, 2010; Shao *et al.*, 2013). Renal cells have a well-developed ER, turning them particularly sensitive to perturbations in ER function. Indeed, studies in proximal tubular cells revealed upregulation of GRP78/BiP by albumin overload (Ohse *et al.*, 2006).

Recent studies have been indicated that ER stress response is activated in T1DM kidneys, and can be responsible for renal cells apoptosis mediated by activation of CHOP, JNK and caspase-12 proteins (Liu *et al.*, 2008). Renal proximal tubular cells apoptosis is also linked to ER stress response activation (Lim *et al.*, 2010). In addition, ROS seem to play a crucial role in podocyte injury by CHOP protein upregulation (Bek *et al.*, 2006).

During recent years, accumulating evidences suggest the relevance of ER stress in the development and pathogenesis of DN. Therefore, it is crucial to better understand the role of ER stress in renal lesions due to diabetes.

4. Diabetes therapy – dipeptidyl peptidase-IV inhibitors

A new class of oral anti-diabetic agents, the dipeptidyl peptidase-IV (DPP-IV) inhibitors or gliptins, such as sitagliptin, has increasingly gained emphasis in the therapeutic managing of T2DM patients. These inhibitors are mainly responsible for lowering blood glucose levels by potentiating incretins action, stimulating insulin secretion and inhibiting glucagon activity, in response to chronic hyperglycaemia.

4.1. Incretins

Incretins are peptide hormones that are involved in the physiologic regulation of glucose homeostasis, as exemplified by glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) (Barnett, 2006; Drucker, 2006). Incretin hormones are secreted from the gastrointestinal tract after food intake, in a nutrient-dependent manner, and stimulate glucose-dependent insulin secretion. The insulin secretory response of incretins is called *incretin effect*, and accounts for at least 50% of total insulin secreted after oral glucose. Therefore, incretin hormones are insulinotropic (Kim and Egan, 2008).

The first incretin hormone described was GIP. GIP is a 42 amino acid peptide produced predominantly in duodenal K cells in the proximal small intestine (duodenum and jejunum) (Buchan *et al.*, 1978; Takeda *et al.*, 1987). The predominant stimulus for GIP secretion is nutrient intake, namely absorbable carbohydrates and lipids. Circulating levels of GIP are therefore greatly increased in response to food ingestion (Holst and Gromada, 2004). In addition to being insulinotropic, GIP also regulates fat metabolism in adipocytes, including insulin-stimulated incorporation of fatty acids into triglycerides. Additionally, it stimulates lipoprotein lipase activity, modulates fatty acid synthesis, promotes β-cell proliferation and cell survival (Drucker, 2003).

GLP-1 was the second incretin hormone identified. GLP-1 is produced in enteroendocrine L-cells in the distal small bowel and colon, where is secreted into the bloodstream in response to nutrient ingestion (Jang *et al.*, 2007). Like GIP, plasma levels of GLP-1 also rise rapidly minutes after food intake. GLP-1 is thought to increase insulin biosynthesis trough induction of Pdx-1 transcription, leading to enhanced insulin gene expression (Wang *et al.*, 1999). In addition to its insulinotropic effects (Nauck *et al.*, 1993), GLP-1 also inhibits gastric emptying which decreases food intake (Meier *et al.*,

2003), suppresses the elevated glucagon secretion (Komatsu *et al.*, 1989) and slows the rate of endogenous glucose production (Prigeon *et al.*, 2003). It has also been shown that GLP-1 preserves or enhances β-cells function as a result of β-cell proliferation and inhibition of apoptosis (Farilla *et al.*, 2002). GLP-1 appears to be responsible for the majority of the incretin effect on pancreatic β-cell function and is responsible for a substantial part of the insulin response to oral glucose. Therefore, it has become a favored potential therapeutic target.

GIP and GLP-1 achieve their insulinotropic effects by binding to their specific receptors, which are coupled to G protein (Figure 8). GIP receptor (GIPR) is expressed in pancreatic islets and also in the gut, adipose tissue, heart and brain (Holst and Gromada, 2004; Drucker, 2006). On the other hand, GLP-1 receptor (GLP-1R) is widely expressed in the kidney, lung, heart, and also in central nervous system and pancreatic islets, mainly in β-cells (Drucker, 2006). Binding of GIP and GLP-1 to the receptors causes activation of adenylate cyclase which results in the formation of intracellular cyclic adenosine monophosphate (cAMP), leading to PKA and cAMP-regulated guanine nucleotide exchange factor II (cAMP-GEFII) activation. This leads to several events, including altered ion channel activity, increased intracellular calcium concentration and enhanced exocytosis of insulin-containing granules (Figure 8) (Holz, 2004). Furthermore, GLP-1R activation also leads to induction of insulin gene transcription (Drucker *et al.*, 1987) and increased pancreatic insulin biosynthesis (Hosokawa *et al.*, 1996).

The incretin effect is restricted by GIP and GLP-1 short half-life, that is about 1 to 2 minutes in rodents (Kieffer *et al.*, 1995), 7 minutes in healthy humans and 5 minutes in T2DM patients for exogenous GIP (Deacon *et al.*,, 2000), and about 2 minutes in rodents and humans for exogenous GLP-1 (Deacon *et al.*, 1995). The incretin degradation is remarkably rapid due to its inactivation by DPP-IV.
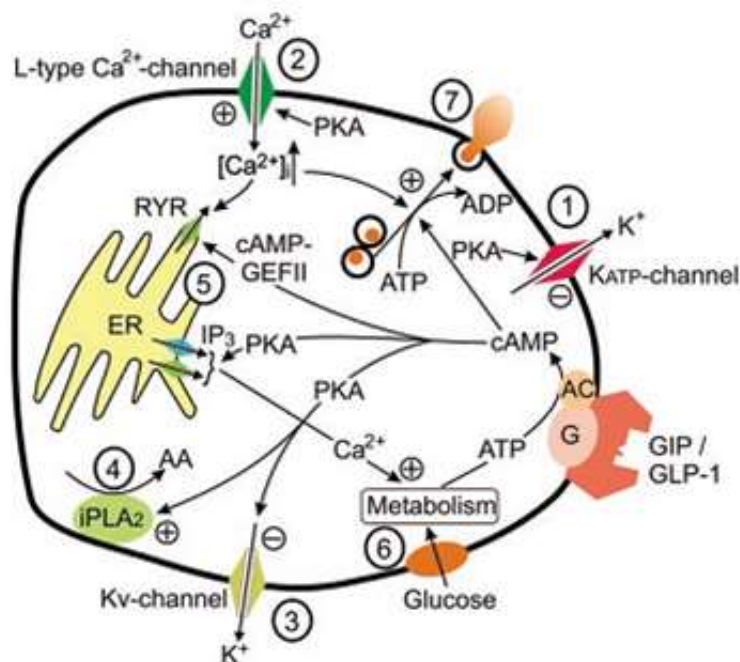
Figure 8. GLP-1R and GIPR activation, and main molecular events during incretin-induced insulin secretion from β-cell. Binding of GLP-1 and GIP to their receptors couples to activation of adenylate cyclase, and intracellular cAMP levels are elevated, leading to activation of PKA and cAMP-GEFII, which leads to elevation of intracellular Ca^{2+} levels via a depolarization of plasma membrane by inhibition of K_{ATP} and K_V channels, after ATP generation from glucose and consequent opening of voltage-gate L-type Ca^{2+}-channel. Intracellular Ca^{2+} levels are further increased via stimulation of IP_3R and RyR on the ER. In addition, L-type Ca^{2+} channels are phosphorylated by PKA, resulting in an increase of their open probability and thus facilitation of enhanced Ca^{2+} influx. The changes in intracellular Ca^{2+} concentrations lead to fusion of insulin-containing vesicles to the plasma membrane and subsequent rapid exocytosis of insulin from β-cells. The exocytosis of insulin-containing vesicles is also potentiated by increased cAMP levels, due to the ability of cAMP to accelerate granule mobilization, resulting in an increased size of the pools of granules that are immediately available for release. (Image taken from Holst and Gromada, 2004)

4.2. Dipeptidyl peptidase-IV (DPP-IV)

DPP-IV is the primary enzyme responsible for inactivating both GIP and GLP-1, by cleavage of the penultimate alanine residue in N-terminal of full-length (Mentlein *et al.*, 1993), which converts them to bioinactive metabolites. DPP-IV, also known as CD26, is a membrane-associated peptidase of 766 amino acids that is widely distributed in numerous tissues and cells, such as lymphocytes, endothelial cells, gut, liver, pancreas and kidney, as well as being present in soluble form in the circulation in plasma (Pauly *et al.*, 1996; Barnett, 2006; Kim and Egan, 2008; Kirino *et al.*, 2009). DPP-IV has a serine exopeptidase activity that cleaves X-proline dipeptides from the N-terminus of polypeptides, and the substrates of this enzyme are proline- or alanine-containing peptides, which include numerous neuropeptides, hormones and chemokines (Baggio and Drucker, 2007). Therefore, DPP-IV inhibition has a range of possible pleiotropic effects,

well beyond GLP-1 modulation, that may or may not provide additional positive effects for diabetes treatment.

Insight into the role of DPP-IV in the control of incretin biology has been derived from several studies with inactivating mutations in the DPP-IV gene and from the results of experiments using inhibitors of DPP-IV activity, which potentiates endogenous GLP-1 and GIP action by reduction of incretin degradation. In DPP-IV knockout mice, it was observed increased levels of GIP and GLP-1, as well as an improvement in glucose tolerance and enhanced insulin secretion (Marguet *et al.*, 2000). Additionally, studies using DPP-IV inhibitors to treat rodents with experimental diabetes also showed significant improvements in glucose tolerance, preservation of GLP-1 levels and increased levels of plasma insulin after oral glucose (Baggio and Drucker, 2007). Moreover, it has been shown that treatment with DPP-IV inhibitors in animal models of type 1 and 2 diabetes preserves islet function and increases pancreatic insulin content, through increasing proliferation, neogenesis and apoptosis resistance of β-cells (Reimer *et al.*, 2002; Popisilik *et al.*, 2003).

Clinical trials in type 2 diabetic patients demonstrated that the treatment with DPP-IV inhibitors improve postprandial and fasting glycaemic control, in association with increased GLP-1 levels and reduced glucagon levels (Ahren *et al.*, 2002). Complementary studies also demonstrated that DPP-IV inhibitors increase the insulin secretory rate in type 2 diabetic patients, which suggests an improvement in β-cell function (Mari *et al.*, 2005).

4.3. Sitagliptin

Many DPP-IV inhibitors have entered clinical trials in diabetic patients, namely sitagliptin, the first one, vidaglitin, saxagliptin and others that were further developed (Barnett, 2006). Sitagliptin is an oral, once-daily, potent and highly selective DPP-IV inhibitor for treatment of T2DM, improving fasting and postprandial glycaemia and deceasing glycosylated hemoglobin (HbA1c) levels (Herman *et al.*, 2007). Sitagliptin is a well-tolerated anti-diabetic drug, without weight changes and with low propensity to cause hypoglycaemia (Drucker and Nauck, 2006).

The DPP-IV enzyme contains two glutamate residues (Glu[205] and Glu[206]) highly conserved, that are essential for its enzymatic activity (Abbott *et al.*, 1999), and has been demonstrated experimentally that the amino groups contained within the DPP-IV

inhibitor sitagliptin interact with these two glutamate residues (Biftu *et al.*, 2007), which inhibits the enzymatic activity of DPP-IV.

