ROLE OF THE CAROTID BODY IN METABOLIC DISTURBANCES

MARIA JOÃO RODRIGUES FERREIRA RIBEIRO

Tese para obtenção do grau de Doutor em Ciências da Vida

na Especialidade em Biomedicina

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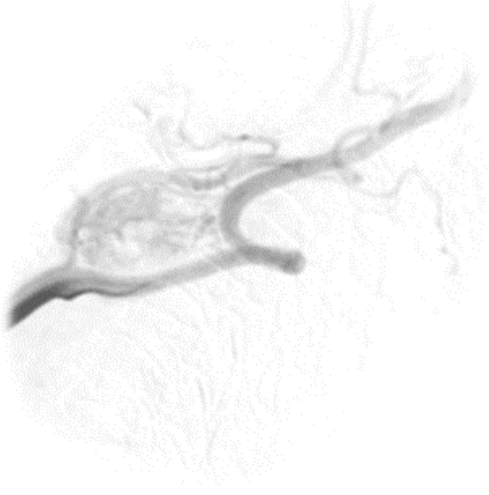
- Aos meus pais Leonida e João,
Ao meu sobrinho Afonso -



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Thesis Publications

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Conde SV, Sacramento JF, Guarino MP, Gonzalez C, Obeso A, Diogo LN, Monteiro EC, **Ribeiro MJ** (2015) Carotid body, insulin, and metabolic diseases: unraveling the links. Frontiers in Physiology, 5(418): 1-14.

Vera-Cruz P, Guerreiro F, **Ribeiro MJ**, Guarino MP and Conde SV (2015) Hyperbaric oxygen therapy improves glucose homeostasis in type 2 diabetes patients: a likely involvement of the carotid bodies. Advance in Experimental Medicine and Biology, 860:221-225.

Conde SV, **Ribeiro MJ**, Melo BF, Guarino MP and Sacramento JF (2016) Insulin resistance: a new consequence of altered carotid bodychemoreflex? J Physio 2016; doi: 10.1113/JP271684.

Ribeiro MJ, Lima PA, Obeso A, Patricio C, Conde SV (2016) Kv1.3 channels mediate insulin action in the rat carotid body (Submitted to Journal of General Physiology).

Was presented in Scientific Meetings as:

Oral Communications:

In International Congresses:

Ribeiro MJ, Gonzalez C, Guarino MP, Monteiro EC, Conde SV (2012) Is insulin a stimulus for carotid body activation? 6th European Congress of Pharmacology, 17 – 20th July, Granada, Spain.

Ribeiro MJ, Sacramento JF, Monteiro EC and Conde SV (2012) Chronic carotid sinus nerve resection prevents the development of insulin resistance in rats fed with hypercaloric diets. 48th Annual Meeting of the European Association for the Study of Diabetes (EASD). 1-5th October Berlin, Germany.

Ribeiro MJ, Lima PA, Obeso A, Patricio C, Gonzalez C, Conde SV (2014) Kv1.3 mediates insulin signaling in the rat carotid body. 19th International Society for Arterial Chemoreception (ISAC) Congress, 29-3rd Leeds, United Kingdom.

In National Congresses:

Ribeiro MJ, Gonzalez C, Guarino MP, Monteiro EC, Conde SV (2012) Insulin triggers carotid body activation. XLII Anual Reunion of Portuguese Society of Pharmacology/ XI Reunion of Clinical Pharmacology, 1-3rd February Lisbon, Portugal.

Ribeiro MJ, Sacramento JF, Gonzalez C, Guarino MP, Monteiro EC, Conde SV (2013). Carotid body is involved in the development of insulin resistance in Wistar rats: an effect mediated by sympathetic activation. XLIII Reunion of Portuguese Society of Pharmacology/XII Reunion of Clinical Pharmacology, 6-8th February Oporto, Portugal

Poster Presentations:

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Ribeiro MJ, Sacramento JF, Guarino MP, Conde SV (2013) Carotid body activity is increased in rat hypercaloric models of insulin resistance and hypertension". 37th Congress of the International Union of Physiological Sciences, 21-26th, Birmingham, United Kingdom.

Ribeiro MJ, Sacramento JF, Guarino MP, Conde SV (2013) Can carotid sinus nerve resection be a therapeutic approach for the treatment of insulin resistance?" 49th European Association for the Study of Diabetes (EASD), 23-27th, Barcelona, Spain.

Note: The results presented herein, in chapters III-V are formatted according to the style of the journal where the papers were published or submitted for publication, with minor modifications.

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Abbreviations List

4-AP: 4-aminopyridine

5-HT: 5-hydroxytryptamine

β-ADR: Beta-adrenoceptors

AACE: American Association of Clinical Endocrinology

ACh: Acetylcholine

ADP: Adenosine diphosphate

AgTx2: Agitoxin-2

AMPK: 5' AMP-activated protein kinase

aPKC: Atypical protein kinase C

ATA: Absolute atmosphere

ATP: Adenosine triphosphate

BMI: Body mass index

BK_{Ca}: Large conductance K_{Ca} channel

[Ca2+]i: Intracellular calcium

cAMP: Cyclic adenosine monophosphate

CA: Catecholamine

CB: Carotid body

CCA: Common carotid artery

CO: Carbon monoxide

CSN: Carotid sinus nerve

CN: Cyanide

DA: Dopamine

DAPI: 4-6 diamina-2-phenylndole

DOPAC: 3, 4-Dihydroxyphenylacetic acid

EAG: Ether-á-go-go channel

EDTA: Ethylenediamine tetraacetic acid

EGIR: European Group for the study of Insulin Resistance

ENT: Equilibrative nucleoside transporter

ERK: Extracellular signal –regulated kinase

F (1, 6)/ (2, 6) bip: Fructose (1, 6)/ (2, 6) bisphosphate

F-6-P: Fructose-6-phosphate

FbiPase: Fructose (1, 6)/ (2, 6) biphosphatase

FFA: Free fatty acid

FPG: Fasting plasma glucose

FITC: Fluorescein isothiocyanate

G-1-P: Glucose -1-phosphate

G1Pase: Glucose-1-phosphatase

G-6-P: Glucose-6-phosphate

G6Pase: Glucose 6-phosphatase

GIP: Glucose dependent insulinotropic polypeptide

GK: Glucokinase

GLP-1: Glucagon-like peptide 1

GLUT: Glucose transporter

GLUT1-13: Glucose transporters 1 to 13

GP: Glycogen phosphorylase

GS: Glycogen synthase

GSK3: Glycogen synthase kinase 3

GTP: Guanosine-5'-triphosphate

HbA1c: Glycated haemoglobin

HBOT: Hyperbaric Oxygen Therapy

HDL: High density protein

HF: High fat

HMIT: Proton driven myoinositol transporter

HRP: Horseradish peroxidase

HSu: High sucrose

HT: Hypertension

IDF: International Diabetes Federation

IGT: Impaired glucose tolerance

IL-6: Interleukin-6

IL-1β: Interleukin 1beta

InsR: Insulin Receptor

IR: Insulin resistance

IRS: Insulin receptor substrate protein

IRS-1: Insulin receptor substrate 1

IRS-2: Insulin receptor substrate 2

ITT: Insulin tolerance test

JNK: C jun amino terminal kinase

K_{ATP}: ATP-sensitive K^+ channel

Kir: Inward-rectifier channel

Kv: Voltage-gated K^+ channel

K_{Ca}: Ca^{2+}-activated channel

K_{ITT}: Constant of insulin tolerance test

LDL: Low-density protein

MAP: Mean arterial pressure

MAPK: Mitogen activated protein kinase

MgTx: Margatoxin

mRNA: messenger RNA

MS: Metabolic Syndrome

MSNA: Muscle sympathetic nerve activity

NCEP: ATPIII: National Cholesterol Education Program Adult Treatment Panel III

NDGU: Non-insulin-dependent glucose uptake

NST: Nucleus solitary tract

OB: Olfactory bulb

OCC: Occlusion of common carotid

PAI-1: Pasminogen activator inhibitor 1

PCA: Perchloric acid

PBS: Phosphate-buffered saline

PDK1: Phosphoinositide-dependent kinase-1

PEP: Phosphoenolpyruvate

PEPCK: Phosphoenolpyruvate carboxykinase

PFK1: Phosphofructose-1-kinase

PI3K: 1A phosphatidylinositol 3-kinase