Sitagliptin is a small non-peptide-based active molecule with 523.32 Da of molecular weight, corresponding chemically *7-[(3R)-3-amino-1-oxo-4-(2,4,5-trifluorophenyl)butyl]-5,6,7,8-tetrahydro-3-(trifluormethyl)-1,2,4-triazolo[4,3-a]pyrazine phosphate (1:1)monohydrate* (Figure 9). Sitagliptin was approved by INFARMED for the treatment of T2DM in Portugal in November 2007.
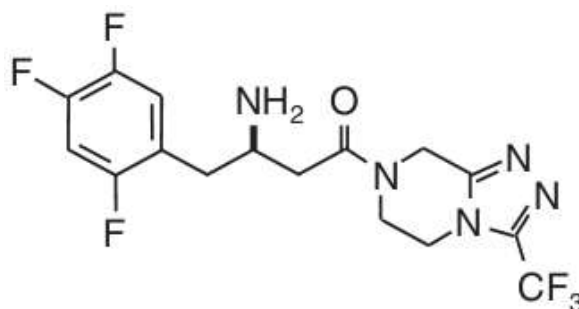
Figure 9. Chemical struture of Sitagliptin (Adaptef from Barnett, 2006)

Oral sitagliptin is rapidly absorbed and achieves peak plasma levels in 1 to 6 hours, and the administration can be in the presence or absence of food. Its half-life is 8 to 14 hours and its bioavailability is approximately 87% (Kim and Egan, 2008). Approximately 80% of the dose is excreted unchanged by the kidney and a small amount (15%) is metabolized in the liver (Bergman et al.*,* 2007; Kim and Egan, 2008). The recommended dose is 100 mg daily, which inhibits DPP-IV activity at least 80% over a 24 hours period (Aschner *et al.*, 2006). Sitagliptin was evaluated as monotherapy or in combination with other anti-diabetic drugs, such as metformin, showing that sitagliptin alone significantly reduces HbA1C levels about 0.79% in type 2 diabetic patients, with 100 mg once daily treatment for 24 weeks (Aschner *et al.*, 2006). Several clinical trials in patients with T2DM showed that sitagliptin was well tolerated and significantly improves glycaemic levels, by lowering blood glucose and HbA1C levels (Ahren *et al.*, 2002; Kim *et al.*, 2005; Aschner *et al.*, 2006; Raz *et al.*, 2006; Nonaka *et al.*, 2007).

Recent studies demonstrated that sitagliptin have high selectivity for DPP-IV (IC_{50}: 18 nM) (Weber *et al.*, 2004), which is required for an acceptable safety and tolerability treatment. At least two dipeptidyl peptidases, such as dipeptidyl peptidases VIII (DPP-VIII) and dipeptidyl peptidases IX (DPP-IX), are structurally closely related to DPP-IV, but sitagliptin have low affinity for these peptidases, with IC_{50}: 48000 nM for

DPP-VIII and IC$_{50}$: >100000 nM for DPP-IX (Kim *et al.*, 2005). Acute toxicity in animal models was reported with DPP-VIII and DPP-IX inhibition (Lankas *et al.*, 2005); by that, the low selectivity of sitagliptin for DPP-VIII and DPP-IX enhances the safety of the treatment.

Given that sitagliptin is orally bioavailable might offer greater benefits compared with injectable agents (e.g. insulin) in terms of patient compliance. Moreover, sitagliptin significantly improves glycaemic levels, promotes β-cell survival and enhances insulin secretion, as well as offers low risk of hypoglycaemia and no weight gain. All these potential advantages enhance the therapeutic benefits of sitagliptin when related to other anti-diabetic drugs, turning it a promising therapeutic intervention for T2DM treatment, not only by it direct effects on glycaemic control, but also by the pleiotropic effects of incretins (Figure 10).
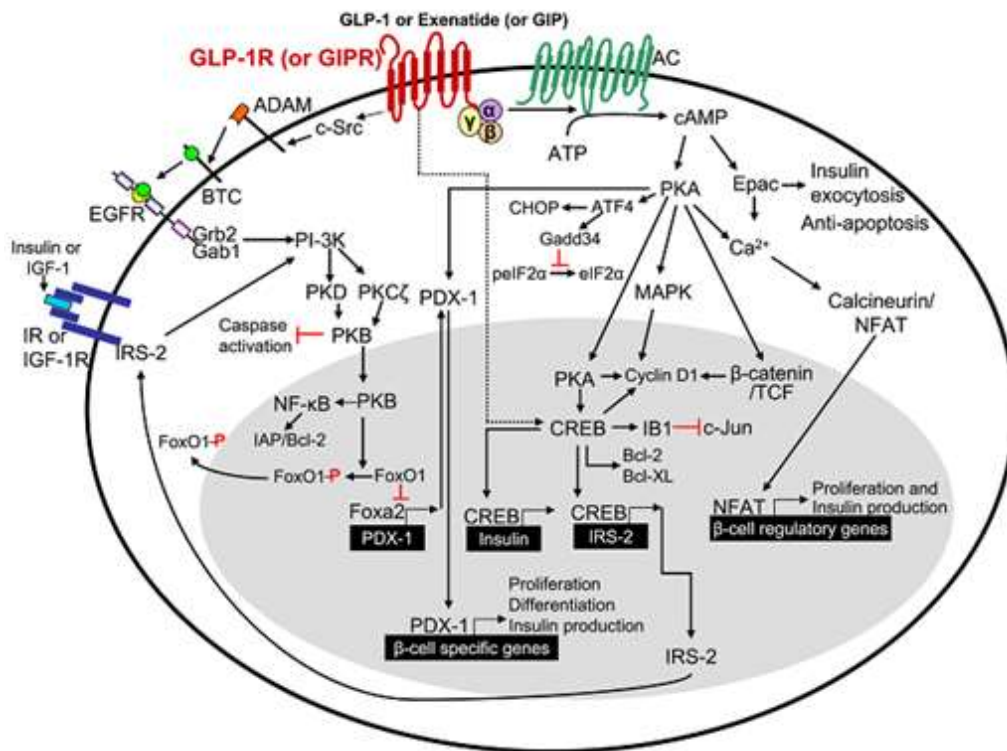
Figure 10. Pleiotropic effects of GLP-1. Schema outlining the incretin downstream signal transduction pathways in a β-cell. GLP-1R activation (and GIPR activation to some extent) recruits signaling mechanism that considerably overlap, leading to promotion of β-cell proliferation and prevention of β-cell apoptosis. Dashed line indicates mechanism that is not fully delineated. (Image taken from Kim and Egan, 2008)

5. Aims of this thesis

Several authors have been exploiting the cytoprotective actions of DPP-IV inhibitors in distinct organs and conditions, including pancreas, retina and heart (Mu *et al.*, 2006; Gonçalves *et al.*, 2010; Read *et al.*, 2010). However, until now, few studies have addresses the putative beneficial impact of these agents, including sitagliptin, on DN (Vaghasiya *et al.*, 2010; Liu *et al.*, 2011; Mega *et al.*, 2011).

In addition, the impact of sitagliptin therapy on ER stress, inflammation and apoptosis underlying DN development remains relatively unexploited. Therefore, the general goal of this study is to evaluate the efficacy of sitagliptin, a DPP-IV inhibitor, in preventing the deleterious effects of diabetes on the kidney of *Zucker Diabetic Fatty* (ZDF) rats.

To accomplish the main purpose, specific approaches have been devised as follows:

- ✓ Characterization of glycaemic profile of non-diabetic ZDF (+/+) and diabetic ZDF (*fa/fa*) non-treated and treated with sitagliptin, by measuring blood glucose, HbA1c and insulin levels.

- ✓ Assessment of the incretin signaling pathway: DPP-IV/CD26, GLP-1 and GLP-1R protein levels in the kidney will be determined.

- ✓ Evaluation of Akt survival pathway as an indicator of ECM expansion and mesangial hypertrophy in the kidney.

- ✓ Analysis of the inflammatory state: proinflammatory cytokines TNF and IL-1β distribution in kidney sections will be evaluated.

- ✓ Evaluation of the apoptotic state: BAX/Bcl-2 ratio and pro-apoptotic Bid protein levels in the kidney will be determined.

- ✓ Assessment of the ER stress response: CHOP, GRP78/BiP and ATF4 protein levels in the kidney will be analyzed.

CHAPTER 2

MATERIAL AND METHODS

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1. Animal model and groups

In this study, we used a rodent model of obese T2DM, the *Zucker Diabetic Fatty* (ZDF) rats, which develop obesity, hyperlipidaemia, fasting hyperglycaemia, hyperinsulinaemia and insulin resistance (Peterson *et al.*, 1990).

Obese ZDF rats (ZDF, *fa/fa*) and non-obese non-diabetic controls (ZDF, +/+) with 6 weeks age were obtained from Charles River Laboratories (Barcelona, Spain), and before starting the study, there was 2 weeks of acclimatization. Rats were housed under controlled temperature (23±1ºC) and relative humidity (60%), and a 12-h light-12-h dark cycle. The animals were fed distilled water *ad libitum* and rodent maintenance chow (A-04 Panlab, Barcelona, Spain) containing 15.4% of protein and 2.9% of lipids. In this study, diabetic ZDF (fa/fa) and non-diabetic ZDF (+/+) rats with 20 weeks age were used.

All procedures involving animals were performed according to the National and European Communities Council Directives on Animal Care.

At 20 weeks of age, ZDF (*fa/fa*) rats were divided into two groups (n=6-8 rats per group) and were orally treated during 6 weeks with vehicle (orange juice) or sitagliptin (Januvia®, MSD, Portugal) 10 mg/kg/day. Therefore, we had three groups of animals: (1) control ZDF (+/+) group treated with vehicle; (2) diabetic ZDF (fa/fa) group with vehicle treatment and (3) diabetic ZDF (fa/fa) group under sitagliptin therapy.

The body weight of each animal was recorded weekly during the experimental period, using an analytical balance (KERN CB 6 K1, Alemanha).

1.1. Sample collection

At the end of the treatment, rats were anaesthetized (2 mg/kg intraperitoneal cocktail of a 2:1 50 mg/ml ketamine solution in 2.5% chlorpromazine) and blood from the jugular vein was collected. After that, rats were sacrificed and the kidneys immediately removed and carefully cleaned of adherent fat and connective tissue. The kidneys were embedded in OCT tissue embedding matrix (Thermo Scientific, Waltham, MA, USA) at -50ºC, for immunohistochemistry and fluorescence microscopy studies, or divided in sections, that were frozen in liquid nitrogen and then stored at -80ºC, for immunoblot analysis.

1.2. Biochemical data

Serum glucose levels were measured through blood samples from the tail vein using a glucose oxidase commercial kit (Sigma, St. Louis, Mo, USA) and HbA1c levels were measured in total blood by DCA 2000+ Analyser (Bayer Diagnostics, Barcelona, Spain).

2. Protein extraction from rat kidney

Kidney sections were weighted, cut into small pieces and homogenized by mechanical dissociation using a Potter-Elvehjem, at 4ºC, in 5 volumes of RIPA lysis buffer (150 mM NaCl, 50 mM Tris (pH 7.5), 5 mM ethylene glycol tetraacetic acid (EGTA), 1% (v/v) Triton X-100 (Tx-100), 0.5% (m/v) sodium deoxycholate (DOC) and 0.1% (m/v) sodium dodecyl sulfate (SDS), supplemented with 2 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM iodoacetamide (IAD), 30 mM NaF, 1 mM sodium orthovanadate and 1x protease inhibitor cocktail (Roche, Indianapolis, IN, USA)). After incubation on ice for 1h, the lysates were sonicated and then centrifuged at 16,000×g, for 15 min, at 4ºC.

After centrifugation, the resulting supernatant fraction (corresponding to total extract) was collected and protein concentration was determined using the bicinchoninic acid assay (Pierce, Rockfor, IL, USA). Sample were then denatured with 6x Laemmli buffer (350 mM Tris-HCl (pH 6.8), 30% (m/v) glycerol, 10% (m/v) SDS, 600 mM DL-dithiothreitol (DTT) and 0.012% (m/v) bromophenol blue) for 1h at 37ºC.

3. Subcellular fractionation by sucrose gradient

Rat kidney subcellular fractions were prepared as previously described by Mosevitsky and Silicheva (2009). Kidney sections were weighted, cut into small pieces and homogenized by mechanical dissociation using a Potter-Elvehjem, at 4ºC, in 4 volumes of hypotonic buffer A (20 mM Tris-HCl (pH 7.4), 4 mM NaCl and 1 mM ethylene diamine tetraacetic acid (EDTA), supplemented with 2 mM PMSF, 30 mM NaF, 2 mM IAD, 1 mM sodium orthovanadate and 1x protease inhibitor cocktail (Roche, Indianapolis, IN, USA) with 10% (m/v) sucrose. The homogenate obtained was filtered with a nylon mesh (250 μm) and the filtrate was centrifuged at 3,000×g for 15 min, at

4ºC. The supernatant (cytoplasmic fraction) was collected and then pellet was resuspended in hypotonic buffer A (2.4:1) with 10% sucrose and 0.5% (v/v) Triton X-100. After incubation for 5 min in an ice bath, the suspension was layered on the top of 40% (m/v) sucrose in hypotonic buffer B (20 mM tris-HCl (pH 7.4), 4 mM NaCl and 1 mM EDTA, supplemented with 2 mM PMSF and 2 mM IAD and centrifuged at 7000 g for 20 min, at 4ºC. The pellet was gently resuspended in hypotonic buffer A (2.4:1) with 10% sucrose and 0.5% (v/v) Triton X-100, and the centrifugation was repeated. The dense white pellet (cell nuclei) obtained after centrifugation was finally resuspended in RIPA lysis buffer (2.4:1) (150 mM NaCl, 50 mM Tris (pH 7.5), 5 mM EGTA, 1% (v/v) Triton X-100 (Tx-100), 0.5% (m/v) sodium deoxycholate and 0.1% (m/v) SDS, supplemented with 2 mM PMSF, 2 mM IAD, 30 mM NaF, 1 mM sodium orthovanadate and 1x protease inhibitor cocktail (Roche, Indianapolis, IN, USA)). After incubation on ice for 1h, the lysates were sonicated and then centrifuged at 14.000g for 10 min, at 4ºC, and the supernatant was collected (nuclei fraction).

Protein concentration of cytoplasmic and nuclei fractions were determined using the bicinchoninic acid assay (Pierce, Rockfor, IL, USA), and then were denatured as previous described.

4. Polyacrylamide gel electrophoresis and immunodetection (*Western blotting*)

For the western blot analysis, 40 to 80 µg of protein were loaded per lane and separated by electrophoresis on SDS-12% or SDS-10% polyacrylamide gel in buffer 25 mM Tris-HCl (pH 8.0-8.5) containing 192 mM glycine and 0.1% (m/v) SDS, at 160 V. After electrophoresis, protein were electro-transferred to polyvinylidene difluoride (PVDF) (Immobilon®-P PVDF transfer membranes 0,45 µm, Millipore) or nitrocellulose (Trans-Blot®Turbo™ Midi Nitrocellulose Transfer Pack 0.20 µm membranes, Bio-Rad Life Science) membranes. Western blot transfer was performed using 25 mM Tris-HCl (pH 8.0-8.5) containing 192 mM glycine, 20% (v/v) methanol and 0.005% (m/v) SDS, for 1h 30min at 260 mA, at 4ºC. After transfer, membranes were blocked with 5% (m/v) non-fat milk in Tris-buffered saline (TBS: 20 mM Tris (pH 7.6) and 150 mM NaCl, containing 0.1% (v/v) Tween-20 (TBS-T)), for 1h with agitation, at room temperature. Membranes were incubated with primary antibodies (Table 1), diluted in TBS-T supplemented with 5% non-fat milk or 5% (m/v) bovine serum albumin (BSA), overnight

at 4ºC. Membranes were washed three times with TBS-T for 30 min and then incubated with adequate horseradish peroxidase (HRP) conjugated secondary antibodies (Table 1) with agitation for 1h, at room temperature. After secondary antibody incubation, membranes were washed again for 30 min with TBS-T and reprobed with enhanced chemiluminescence (ECL) reagent (Bio-Rad Life Science). To confirm equal protein loading and sample transfer, blots were reprobed with mouse anti- β-actin, rabbit anti-Lamin B1 or goat anti-GAPDH antibodies.

Immunoreactive bands were revealed by scanning blots using a VersaDoc (Bio-Rad Life Science) imaging system. Densitometric analyses were performed using the ImageJ 1.42n software.

Table 1. Primary and secondary antibodies used for Western Blotting analysis.

Antibody	Dilution	Company
Mouse monoclonal antibody anti-Bcl-2	1:200	Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA)
Rabbit polyclonal antibody anti-Bax	1:200	Santa Cruz Biotechnology, Inc.
Rabbit polyclonal antibody anti-Bid	1:500	Chemicon (CA, USA)
Rabbit polyclonal antibody anti-CD26	1:4000	Abcam (Cambridge, UK)
Mouse monoclonal antibody anti-GLP-1	1:1000	Abcam
Mouse anti-GRP78/BiP	1:500	BD Transduction Laboratories (San Jose, CA, USA)
Goat polyclonal antibody anti-ATF4	1:166.7	Sigma-Aldrich Co. (St. Louis, MO, USA)
Mouse monoclonal antibody anti-CHOP	1:1000	Cell Signaling (Danvers, MA, USA.)
Rabbit monoclonal antibody anti-P-Akt	1:2000	Cell Signaling
Rabbit polyclonal antibody anti-Lamin B1	1:4000	Abcam
Goat polyclonal antibody anti-GAPDH	1:10000	Sicgen (Coimbra, Portugal)
Mouse monoclonal antibody anti-β-Actin	1:10000	Sigma-Aldrich Co.
HRP secondary anti-mouse	1:10000	Bio-Rad (Hercules, CA, USA)
HRP secondary anti-rabbit	1:10000	Bio-Rad
HRP secondary anti-goat	1:10000	Bio-Rad

5. Immunohistochemistry

Cross sections of rat kidney (6 μm) were cut in a cryostat (Leica CM3050S, Nussloch, Germany). Transverse sections of rat kidney were fixed with cold acetone for 10 min, and then washed, with phosphate-buffered saline (PBS: 137 mM NaCl, 27 mM KCl, 81 mM Na_2HPO_4, 15 mM KH_2PO_4, pH 7.3) (3×5 min). After washing, sections were permeabilized for 30 min with 0.25% (v/v) Triton X-100 in PBS and blocked for 40 min with 10% (v/v) normal goat serum or with 5% (m/v) BSA, prior to incubation with the primary antibodies (Table 2) diluted in PBS with 0.25% Triton X-100, overnight at 4ºC. Samples were washed with washing buffer (3×5 min). Sections were incubated with the secondary fluorescent antibodies (Table 2) and 1μg/ml 4',6-diamidino-2-phenylindole (DAPI) for 1h at room temperature in a humidified chamber in the dark. After incubation, sections were washed with PBS three times of 5 min each, and the slides were mounted using the Glycergel mouting medium (Dako, Carpinteria, CA, USA) and sealed with nail polish. The samples were stored at 4ºC until acquisition of images in the confocal fluorescence microscope (LSM 710, Carl Zeiss, Gottingen, Germany). For a negative experiment, the primary antibody was omitted.

Table 2. Primary and secondary antibodies used in the immunofluorescence assays.

Antibody	Dilution	Company
Rabbit polyclonal antibody anti-Bax	1:50	Santa Cruz Biotechnology, Inc.
Rabbit polyclonal antibody anti-CD26	1:150	Abcam
Rabbit polyclonal antibody anti-GLP-1R	1:200	Abcam
Mouse monoclonal antibody anti-GLP-1	1:250	Abcam
Mouse monoclonal antibody anti-CHOP	1:150	Cell Signaling
Goat polyclonal antibody anti-IL-1β	1:100	R&D Systems (Minneapolis, MN, USA.)
Rabbit polyclonal antibody anti-TNF	1:500	R&D Systems
Alexa Fluor 568 goat anti-mouse	1:200	Molecular Probes Inc. (OR, USA)
Alexa Fluor 488 goat anti-rabbit	1:300	Molecular Probes Inc.
Alexa Fluor 594 donkey anti-goat	1:100	Molecular Probes Inc.

6. Statistical analysis

Results were statistically analyzed using GraphPad Prism (GraphPad Prism 5.0 software, La Jolla, CA, USA), and expressed as mean ± standard errors of the mean (SEM). The comparison of values between groups was performed by using analysis of variance (ANOVA) followed by Bonferroni´s *Post-hoc* test. Values of $p<0.05$ were considered statistically significant.

CHAPTER 3

RESULTS

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1. Sitagliptin prevents the weight loss and decreases glucose and HbA1c blood levels in the diabetic animals

Rodent models of T2DM are frequently used to clarify the mechanisms responsible for the pathophysiology of diabetes evolution, as well as its complications, such as DN. The T2DM animal model used in this study was the *Zucker Diabetic Fatty* (ZDF) rat, which presents a mutation in the gene encoding the leptin receptor (*fa/fa*) resulting in obesity, insulin resistance, reduced glucose tolerance, hypertension, and renal and cardiovascular disease (Peterson *et al.*, 1990; Janssen *et al.*, 1999; Phillips *et al.*, 1999). Thus, this animal model of T2DM develops a phenotype very similar to T2DM occurring in humans (Peterson *et al.*, 1990), including the presence of microvascular complications, such as DN (Janssen *et al.*, 1999; Phillips *et al.*, 1999). Non obese and non-diabetic ZDF (+/+) rats were used as controls. The ZDF (+/+) rats remain normoglycaemic over time and do not display the metabolic and anatomic changes observed in the diabetic rats.

With the purpose to evaluate the therapeutic effect of sitagliptin as an anti-diabetic drug, in reverting/ameliorating some of the kidney lesions in diabetes, we treated diabetic ZDF rats with sitagliptin. It was shown that this oral anti-diabetic agent ameliorates the glycaemic control in T2DM patients and improves fasting and postprandial glycaemia by glucose-dependent insulin secretion (Ahren *et al.*, 2002; Kim *et al.*, 2005; Aschner *et al.*, 2006; Raz *et al.*, 2006; Nonaka *et al.*, 2007).

Body weight and glycaemia of diabetic (ZDF (*fa/fa*)) and non-diabetic (ZDF (+/+)) rats were monitored at the beginning (20 weeks old) and at the end (26 weeks old) of the study (Table 3). At 20 weeks, no significant differences in the body weight were observed between the groups. At the end of the study, 26 weeks of age, the diabetic animals presented a decrease of about 9% in their body weight (p<0.001 vs 20 weeks old ZDF (*fa/fa*), nevertheless the control animals ZDF (+/+) continued to gain weight. Treatment with sitagliptin stabilized the weight loss in the diabetic animals, preventing part of the body weight loss when compared with the ones without treatment (Table 3).

No differences in food intake were found within each group throughout the treatment (data not shown).

At the beginning of the study (20-weeks-old animals), the blood glucose levels of diabetic rats (304±17 mg/dl, p<0.001) were significantly higher when compared to their

age-matched controls (92±2 mg/dl). Hyperglycaemia was accompanied by a decline in insulin secretion as previously reported (Ferreira *et al.*, 2010). At the beginning of the study, insulin levels were already significant lower in diabetic animals (13.7±0.9, p<0.01) when compared to their age-matched controls (15.0±5.9), and at the final time, the diabetic ZDF (*fa/fa*) rats exhibit relative insulinopaenia, comparatively to non-diabetic ZDF (+/+) rats. After the oral treatment with sitagliptin, blood glucose levels of diabetic animals (486±19 mg/dl, p<0.01) decreased significantly when compared to non-treated diabetic animals (523±16 mg/dl), as well as the insulinopaenic profile of diabetic animals (7.6±1.5, p<0.001) were significantly prevented (10.6±1.8) (Table 3).

Table 3. Body weight and blood glucose levels throughout study in non-diabetic ZDF (+/+) and diabetic ZDF (*fa/fa*) rats non-treated and treated with 10 mg/kg/day sitagliptin, for 6 weeks.

	Cont		Diab		Diab+Sita
Age (weeks)	20	26	20	26	26
Weight (g)	407±7	446±8	388±9	354±9***	380±14
Blood glucose (mg/dl)	92±2	89±3	304±17***	523±16***	486±19##
Insulin (mU/L)	15.0±5.9	15.8±3.0	13.7±0.9**	7.6±1.5***	10.6±1.8###

Data are expressed as mean ± SEM (n ≥ 6 per group), *** p<0.001 and **p<0.01 significantly different from age-matched control, ## p<0.01 and ### p<0.001 significantly different from age-matched diabetic. ANOVA followed by Bonferroni's *post hoc* test.