PIP3: Phosphotidylinositol (3, 4, 5) – triphosphate

PK: Pyruvate kinase

PKA: Protein kinase A

PKB: Protein kinase B

SCH: Src homology

SDS: Dodecyl sulfate

SKCa, Small conductance KCa channel

SGLT: Sodium-driven sugar co-transporter

SHK: Stichodactyla toxin

SUR: Sulfonylurea receptor

T2D: Type 2 Diabetes

TEA: Tetreathylammonium

OGTT: Oral glucose tolerance test

OSA: Obstructive Sleep Apnea

ROS: Reactive Oxygen Species

TASK: Tandem-pore domain K^+ channel

TBST: Tris-buffered saline tween

TCA: Tricarboxylic acid

TEA: Tetraethylammonium

TNFα: Tumor necrosis factor alfa

TV: Tidal volume

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Resumo

A resistência à insulina (IR) é uma condição patológica característica de doenças de elevada prevalência na atualidade, tais como a Síndrome Metabólica (MS) e a Diabetes Tipo 2 (T2D). Em associação com a obesidade, é uma das principais causas que leva ao desenvolvimento de distúrbios metabólicos e suas co-morbilidades, tais como a hipertensão arterial (HT), a dislipidemia e as doenças cardiovasculares. Atualmente, é consensual que a ativação crónica do sistema nervoso simpático desempenha um papel crucial no desenvolvimento da IR, no entanto, ainda permanece por esclarecer qual o mecanismo responsável pela sobreactivação crónica simpática nos distúrbios metabólicos. Na apneia obstrutiva do sono (OSA), um dos mecanismos que induz o desenvolvimento de HT centra-se na ativação do corpo carotideo (CB) e subsequente ativação do sistema nervoso simpático. Os CBs são os principais quimiorreceptores periféricos, localizados na bifurcação da artéria carótida comum (CCA), sendo sensíveis a variações na PO_2 (hipóxia), PCO_2 (hipercapnia), pH (acidose) e temperatura. Quando ativados, as células quimiorrecetoras (tipo I) respondem às variações de gases arteriais libertando neurotransmissores que ativam as terminações sensitivas do nervo do seio carotídeo (CSN). A atividade do CSN é integrada ao nível do tronco encefálico traduzindo-se num aumento da frequência respiratória (hiperventilação), assim como na normalização da pressão arterial através da ativação do sistema nervoso simpático. Nos últimos anos o CB foi proposto como um sensor de glucose, como tendo um papel na regulação da homeostasia energética, sugerindo desta maneira uma importância adicional fora do seu contexto clássico.

A presente dissertação assenta em duas hipóteses de trabalho: 1) que uma sobre ativação crónica do CB está na génese do desenvolvimento de IR e HT, através da ativação do sistema nervoso simpático; e 2) que a insulina é um estímulo para ativação do CB, desempenhando a sua ação através dos canais dependentes de voltagem do subtipo 1.3 (Kv1.3) presentes nas células tipo I. Assim, o objetivo geral do presente trabalho foi estudar qual o papel do CB no desenvolvimento da IR e distúrbios metabólicos associados.

No **capítulo I** são introduzidos conceitos gerais sobre a homeostasia da glucose, focando as patologias que têm por base a desregulação desta homeostasia, como a MS e a T2D. Foi também abordado neste capítulo a associação patológica entre o sistema

nervoso simpático e as alterações metabólicas. Para além disso, descreveu-se de uma maneira breve os conceitos chave da função do CB, assim como os seus novos potenciais papéis fisiológicos. Por fim, a oxigeno terapia hiperbárica (HBOT) e as suas aplicações foram abordadas.