The HbA1c levels were determined only at the end of the study, in 26-weeks-old animals (Figure 11). The diabetic group had significantly higher HbA1c levels (10.18±0.29%, p<0.001) than the control group (4.10±0.15%). Treatment with sitagliptin for 6 weeks in diabetic animals promoted a significant decrease of 1.2% (p<0.01) when compared with non-treated diabetic animals (Figure 11).
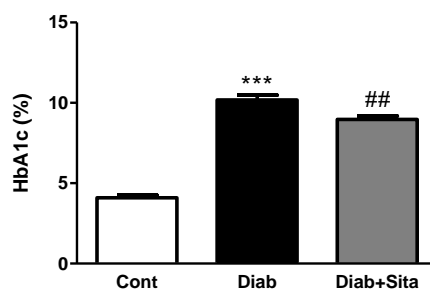
Figure 11. HbA1c levels in non-diabetic ZDF (+/+) and diabetic ZDF (*fa/fa*) rats non-treated and treated with 10 mg/kg/day sitagliptin for 6 weeks, at 26 weeks of age. Data are expressed as mean ± SEM (n ≥ 6 per group), *** p<0.001 significantly different from control, ## p<0.01 significantly different from diabetic, ANOVA followed by Bonferroni's *post hoc* test.

2. Sitagliptin decreases the protein levels of DPP-IV in the kidney of diabetic animals

CD26, the membrane form of DPP-IV enzyme, is widely distributed in numerous tissues, including the kidney. This enzyme is responsible for inactivating both GIP and GLP-1, inactivating the incretin effect (Mentlein *et al.*, 1993). Therefore, DPP-IV inhibition potentiates endogenous incretin action by reduction its degradation. In fact, studies with DPP-IV knockout mice showed an increase in GIP and GLP-1 levels, as well as an improvement in glucose tolerance and enhanced insulin secretion (Marguet *et al.*, 2000).

In order to investigate the effect of sitagliptin on DPP-IV/CD26, its protein levels and distribution in the kidney were evaluated by *Western Blottitng* and immunohistochemistry, respectively (Figure 12).

Diabetes led to increased DPP-IV levels (244.26±29.14% of control, p<0.001) (Figure 12A). Sitagliptin treatment in the diabetic rats prevented the increase in kidney DPP-IV (146.03±15.66%; p<0.01) protein levels, compared to diabetic animals without treatment (Figure 12A).

Immunohistochemistry experiments performed in kidney frozen sections confirmed these results (Figure 12B). Diabetes led to a significant increase in DPP-IV immunoreactivity both in the glomerulus and cells around it, probably tubular cells. No staining was observed when the negative control for DPP-IV was performed, showing that the signal obtained is specific of the primary antibody used. Treatment with sitagliptin markedly decreased the staining for DPP-IV in the kidney of diabetic rats (Figure 12B).
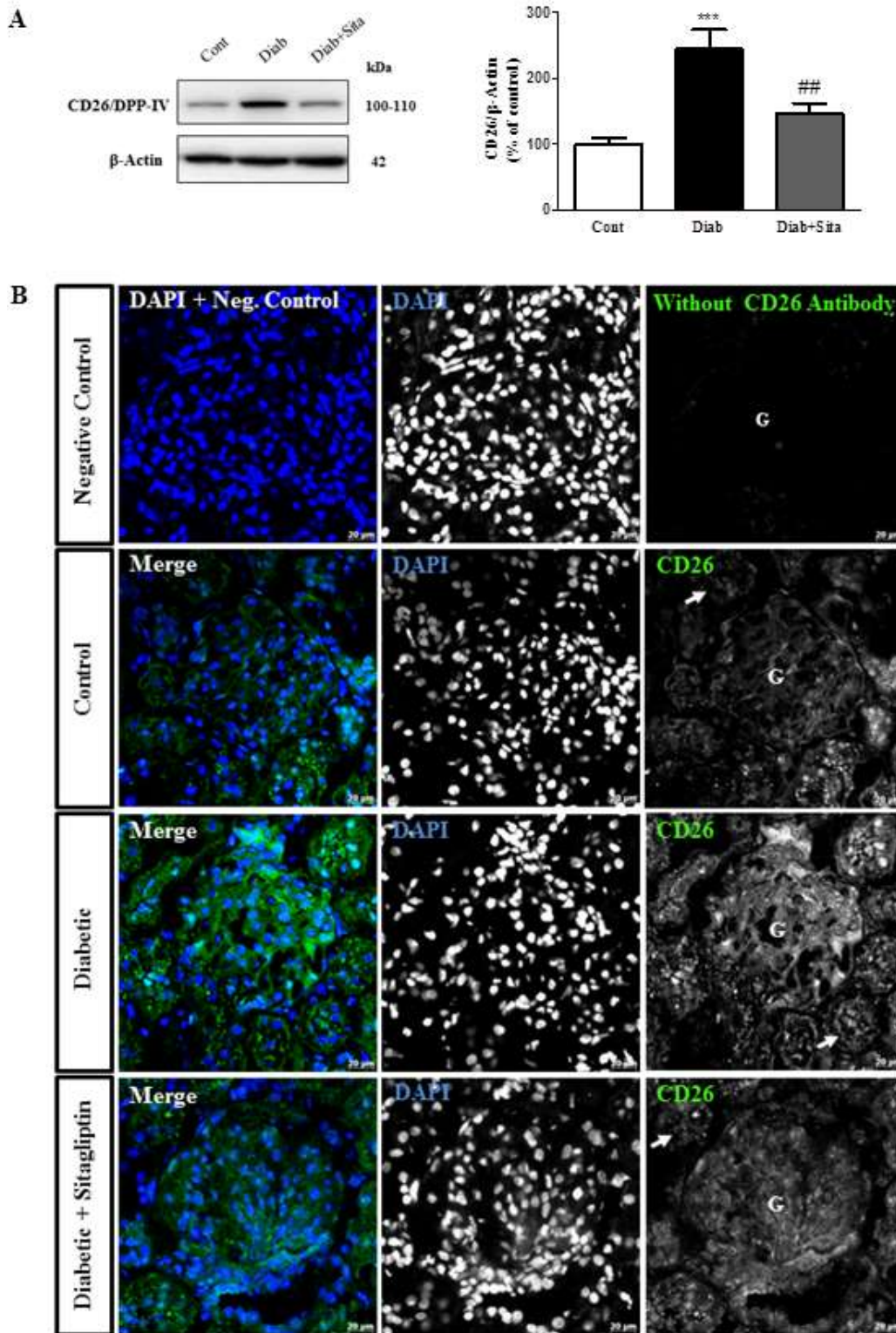
Figure 12. Sitagliptin prevents the upregulation of DPP-IV content in the kidney induced by diabetes.
(A) The protein levels of DPP-IV were assessed in total kidney cell lysates by *Western Blotting* in ZDF
(+/+) and ZDF (*fa/fa*) non-treated or treated with sitagliptin. The *Western Blots* presented are representative
of each group of animals. Data are expressed as percentage of control and represent the mean ± SEM (n ≥ 6
per group), *** $p<0.001$ significantly different from control, ## $p<0.01$ significantly different from
diabetic, ANOVA followed by Bonferroni's *post hoc* test. (B) Rpresentative confocal images for each
group of animals showing DPP-IV immunoreactivity (green) and nuclear staining with DAPI (blue) in
kidney sections. Magnification 400×. Legend: G - glomeruli, Arrows - tubular cells.

3. Sitagliptin modulates the incretin axis in the kidney of diabetic animals

In order to investigate the effect of DPP-IV inhibition in the incretin axis, the content of GLP-1 protein was determined in total kidney extract by *Western Blotting*, as well as the distribution of this protein and their respective receptor (GLP-1R) in frozen kidney sections by immunohistochemistry (Figure 13).

In diabetic animals, GLP-1 levels show a tendency to decrease (83.36±7.01%) compared to control group (100.00±5.78%), of about 16.64%. With sitagliptin administration, GLP-1 levels increased about 69.8% (159.74±28.39%, p<0.05) in diabetic animals, relatively to non-treated diabetic group (Figure 13A).

By immunohistochemistry, we also observed a decreased immunoreactivity for GLP-1 in the diabetic kidneys. Treatment with sitagliptin increased GLP- 1 staining in diabetic animals (Figure 13B). Contrary to expected, although GLP-1R immunoreactivity is decreased in the kidney in response to diabetes, treatment with sitagliptin even accentuates this decrease in the glomeruli of the diabetic kidneys (Figure 13B). Additionally, the analysis of GLP-1 and GLP-1R co-staining also show some co-localization of the two proteins, even in diabetic rats treated with sitagliptin (Figure 13C). This eventually suggests a possible binding of GLP-1 to its receptor, which can lead to activation of incretin downstream signaling from the receptor.
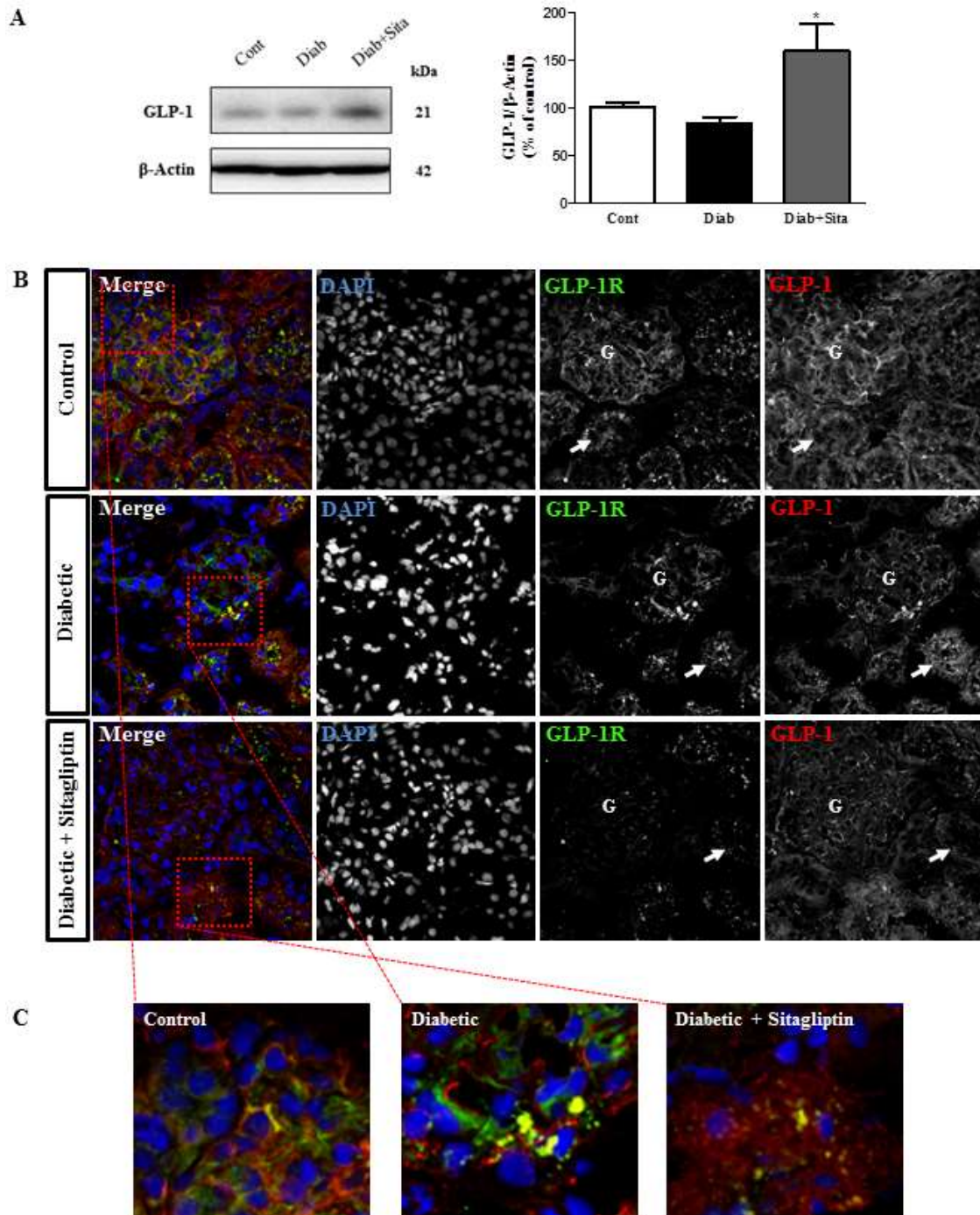
Figure 13. Effect of sitagliptin treatment in protein levels and distribution of GLP-1 and GLP-1R in the diabetic kidney. (A) The protein levels of GLP-1 were assessed in total kidney cell lysates by *Western Blotting* in ZDF (+/+) and ZDF (*fa/fa*) non-treated or treated with sitagliptin. The *Western Blots* presented are representative of each group of animals. Data are expressed as percentage of control and represent the mean ± SEM (n ≥ 6 per group), *p<0.05 significantly different from diabetic, ANOVA followed by Bonferroni's *post hoc* test. (B) Representative confocal images for each group of animals showing GLP-1R immunoreactivity (green), GLP-1 immunorectivity (red) and nuclear staining with DAPI (blue) in the kidney sections. (C) Magnification of co-localization areas (Yellow). Original magnification 400×. Legend: G - glomeruli, Arrows - tubular cells.