No **capítulo II** encontram-se sumariados os objectivos do trabalho.

No **capítulo III** estudou-se o papel do CB no desenvolvimento de IR e HT, utilizando-se ratos Wistar submetidos a dois tipos de dietas hipercalóricas, a dieta enriquecida em lípidos (HF) para obtenção de um modelo de obesidade com IR e HT, e a dieta enriquecida em sacarose (HSu),para obter um modelo não obeso com IR e HT. Ambos os modelos patológicos foram comparados com ratos submetidos a uma dieta normocalórica. Observou-se que atividade do CB está aumentada nos modelos animais patológicos o que se traduziu num aumento da ventilação basal e da ventilação em resposta à hipóxia isquémica. Observou-se que a libertação de dopamina (DA) e a expressão da enzima tirosina hidroxilase (TH) no CB se encontram aumentadas nos ratos HF e HSu. Demonstrou-se também, que o CB está envolvido na génese da IR e da HT, uma vez que a desnervação crónica bilateral do CSN preveniu o desenvolvimento de IR e HT, da hiperglicemia e insulinemia em jejum, do aumento dos ácidos gordos livres (FFAs) e dos níveis das catecolaminas (CAs) circulantes e presentes na medula adrenal. Adicionalmente, constatou-se que a insulina ativa o CB sendo capaz de iniciar uma reposta neurosecretora avaliada pelo aumento do cálcio intracelular ($[Ca^{2+}]i$) e pelo aumento da libertação de ATP e dopamina (DA) que se traduziu num aumento da ventilação espontânea. Observou-se também que a ação da insulina no CB ocorre via recetores de Insulina (InsR), uma vez que os InsR presentes no CB são fosforilados na presença de insulina.

No **capítulo IV** foi investigado o papel dos canais Kv1.3, como mediadores da ação da insulina no CB. Neste capítulo descreveu-se a presença de correntes de K^+ dependentes de voltagem sensíveis à insulina, que possuem um perfil farmacológico característico de canais Kv1.3, uma vez que a Margatoxina (MgTx) e o ShK-Dap[22] (bloqueadores específicos dos canais Kv1.3) mimetizam a ação da insulina nestas correntes. Observou-se por imunocitoquímica a presença de canais Kv1.3 nas células tipo I, sendo estes fosforilados na presença de insulina, o que sugere que a fosforilação é um dos mecanismos através do qual a insulina modula a atividade destes canais. Para além

disso, constatou-se que os canais Kv1.3 estão envolvidos na resposta neurosecretora induzida pela insulina, visto que a MgTx mimetizou o efeito da insulina na libertação de DA do CB.

No **capítulo V** encontra-se descrito o estudo clínico, no qual foi avaliado o efeito da HBOT na tolerância à glucose. Para tal, foram recrutados voluntários não diabéticos e com T2D com indicação para terapêutica com HBOT, no centro português de Medicina Subaquática e Hiperbárica e submetidos a 20 sessões de HBOT. Observou-se que a HBOT melhora a glicemia em jejum e a tolerância à glucose em doentes diabéticos, sem quaisquer alterações nos voluntários não diabéticos.

Por fim, no **capítulo VI** efetuou-se uma discussão geral e integrada de maneira a posicionar o presente trabalho no contexto científico atual. Em conclusão, os dados apresentados neste trabalho clarificam o papel do CB na patogénese da IR e HT e sugerem que o CB pode ser um alvo terapêutico promissor para intervenção na T2D, MS e OSA.

Abstract

Insulin Resistance (IR) is a pathogenic feature present in highly prevalent diseases, like metabolic syndrome (MS) and Type 2 Diabetes (T2D). Together with obesity they are the major causes that predispose and/or trigger metabolic dysfunctions that contribute to the development metabolic disturbances and related comorbidities, such as, hypertension (HT), dyslipidemia and other cardiovascular diseases. It is now becoming consensual that chronic sympathetic nervous system activation plays a role in the development of IR, however the mechanisms leading to its sustained activation in metabolic disturbances are still unknown. In obstructive sleep apnea (OSA) several reports have linked HT and the increased sympathetic nervous system activity with the overactivation of carotid body (CB). The CB is a major peripheral chemoreceptor organ that sense changes in blood O_2, (hypoxia) CO_2, (hypercapnia) and pH levels (acidosis). Hypoxia and acidosis/hypercapnia activate CB type I cells, which respond by releasing neurotransmitters in order to increase the action potential frequency in their sensory nerve, the carotid sinus nerve (CSN). The CSN activity is integrated in brainstem to induce a fan of respiratory reflexes aimed, primarily to normalize the altered blood gases via hyperventilation and to regulate blood pressure and cardiac performance via activation of the sympathetic nervous system. Recently, the CB was proposed to be a glucose sensor and to be implicated in the regulation of energy homeostasis control, therefore demonstrating new roles for this organ outside its classical function.

Herein the hypothesis that CB overactivation is involved in the genesis of metabolic dysfunction through sympathetic nervous system activation was investigated. Additionally, the hypothesis that insulin stimulates the CB via Kv1.3 channels present at type I cells was studied. The **general aim** of this work was to establish the role of CB in the development of IR and related metabolic dysfunctions.

Chapter I introduces general concepts of glucose homeostasis and the diseases where glucose metabolism is deregulated, such as, MS and T2D. Additionally the link between sympathetic nervous system and dysmetabolism is focused herein. Moreover, key concepts of CB function as well as its potential new physiological roles are debated. Hyperbaric oxygen therapy (HBOT) and its applications are introduced.

In **chapter II** are described the general and specific objectives of this work.

In **chapter III** we have investigated the role of CB in the development of IR and HT. For that, we have used Wistar rats submitted to two hypercaloric diets: the high-fat (HF) diet, which is a combined model of IR, HT and obesity and the high-sucrose (HSu) diet, which is a lean model of IR and HT. Pathological animal models have been compared with aged-matched controls. In this chapter we have demonstrated that CB activity is increased in pathological animal models, since CB-mediated basal ventilation and ventilation in response to ischemic-hypoxia were increased, as well as the CB type I cell function-assessed both as hypoxia-evoked release of dopamine and tyrosine hidroxilase (TH) expression. It was also demonstrated that CSN bilateral resection prevented diet-induced IR and HT, as well as increased fasting glycemia, fasting insulinemia, free fatty acids (FFAs) and sympathoadrenal activity. Additionally, it was shown that insulin triggers CB activation through the presence of insulin receptors (InsR) that were phosporylated in the presence of insulin allowing the CB neurosecretory response, measured as the increase in $[Ca^{2+}]i$ and the release of ATP and dopamine that is transduced into an increase in ventilation.

In **chapter IV** the role of Kv1.3 channels as effectors of insulin action on rat CB was studied. Carotid body type I cells and whole-CB from animals submitted to standard-diet were used. We demonstrated that CB type I cells exhibit insulin sensitive voltage activated K^+ currents, that are mediated by Kv1.3 channels since Margatoxin (MgTx) and ShK-Dap^{22} (specific blockers to Kv1.3 channels) inhibit voltage K^+ activated currents and the action of insulin. Additionally, we have observed that Kv1.3 channels were expressed in CB type I cells and showed that phosporylation could be one of the mechanisms by which insulin modulates the Kv1.3 activity in rat CB. Moreover, we have found that Kv1.3 channels are involved in the release of catecholamines (CAs from rat CB, since MgTx mimics the effect of insulin on the release of CAs.

The **chapter V** presents a clinical study, where the effect of HBOT on glucose tolerance in T2D patients was evaluated. T2D and non-diabetic volunteers, both with indication for HBOT have been recruited at the Subaquatic and Hyperbaric Medicine Center of Portuguese Navy and submitted to 20 sessions of HBOT. We demonstrated that HBOT ameliorates fasting glycemia and glucose tolerance in T2D patients, without altering glucose homeostasis in non-diabetic patients.