4. Sitagliptin decreases the Akt activity in the kidney of diabetic animals

Previous studies from our group revealed that the obese diabetic (ZDF, *fa/fa*) rats of 26 weeks of age had a significant ($p<0.001$) increase in glomerular basement membrane thickening and glomerular atrophy, which was accompanied by an intense expression of mesangial expansion and Bowman's capsule thickening ($p<0.01$), when compared with control animals (ZDF (+/+)) (Mega *et al.*, 2011). In the group of diabetic animals treated with sitagliptin, there was an amelioration of these lesions, when compared to non-treated diabetic animals. Since it has previously described that mesangial cells hypertrophy and ECM expansion is associated with Akt activation (Zdychova and Komers, 2004; Alique *et al.*, 2011; Kattla *et al.*, 2008), we next intended to evaluate whether the improvement mediated by sitagliptin in renal lesions is mediated by this signaling pathway. In this context, we evaluated the P-Akt levels in the kidney, by *Western Blotting* (Figure 14).

As expected, the diabetic animals showed a significant increase in P-Akt protein levels ($287.58\pm38.02\%$, $p<0.001$) relatively to control animals ($100.00\pm18.43\%$), and the administration of sitagliptin decreased the P-Akt of about 110% (to $177.51\pm20.79\%$, $p<0.05$) as compared to non-treated diabetic animals (Figure 14), showing a possible connection between Akt signaling and renal lesions evolution, and also suggesting that sitagliptin could prevent this lesions by modulation of Akt activity.
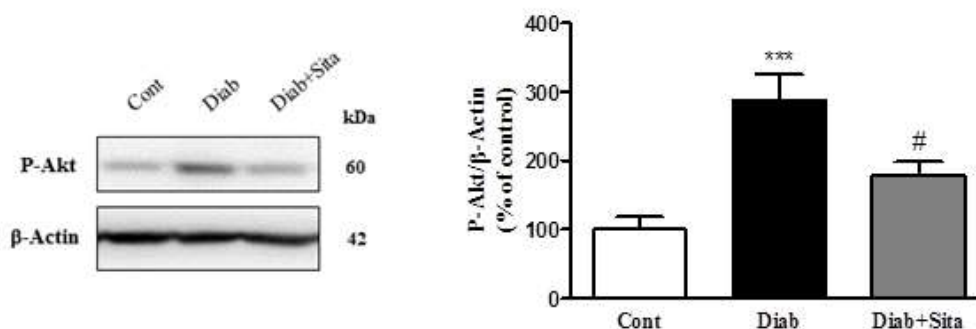
Figure 14. Effect of sitagliptin in P-Akt protein levels in the diabetic kidney. The protein levels of P-Akt were assessed in total kidney cell lysates by *Western Blotting* in ZDF (+/+) and ZDF (*fa/fa*) non-treated or treated with sitagliptin, in, total kidney cell lysates. The *Western Blots* presented are representative of each group of animals. Data are expressed as percentage of control and represent the mean ± SEM ($n \geq 6$ per group), ***$p<0.001$ significantly different from control, # $p<0.05$ significantly different from diabetic, ANOVA followed by Bonferroni's *post hoc* test.

5. Sitagliptin decreases the inflammatory state in the diabetic kidney

Several studies suggest that high glucose and lipid concentrations stimulate the production of proinflammatory cytokines (Donath *et al.*, 2008; Ferreira *et al.*, 2010). Moreover, it has been shown that the inflammatory state is relevant in the pathogenesis of DN (Saraheimo *et al.*, 2003; Dalla Vestra *et al.*, 2005) and improvement of renal lesions has been described as being associated with an amelioration of the inflammatory state (Ferreira *et al.*, 2010). Recent studies performed in our laboratory, in the same animal model, demonstrated that diabetes increased serum levels of IL-1β and TNF, and that sitagliptin administration significantly decreased the IL-1β levels (Ferreira *et al.*, 2010).

The proinflammatory cytokines IL-1β and TNF are thought to contribute to an inflammatory response in diabetic kidney (Hasegawa *et al*., 1995). Therefore, we performed a qualitative analysis to evaluate the cellular distribution for these proinflammatory cytokines in kidney frozen sections by immunohistochemistry (Figure 15).

Diabetes markedly increased the immunoreactivity of IL-1β and TNF in cells around the glomeruli that are probably tubular cells and/or accumulation of interstitial inflammatory cells. The administration of sitagliptin improves the protein levels of IL-1β and TNF in the diabetic kidney (Figure 15). Furthermore, the increase in the immunoreactivity for these cytokines in the kidney of diabetic rats may also be due to the recruitment and accumulation of interstitial inflammatory cells infiltrations, such as macrophages, monocytes and T cells (Chow *et al.*, 2004; Xiao *et al.*, 2009).
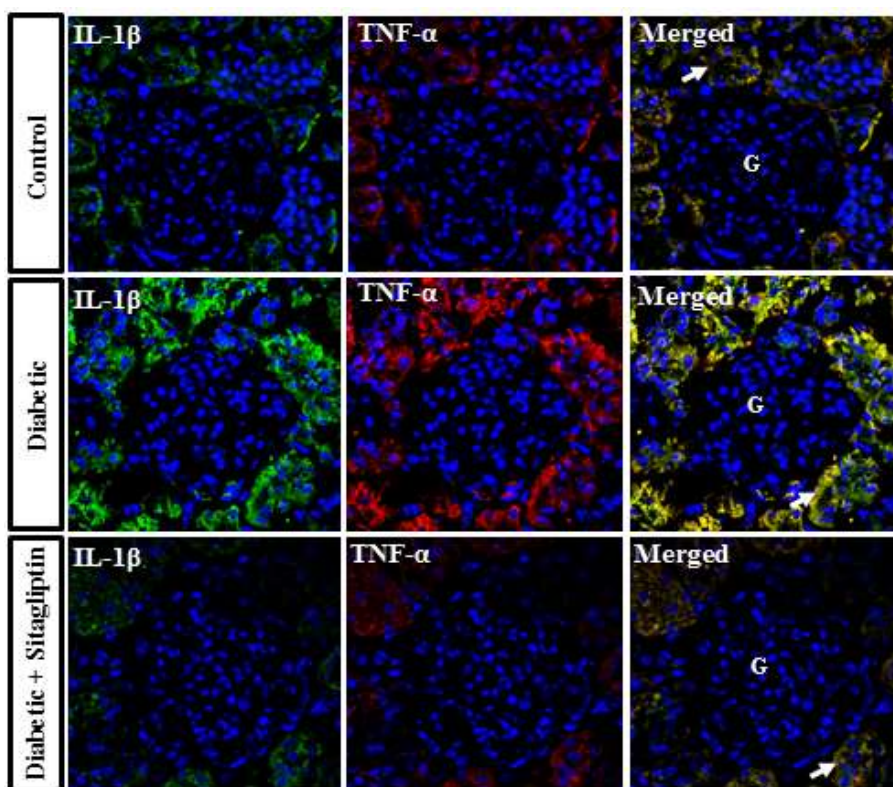
Figure 15. Sitagliptin decreases the proinflammatory cytokines IL-1β and TNF-α in the diabetic kidney. Representative confocal images for ZDF (+/+) and ZDF (*fa/fa*) non-treated or treated with sitagliptin, showing IL-1β immunoreactivity (green), TNF-α immunorectivity (red) and nuclear staining with DAPI (blue) in kidney sections. Magnification 400×. Legend: G - glomeruli, Arrows - tubular cells.

6. Effects of diabetes and sitagliptin treatment in ER stress response in the kidney

There are several reports in the literature implicating the ER stress in several pathologies, including diabetes, by being involved in pancreatic β-cells loss and insulin resistance (Araki *et al.*, 2003). Furthermore, altered metabolic conditions associated with diabetes can lead to UPR response, leading to apoptosis and inflammation (Kaneto *et al.*, 2006), which contribute for the development of diabetic complications, namely DN (Lindenmeyer *et al.*, 2008). Therefore, we want evaluate the protein levels of CHOP, since this transcription factor plays an important role in ER stress-mediated apoptosis (Nishitoh, 2012), whose induction strongly depends on ATF4, another transcription factor activated by PERK pathway. Furthermore, we also intended to evaluate how GRP78/BiP is regulated, which is an important ER chaperone involved in UPR activation.

As a first approach, we performed a quantitative analysis for CHOP protein by *Western Blotting*, in the kidney total extracts of non-diabetic ZDF (+/+) rats and diabetic ZDF (*fa/fa*) rats treated and non-treated with sitagliptin (Figure 16).

Contrary to the expected, the administration of sitagliptin significantly increase CHOP levels in the diabetic ZDF (*fa/fa*) rats (163.26±11.03%, p<0.001), comparatively to non-treated diabetic (107.27±5.47%) and non-diabetic animals (100.00±6.09%) (Figure 16A). These results were confirmed by immunohistochemistry analysis in the kidney, showing that, although diabetes induced a slightly increase in immunoreactivity for CHOP, the increase in the staining was more evident when diabetic rats were treated with sitagliptin (Figure 16B).
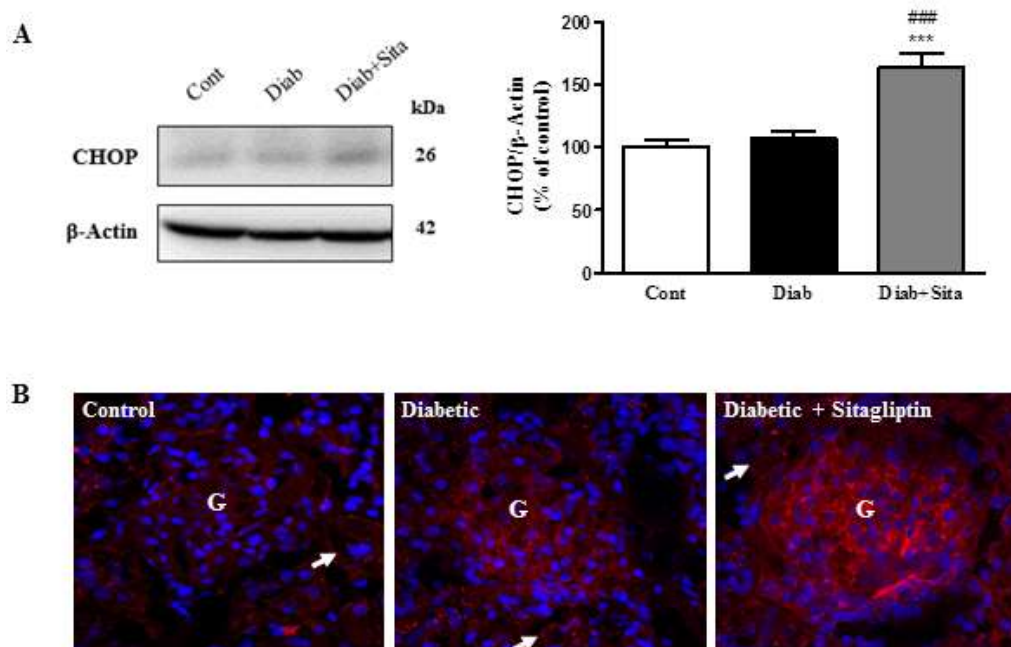
Figure 16. Effect of sitagliptin treatment in protein levels and distribution of CHOP in diabetic kidney. (A) The protein levels of CHOP were assessed in kidney total cell lysates by *Western Blotting*, in ZDF (+/+) and ZDF (*fa/fa*), non-treated or treated with sitagliptin, total kidney cell lysates. The *Western Blots* presented are representative of each group of animals. Data are expressed as percentage of control and represent the mean ± SEM (n ≥ 6 per group), ***p<0.001 significantly different from control, ### p<0.001 significantly different from diabetic, ANOVA followed by Bonferroni's *post hoc* test. (B) Representative confocal images for each group of animals showing CHOP immunoreactivity (red) and nuclear staining with DAPI (blue) in the kidney sections. Magnification 400×. Legend: G - glomeruli, Arrows - tubular cells.

Since we did not observe significant changes in total CHOP protein levels in response to diabetes, we hypothesized that this protein, which is a transcription factor involved in the ER stress response, could be redistributed in the cell, increasing its content in the nuclear fraction. In order to test this hypothesis, we performed a subcellular

fractionation of the kidney and evaluated the levels of CHOP and another ER stress transcription factor, ATF4, in nuclear and cytoplasmic fractions in the kidney of the three groups of animals.

Preliminary results seemed to indicate that there was a subcellular redistribution of CHOP in response to diabetes (Figure 17). There was an increase in CHOP protein levels in the nuclear fraction of diabetic kidney with a paralleled decrease in its content in the cytosolic fraction (Figure 17). We also observed similar results for another transcription factor associated with the ER stress, ATF4 (Figure 17). Although there was an increase in the total amount of CHOP protein in diabetic rats treated with sitagliptin, results of cell fractionation indicated that CHOP, and also ATF4, seemed to accumulate preferentially in the cytosolic fraction (Figure 17).
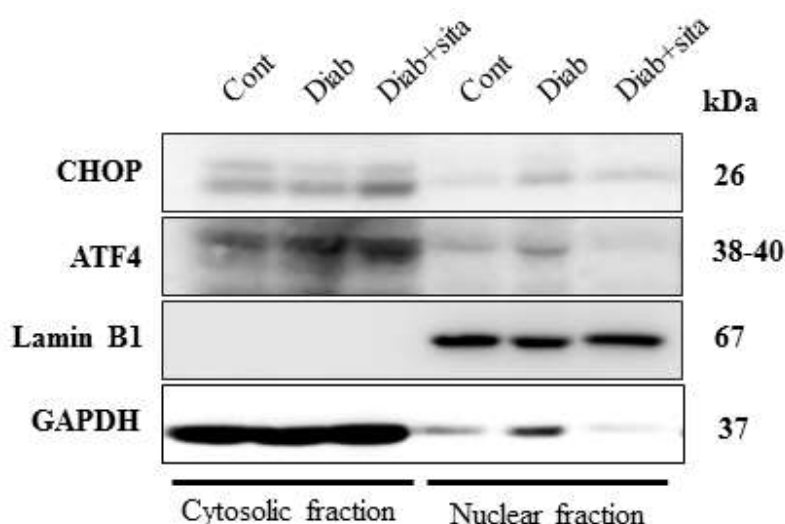
Figure 17. Effect of diabetes and sitagliptin in intracellular distribution of CHOP and ATF4 in the kidney. The protein levels of CHOP and ATF4 were assessed in subcellular fractions of the kidney by *Western Blotting* in ZDF (+/+) and ZDF (*fa/fa*) non-treated or treated with sitagliptin. The *Western Blots* presented are representative of each group of animals. Preliminary data (n = 1 per group).

GRP78/BiP is essential for UPR signaling cascade activation, through activation of the three ER stress sensors, namely IRE1α, PERK and ATF6, and is the central regulator of ER function and homeostasis (Schröder and Kaufman, 2005; Ni and Lee, 2007). There has been described that during ER stress response, the protein levels of GRP78/BiP increase, since the UPR is associated with an up-regulation of ER chaperone proteins, such as GRP78/BiP (Kozutsumi *et al.*, 1988).

In an attempt to correlate the observed protein levels in kidney for CHOP with the UPR signaling cascade activation, a *Western Blotting* analysis to evaluate the protein

levels of GRP78/BiP was performed. However, no significant differences were observed between groups (Figure 18).

Figure 18. Effect sitagliptin treatment in protein levels of GRP78/BiP in the diabetic kidney. (A) The protein levels of GRP78/BiP were assessed in kidney total cell lysates by *Western Blotting* in ZDF (+/+) and ZDF (*fa/fa*) non-treated or treated with sitagliptin. The *Western Blots* presented are representative of each group of animals. Data are expressed as percentage of control and represent the mean ± SEM (n ≥ 6 per group), ANOVA followed by Bonferroni's *post hoc* test.

7. Sitagliptin protects the diabetic kidney against apoptotic cell death induced by diabetes

The progressive decline in renal function is associated with renal cells loss, such as glomerular and tubular cells. Therefore, in order to evaluate the apoptotic state in the diabetic kidney, the content of the pro-apoptotic proteins BAX and Bid, as well as the anti-apoptotic protein Bcl-2 was determined.

It is well established that the ratio between BAX and Bcl-2 determines the response to a cell death signal, being considered an indicator for the activation of apoptosis (Xiang *et al.*, 1996). The levels and subcellular distribution of pro-apoptotic protein BAX were determined by *Western Blotting* and immunohistochemistry, and the content of anti-apoptotic protein Bcl-2 was determined by *Western Blotting*, in total kidney of ZDF (+/+) and ZDF (*fa/fa*) rats.

The results exhibited in Figure 19 show a significant increase ($p < 0.01$) in BAX/Bcl-2 ratio in diabetic animals when compared to control group. The administration of sitagliptin improves ($p < 0.05$) the pro-apoptotic state induced by diabetes (Figure 19A). A significant increase in immunoreactivity for the pro-apoptotic protein BAX in the kidney of diabetic ZDF (*fa/*fa) rats was observed, and there seems to be a subcellular redistribution of protein, which decreases with sitagliptin treatment, mainly in glomerulus (Figure 19B).
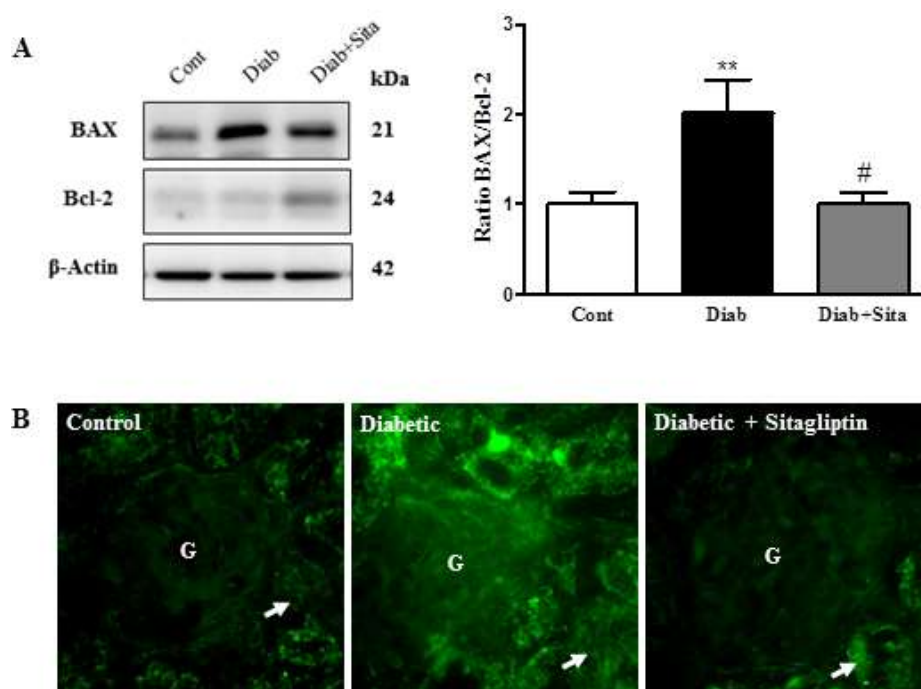
Figure 19. Effect of sitagliptin treatment in protein levels of BAX and Bcl-2 and subcellular distribution of BAX in the diabetic kidney. (A) The protein levels of BAX and Bcl-2 were assessed in kidney total cell lysates by *Western Blotting* in ZDF (+/+) and ZDF (*fa/fa*) non-treated or treated with sitagliptin. The *Western Blots* presented are representative of each group of animals. Data are expressed as the mean ± SEM (n ≥ 6 per group), **p<0.01 significantly different from control, # p<0.05 significantly different from diabetic, ANOVA followed by Bonferroni's *post hoc* test. (B) Representative confocal images for each group of animals showing BAX immunoreactivity (green) in the kidney sections. Magnification 400×. Legend: G - glomeruli, Arrows - tubular cells.

During apoptosis, an important pathway leading to caspase activation involves the release of cytochrome c from the intermembrane space of mitochondria, by permeabilization of the outer mitochondrial membrane, and it has been described that Bid protein, a pro-apoptotic member of the Bcl-2 family, induces the release of cytochrome c, leading to cell apoptosis (Kluck *et al.*, 1999).

By *Western Blotting* analysis, it was observed that diabetes also induces a significant increase in Bid levels (173.57±22.64%, p<0.01), as observed for BAX, of about 73.57% comparatively to non-diabetic ZDF (+/+) rats (100.00±15.33%). Treatment with sitagliptin decreased the levels of Bid in diabetic animals by about 60.55% (113.02±9.43%), relatively to non-treated animals (p<0.05) (Figure 20).
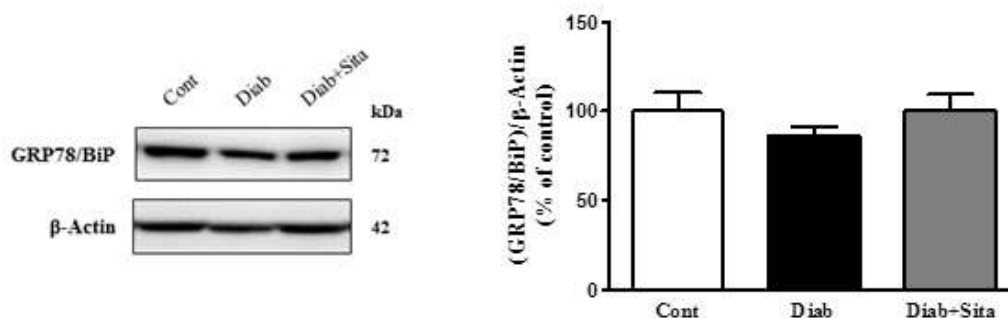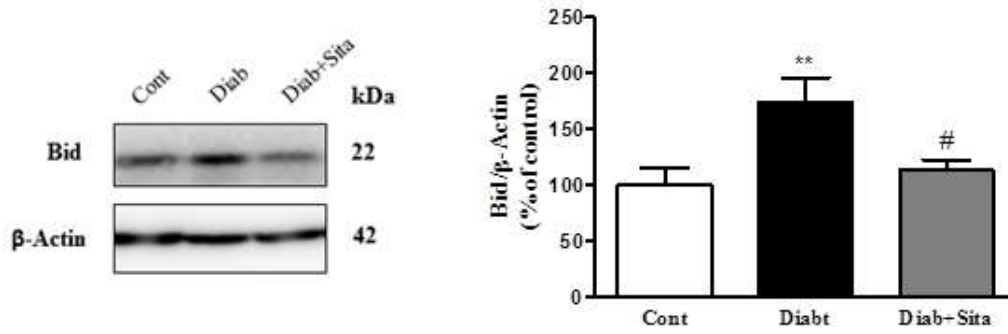
Figure 20. Effect of sitagliptin in protein levels of Bid in the diabetic kidney. The protein levels of Bid were assessed in kidney total cell lysates by *Western Blotting* in ZDF (+/+) and ZDF (*fa/fa*) non- treated or treated with sitagliptin. The *Western Blots* presented are representative of each group of animals. Data are expressed as percentage of control and represent the mean ± SEM (n ≥ 6 per group), **p<0.01 significantly different from control, # p<0.05 significantly different from diabetic, ANOVA followed by Bonferroni's *post hoc* test.

CHAPTER 4

DISCUSSION

Discussion

Diabetic nephropathy (DN) is one of the major microvascular complications of diabetes and it is currently the leading cause of ESRD (Dronavalli *et al.*, 2008; Kanwar *et al.*, 2008), conferring high morbidity and mortality rates of diabetic patients. About 20-40% of patients with diabetes develop evidence of nephropathy (Dronavalli *et al.,* 2008), and due to the increase of global prevalence of DM in recent years (IDF, 2012), it becomes imperative to implement effective therapeutic strategies for diabetes treatment. Given that a good glycaemic control reduces the risk for the development of diabetic complications, the search of new glucose-lowering agents for treatment for T2DM is particularly highly regarded.

GLP-1 is responsible for most of the incretin effect, which in non-diabetic individuals is a physiological action. However, in T2DM patients the incretin effect is blunted "incretin defect". The "incretin defect", a metabolic deterioration associated with T2DM was demonstrated by Nauck *et al.*, (1986). In their study, oral and intravenous glucose caused similar changes in plasma glucose concentration in subjects with T2DM. In healthy individuals, insulin secretory response after oral glucose ingestion exceeded the response elicited by intravenous administration of an equal amount of glucose. So, in non-diabetic individuals, there was a difference between the response to an intravenous and an oral glucose administration due to the "incretin effect". For individuals with T2DM, this effect was diminished after oral administration of glucose and the plasma glucose values obtained by oral and intravenous administration were similar. Taking into consideration that in diabetes there is an increased activity of DPP-IV (enzyme responsible for incretin degradation) and consequently, a remarkably decrease of incretin action, the use of incretin mimetics, such as GLP-1 analogues, or incretin enhancers, such as DPP-IV inhibitors, would cope with the deficit in incretin system. Sitagliptin is a selective DPP-IV inhibitor for treatment of patients with T2DM, improving fasting and postprandial glycaemia and deceasing HbA1c levels (Herman *et al.*, 2007). Sitagliptin is a well-tolerated oral anti-diabetic agent, neutral concerning changes of weight with low propensity to cause hypoglycaemia (Drucker and Nauck, 2006). However, the sitagliptin efficacy in restoring kidney function, in conditions of chronic hyperglycaemia, in terms of improvement of inflammatory state and cell death, as well as in modulation of ER stress response, either by decreasing blood glucose levels or by extrapancreatic action of incretins in an independent manner of increased insulin secretion, remains unclear.

Our group has recently shown that the DPP-IV inhibitor sitagliptin has a positive impact on the retinal barrier and prevents inflammatory and pro-apoptotic states triggered by diabetes in the retina (Gonçalves *et al.*, 2011). Other authors have already suggested cytoprotective effects of sitagliptin on other tissues and conditions, including on cardiovascular disorders as well as on kidney disease (Read *et al.*, 2010; Vaghasiya *et al.*, 2010; Mega *et al.*, 2011; Matsubara *et al.*, 2013; Apaijai *et al.*, 2013). This study aims to determine the putative benefits of sitagliptin treatment in the diabetic kidney, and the underlined mechanisms, using the *Zucker Diabetic Fatty* (ZDF) rats as T2DM model, the same diabetic animal model that was used in the work described before.

DN in human patients is characterized by renal hypertrophy, hyperfiltration, proteinuria and progressive glomerulosclerosis (Kanwar *et al.*, 2008). Accordingly to previous studies, ZDF (*fa/fa*) rats show a significant renal hypertrophy at 20 weeks of age, as well as loss of kidney function (Mega *et al.*, 2011), thus, this animal model seems to be useful for evaluation of the effects of sitagliptin treatment in the diabetic kidney and their benefits in the prevention or reduction of some of the lesions present in DN.

Our results show that the chronic treatment with sitagliptin provided a significant decrease of insulin levels, plasma glucose and HbA1c, and also prevent the weight loss, characteristic of a more advanced stage of T2DM, which confirms the results obtained in studies performed in humans (Ahren *et al.*, 2002; Kim *et al.*, 2005; Aschner *et al.*, 2006; Raz *et al.*, 2006; Nonaka *et al.*, 2007) and rats (Mega *et al.*, 2011).

In an attempt to clarify the hypothesis that sitagliptin improves the glycaemic control by inhibition of DPP-IV, leading to enhance of incretins effect, we started to evaluate the DPP-IV protein levels and the subcellular distribution in the kidney sections, of non-diabetic ZDF(+/+) rats, and treated and untreated diabetic ZDF(*fa/fa*) rats. DPP-IV is widely distributed in numerous tissues and cells, and its enzymatic activity is exhibited in both membrane-anchored cell-surface peptidase and as a smaller soluble form in blood plasma. In this study, DPP-IV protein levels were assessed in the kidney tissue, through the membrane form, also called CD26. To confirm that DPP-IV inhibition leads to accumulation of GLP-1, we also determine the content of GLP-1 in the kidney, since DPP-IV degrades this incretin hormone.

Our results show that diabetes induces an increase in kidney DPP-IV protein levels, when compared to non-diabetic animals. Accordingly to our results, it was reported in a streptozotocin (STZ)-induced diabetic model that STZ enhanced the expression of *Dpp-IV* mRNA in the kidney tissue (Kirino *et al.*, 2009). There are also

some reports suggesting that microvascular endothelial cells are the main sources of endogenous DPP-IV (Matheeussen *et al.*, 2011). In fact, in an *in vitro* study, both *Dpp-IV* mRNA expression and DPP-IV activity were enhanced by exposure of human glomerular endothelial cells to high glucose (Pala *et al.*, 2003). Furthermore, work performed by Kirino *et al.* (2009) also confirms that STZ-induced diabetes enhances the enzyme activity of circulating DPP-IV. These findings suggest an increase in the plasma DPP-IV activity trough the enhancement of DPP-IV expression induced by high blood glucose. So, although DPP-IV enzyme activity was not evaluated in this study, we can speculate that in diabetic ZDF (*fa/fa*) rats the circulating DPP-IV inhibitory activity might be enhanced, since DPP-IV protein levels were higher than in non-diabetic ZDF (+/+) rats. To prove this hypothesis, we evaluate protein levels and subcellular distribution of GLP-1. The GLP-1 protein levels in diabetic ZDF (*fa/fa*) rats, although with no significant differences from non-diabetic animals, seem that trend to decrease. Kirino *et al.* (2009) study reports a decrease in DPP-IV enzyme activity in the kidney of STZ-induced diabetes, despite increased *Dpp-IV* mRNA expression. This could be explained by the fact that hyperglycaemia-induced renal endothelial cell damage could cause the leakage of microvascular endothelial DPP-IV enzyme of the kidney into the circulation, as suggested by Kirino *et al.* (2009) and other previous reports (Augustyns *et al.*, 1999; Pala *et al.*, 2003). This release of DPP-IV from the kidney into the circulation could contribute to the increase in the circulating DPP-IV enzyme activity, with a paralleled reduction in the kidney tissue. Therefore, this decrease in DPP-IV enzyme activity in the kidney could explain why there are any significant differences in GLP-1 protein levels.

Sitagliptin treatment promoted a significant decrease in DPP-IV protein levels in the diabetic kidney. Given that high glucose levels enhance *Dpp-IV* mRNA expression (Pala *et al.*, 2003), the decrease of DPP-IV levels in the kidney could be related to the sitagliptin-induced decrease in blood glucose levels, through enhancement of incretins insulinotropic effect.

To prove DPP-IV inhibition by sitagliptin we evaluated indirectly DPP-IV enzymatic activity via evaluation of GLP-1 protein levels. As expected, the administration of sitagliptin markedly increases the GLP-1 protein levels in the kidney of diabetic ZDF (*fa/fa*) rats. This could confirm the inhibitory efficacy of DPP-IV induced by sitagliptin, but to establish the relation between GLP-1 degradation to DPP-IV more studies have to be performed, which could be determine with *glp-1* mRNA analysis.

Additionally, our results show that diabetes seems to decrease GLP-1R protein levels in the kidney, accordingly to other reports (Mima *et al.*, 2012). But with administration of sitagliptin the immunoreactivity for GLP-1R in the kidney markedly decreases, which are not in agreement with other reports (Liu *et al.*, 2011). The decreased GLP-1R immunoreactivity could be related to a negative feedback. The markedly increase in GLP-1 protein levels in the diabetic kidney following sitagliptin administration could lead to a decrease in GLP-1R expression and/or degradation, in order to maintain cell homeostasis.

As previously referred, DN is a progressive renal disease, characterized by functional and structural abnormalities in the kidney. The morphological changes of the kidney include glomerular and tubular epithelial hypertrophy, thickening of GBM and expansion of mesangial ECM (Adler, 1994; Chawla *et al.*, 2010). There has been described that Akt signaling pathway has an emerging role in the development of renal lesions, such as mesangial hypertrophy and ECM composition (Zdychova and Komers, 2004; Alique *et al.*, 2011; Kattla *et al.*, 2008).

Akt is a serine/threonine kinase that regulates a variety of cellular processes, including survival, proliferation, protein translation and metabolism (Manning and Cantley, 2007). Akt can be activated in response to insulin or a wide variety of growth stimuli, such as growth factors and insulin, and represent an important intermediate in insulin signaling. Akt activation typically occurs via tyrosine kinase receptors in a phosphatidylinositol 3-kinase (PI3K)-dependent manner (Downward, 1998). The activation of PI3K stimulates the production of phosphatidyl-inositol 3-phosphate (PI3P), which binds to pleckstrin homology (PH) domain of Akt triggering its translocation to the plasma membrane (Franke *et al.*, 1997). Once in correct position in the membrane, Akt can be phosphorylated by 3-phosphoinositide dependent protein kinases (PDKs) at threonine 308 (Thr308) and serine 473 (Ser473) residues (Alessi *et al.*, 1997; Bellacosa *et al.*, 1998). Then, activated (phosphorylated) Akt becomes available for phosphorylation of its downstream targets.

In this study, we observed that P-Akt protein levels significantly increase in the diabetic kidney, in agreement with previous reports (Feliers *et al.*, 2001; Zdychova *et al.*, 2008), suggesting that PI3K/Akt signaling pathway is involved in ECM accumulation and mesangial cells hypertrophy. Being a growth factor, TGF-β1 could activate the PI3K/Akt pathway in various cells, including the kidney. Therefore, the increased in P-Akt protein

levels in DN could be explained by TGF-β1 (Kattla *et al.*, 2008), which is overexpressed in diabetic kidney (Yamamoto *et al.,* 1993). But further studies should address if this mechanism is involved in the data obtained by us.

Previous studies of our group have showed that obese diabetic (ZDF, *fa/fa*) rats develop kidney lesions characterized by increased glomerular basement membrane thickening and glomerular atrophy, accompanied by an intense expression of mesangial expansion and Bowman's capsule thickening (Mega *et al.*, 2011). These lesions showed a trend to improvement in the sitagliptin-treated diabetic animals, when compared to non-treated animals. Therefore, mesangial expansion showed a 37.5% reduction in the most severe grade, glomerular atrophy and glomerular basement membrane presented a 25% and 12.5% reduction, respectively (Mega *et al.*, 2011). Our data demonstrate that sitagliptin-evoked amelioration in renal lesions was accompanied by a decrease of P-Akt levels. Together, these results suggest that the PI3K/Akt signaling pathway might be deeply involved in the progression of renal lesions in the diabetic kidney, and that sitagliptin treatment improved these lesions by mechanisms related to the PI3K/Akt pathway.

Some mechanisms have been proposed to be implicated in Akt-induced renal lesions in DN, such as the Akt/mTOR pathway (Zdychova et al., 2008; Nagai *et al.,* 2005). Mammalian target of rapamycin (mTOR) is one of the down-stream targets of Akt, which directly phosphorylates mTOR; in addition, activated mTOR is involved in the regulation of protein synthesis, promoting cell growth (i.e. an increase in cell mass) (Scott et al.1998). Therefore, several studies show that Akt/mTOR pathway is implicated in mesangial cell hypertrophy (Zdychova et al., 2008; Nagai *et al.,* 2005).

Few is Known about the mechanisms implicated in the putative renoprotective effects of sitagliptin in DN. Our results show that the improvement of renal lesions could be related with the sitagliptin-induced decrease in activated Akt, as well as with the improvement of inflammatory state and cell death by apoptosis, which will be discussed.

Accumulating evidences also points to the critical role of the inflammatory state in the development of diabetic complications, suggesting that microvascular inflammation is a common mechanism in the pathogenesis of DN (Saraheimo *et al.*, 2003; Dalla Vestra *et al.*, 2005). Therefore, there is an increased expression of proinflammatory cytokines in the diabetic kidney, such as IL-1β and TNF (Navarro *et al.*, 2006), leading to enhanced

vascular endothelial permeability, oxidative stress, renal hypertrophy and tubulointerstitial lesions (Lim and Tesch, 2012).

Recently, it has been reported that in the kidney of ZDF (*fa/fa*) rats the expression of VCAM-1 increases with concomitant infiltration of white blood cells, as well as enhanced production of inflammatory cytokines, such as TNF and IL-1β, leading to renal cells injury (Wang *et al.*, 2012). Our study also shows an increase in proinflammatory cytokines, IL-1β and TNF, in the diabetic kidney, mainly in cells around the glomeruli, probably tubular cells and/or accumulation of interstitial inflammatory cells. In the group of diabetic rats under sitagliptin therapy, an accentuated decrease in these proinflammatory cytokines was found. These results, obtained by immunohistochemistry in the kidney sections, seem to be in agreement with results obtained in our group, which report a decreased of IL-1β and TNF serum levels in ZDF (*fa/fa*) rats treated with sitagliptin (Ferreira *et al.*, 2010). Together, these results demonstrate that a chronic sitagliptin treatment corrected the inflammatory state in diabetic kidney, as well as in other diabetic microvascular complications, such as diabetic retinopathy, which was also described by our group (Gonçalves *et al.*, 2011). Moreover, several studies have shown that the decrease in inflammation promotes an amelioration of renal injuries (Yozai *et al.*, 2005; Tone *et al.*, 2005; Wu *et al.*, 2006; Mega *et al.*, 2011).

Inflammatory cells, such as macrophages, lymphocytes and monocytes (Lim and Tesch, 2012), are often found in tubular compartment (Mezzano *et al.*, 2003). In fact, there are reports of infiltration of mononuclear cells in the kidney of patients with DN (Furuta *et al.*, 1993), showing that interstitial inflammatory cells infiltrates are associated with progression of renal injuries in DN (Chow *et al.*, 2004; Ninichuk *et al.*, 2007). MCP-1 played a key role in promoting recruitment and infiltration of macrophage in the diabetic kidney (Tesch *et al.*, 2008), and there has been described that hyperglycaemia increases expression of MCP-1 in tubular cells of the diabetic kidney (Mezzano *et al.*, 2003; Chow *et al.*, 2006). The proinflammatory transcription factor NF-κB was also detected mainly in tubular cells of human and rat kidney, with T2DM and overt nephropathy (Morcos *et al.*, 2002). Furthermore, the NF-κB regulates the gene expression of several molecules involved in inflammation, which includes MCP-1, IL-1β and TNF (Guijarro and Egido, 2001). Based on these evidences, the NF-κB and MCP-1 increased expression in tubular cells of diabetic kidney can be a plausible explanation for our results, but to clarify this hypothesis further studies are required.

ER stress has been suggested as one of the underlying mechanism in the pathogenesis of several diseases, namely atherosclerosis, and neurodegenerative and metabolic diseases, such as diabetes (Malhotra and Kaufman, 2007). Some studies have reported that ER stress-mediated apoptosis is implicated in renal injury onset in diabetes (Liu *et al.*, 2008; Lim *et al.*, 2010). Given that CHOP protein plays an important role in pro-apoptotic pathway of UPR (Nishitoh, 2012), we intended to evaluate the possible association between renal cells apoptosis and ER stress response, by studying CHOP-mediated apoptosis. It has been described that one of the mechanisms involved in CHOP-mediated apoptosis involves increased BAX/Bcl-2 ratio, with down-regulation of Bcl-2 expression, and translocation of BAX to the mitochondria (McCullough *et al.*, 2000; Gotoh *et al.*, 2004). Therefore, as a first approach we determined the BAX/Bcl-2 ratio in the diabetic kidney, whose results demonstrated that diabetes significantly increases this ratio, supporting the hypothesis that diabetes-induced activation of CHOP-mediated apoptosis in the kidney could be implicated in DN progression. However, it is important to note that ER stress-mediated apoptosis could also be related with JNK-mediated and caspase-mediated pathways (reviewed in Inagi, 2009).

ER stress is also implicated in inflammatory state in the kidney, which is proved by the fact that ER stress attenuation decreases the expression of proinflammatory cytokines in diabetic kidney (Qi *et al*., 2010). The signaling pathways in the UPR and inflammation are interconnected through diverse mechanism, including the production of ROS and activation of NF-κB and JNK (Zhang and Kaufman, 2008). Therefore, the increase in the proinflammatory transcription factor NF-κB levels in the kidney, as described above (Morcos *et al.*, 2002), could be linked to ER stress response. Moreover, the increase in the proinflammatory cytokines TNF and IL-1β protein levels in the diabetic kidney could be related to UPR activation. However, in order to clarify this issue further studies are required. Additionally, CHOP has been suggested to be a key molecule, not only in apoptosis, but also in inflammatory response, since UPR-induced CHOP expression is implicated in the processing of pro-IL-1β (Endo *et al.*, 2006).

Given all these findings, we attempted to investigate relationship between apoptosis and inflammation in the diabetic kidney, with the UPR activation, through analysis of CHOP protein levels. Contrary to the expected, there were not any significant differences in the protein levels of CHOP in the total protein extracts of diabetic kidney, comparatively to non-diabetic animals. It is well known that the switch of pro-survival to

pro-apoptotic ER stress response is dependent of the duration and degree of ER stress. In fact, Rutkowski *et al.* (2006) reported in a cell culture system that a chronic exposure to a mild stress can lead to an adaptive ER stress response, as a consequence of the intrinsic instabilities of mRNAs and proteins that promote apoptosis, compared to those that facilitate protein folding and adaptation. Furthermore, this study demonstrates in this cell system that the up-regulation peaked of CHOP expression is after approximately 16h of treatment with stress-inducers (e.g. tunicamycin), and was diminished by 24h. Therefore, we cannot exclude the hypothesis that, in our work, the up-regulation peak of CHOP in the diabetic kidney happened before the end of the study (at 26 weeks of age). In addition, it is also possible that, at this age, CHOP expression in kidney cells is suppressed or attenuated, by degradation of CHOP mRNA and/or CHOP protein (Rutkowski *et al.*, 2006). Although no differences were shown in total protein levels, preliminary data seems to indicate that diabetes favors both CHOP and ATF4 translocation to the nucleus. Despite there is no up-regulation of CHOP expression at this age, this seems to indicate that translocation of this transcription factor to the nucleus may be related to the up-regulation of transcription of pro-apoptotic factors and down-regulation of transcription of anti-apoptotic factors.

Usually, in stress conditions, the GRP78/BiP is up-regulated, due to UPR activation. However, in agreement with the results for CHOP protein levels, the ER chaperone GRP78/BiP did not present significant differences in the kidney of diabetic and non-diabetic animals. GRP78/BiP is the best characterized ER chaperone protein. It is a central regulator of ER function due to its roles in folding and assembly of newly-synthesized proteins, regulating calcium homeostasis and serves as a sensor for ER stress, as well as targeting misfolded protein for proteasome degradation (Lee, 2001). The result for GRP78/BiP protein levels could be related to be related to autophagy, as well as with activation of ERAD, which can lead to an adaptive ER stress response, as said before. ERAD is a process by which misfolded ER proteins are detected, preventing them from processing along secretory pathway, and then performing their retro-translocation to cytosol where they undergo ubiquitin- and proteasome-dependent degradation (Meusser *et al.*, 2005). Autophagy is a cellular process involving the degradation of damage organelles/proteins through the lysossomal machinery, allowing the maintenance of homeostasis by helping the balance between the synthesis, degradation and subsequent recycling of macromolecules and organelles. Therefore, these processes could alleviate the misfolded protein accumulation in the ER lumen leading to amelioration in ER stress,

and so activation of the proximal sensors of ER stress becomes suppressed, as well as GRP78/BiP expression, which could explain the results obtained for GRP78/BiP protein levels in the diabetic kidney. In fact, a recent study has shown that ER stress induces autophagy in tubular cells, as a protection against cell death (Pallet *et al.*, 2008). But further studies are needed to elucidate the role of autophagy associated with ER stress in this animal model, and if autophagy is cytoprotective response to ER stress by removing the overload of unfolded or misfolded protein that exceeds the ER capacity.

Interestingly, the administration of sitagliptin significantly increases CHOP protein levels in the kidney of diabetic ZDF (*fa/fa*) rats. In fact, activation of GLP-1R improved β-cells survival upon induction of ER stress by a mechanism involving increase of ATF4/CHOP expression has been previously reported (Yusta *et al.*, 2006). This result confirms that the enhancement of incretin effect increases cellular defense mechanism, decreasing the pro-apoptotic state, besides the effect in insulin secretion. Our preliminary data of total CHOP protein levels and subcellular fractionation of CHOP and ATF4 proteins, determined by *Western Blotting*, go against this hypothesis. Although sitagliptin administration significantly increased the CHOP protein levels in the kidney of diabetic ZDF (*fa/fa*), the translocation of CHOP and ATF4 to the nucleus decreases. Therefore, the expression of pro-apoptotic factors decreases, accompanied with improvement in cell death by apoptosis, which is consistent with our results. Thereby, we can hypothesize that GLP-1R activation or direct GLP-1 effects, can block CHOP and ATF4 translocation, but to prove that more studies are required.

Based on the study performed by Yusta *et al.* (2006), the up-regulation of CHOP protein that we observed in the kidney of diabetic animals treated with sitagliptin, could be related with the activation of GLP-1R. The GLP-1R activation leads to induction of the transcriptional activator ATF4 in a cAMP- and PKA-dependent manner (Yusta *et al.*, 2006). This will also enhance the expression of its downstream targets, such as CHOP, as well as GADD34, that would lead to eIF2α dephosphorylation and a more rapid recovery of global protein synthesis. However, further experiments have to be performed to prove this hypothesis or determined the mechanism that underlies this fact. But it is noteworthy that this coupling of GLP-1R signaling pathways to modulate ER stress response could represent a promising therapeutic strategy.

CHAPTER 5

CONCLUSION

Conclusion

In the present study, we confirm the deleterious effects of diabetes in the kidney, resulting in cell death by apoptosis, increase inflammation and progression of kidney injuries, namely ECM expansion and mesangial hypertrophy. These are accompanied by increased DPP-IV protein levels. Administration of sitagliptin restored DPP-IV levels towards those in the control group and markedly increased the GLP-1 protein levels in the diabetic kidney.

The sitagliptin treatment was able to ameliorate nephropathy induced by diabetes in ZDF (*fa/fa*) rats, a T2DM model, as shown by significant reduction in inflammatory state and cell death by apoptosis. Sitagliptin seems also to be implicated in ER stress modulation.

In conclusion, it is plausible that the incretin modulator sitagliptin could be used in the prevention/reversion of diabetic nephropathy progression, as well as in the management of T2DM and its complications. However, further studies are needed to better understand the molecular and cellular mechanisms involved in the direct actions mediated sitagliptin in the kidney, which may contribute to the development of new therapeutic strategies more effective and focused on preventing or reducing the injurious effect of chronic hyperglycaemia on cells of the kidney.

CHAPTER 6

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