Finally, a general and integrated discussion is presented in **chapter VI** to positioning the work developed in the present work into the current scientific context. In conclusion the data presented herein clarify the role of the CB in the pathogenesis of diet-induced IR and HT and unveil a new promising target for intervention in T2D, MS, and OSA.

CHAPTER I – GENERAL INTRODUCTION

1. GLUCOSE HOMEOSTASIS – GENERAL CONSIDERATIONS

Among all the nutrients that humans obtain by feeding, glucose represents a large proportion of carbohydrates present in diet, and plays a central role in energy metabolism to produce adenosine triphosphate (ATP). Glucose can be obtained from the dietary intake in gastrointestinal tract and by the endogenous production that occurs, mostly, in the liver and also in the kidney. In the first setting, glucose results from enzymatic digestion of more complex carbohydrates (lactose, sucrose), whereas the endogenous production results from a series of complex biochemical reactions named glycogenolysis (breakdown of glycogen to glucose) and gluconeogenesis (conversion of pyruvate derived from precursors, including lactate and amino acids - specially alanine and glutamine - to glucose) (Gerich, 1993; Saltiel & Kahn, 2001) (see annexes V and II). Once in the bloodstream glucose is taken up by target organs, namely the adipose tissue and skeletal muscle in the periphery, through the action of specific transporters, the glucose transporters (GLUTs) (Wilcox *et al*., 2005; Zhao & Keating, 2007) (**Figure 1.1**). Inside the cell, glucose is phosphorylated and follows different routes: it can be stored as glycogen (in the liver and skeletal muscle) (see annex IV) or it can be metabolized through glycolysis [conversion of glucose-6-phosphate (G-6-P) to pyruvate), which in turn can be reduced to lactate under anaerobic conditions or oxidized, via the tricarboxylic acid (TCA), also known as Krebs cycle, resulting in greater amounts of ATP] (Gerich, 2000; Saltiel & Kahn, 2001) (see annexes I and III).

Plasma glucose concentration is a function of the rate of glucose entering the bloodstream, through intestinal glucose absorption or endogenous glucose production mainly by the liver, balanced by the rate of glucose removal from bloodstream due to its utilization by tissues (Gerich, 2000). The process of maintaining glycemia at a steady-state level is called glucose homeostasis (DeFronzo, 1988). The glycemia need to be upheld within narrow limits by two main reasons: to sustain the brain and the peripheral tissues with their nutrional requirements and also to avoid the toxic effects related to the increases of glycemia (Szablewski, 2011). In healthy humans, glycemia is maintained within a tight physiological range between 3.9 to 5.6 mM (70.2 - 100.8 mg/dl), despite the variations in plasma glucose after a meal, during fasting and also in response to exercise (Roden & Bernroider, 2003). Although with some variations, several studies suggested for any given individual normal blood glucose levels post-absorptively (period between meals in which energy must be supplied by the body´s endogenous fuel

stores) are strictly maintained within ±0.3 mM (5 mg/dl) (DeFronzo, 1983; Kopf *et al*., 1973). Even in the post-prandial state, blood glucose concentrations increase only 1 to 2 mM, and rarely, if ever, exceed 7.8 to 8.4 mM (140-150 mg/dl) (DeFronzo *et al*., 1983; Kopf *et al*., 1973). This is possible due to the cooperation and integration of several physiological systems (such as the sympathetic nervous system and the endocrine system) that have mechanisms that are able to maintain glucose homeostasis (Aronoff, 2004; Roden & Bernroider, 2003). The failure of these mechanisms can lead to hyperglycemia or hypoglycemia and subsequent clinical complications that are characteristic of metabolic disorders, such as diabetes and subsequent cardiomyopathy, angiopathy, nephropathy, retinopathy and stroke (Villeneuve & Natarajan, 2010).

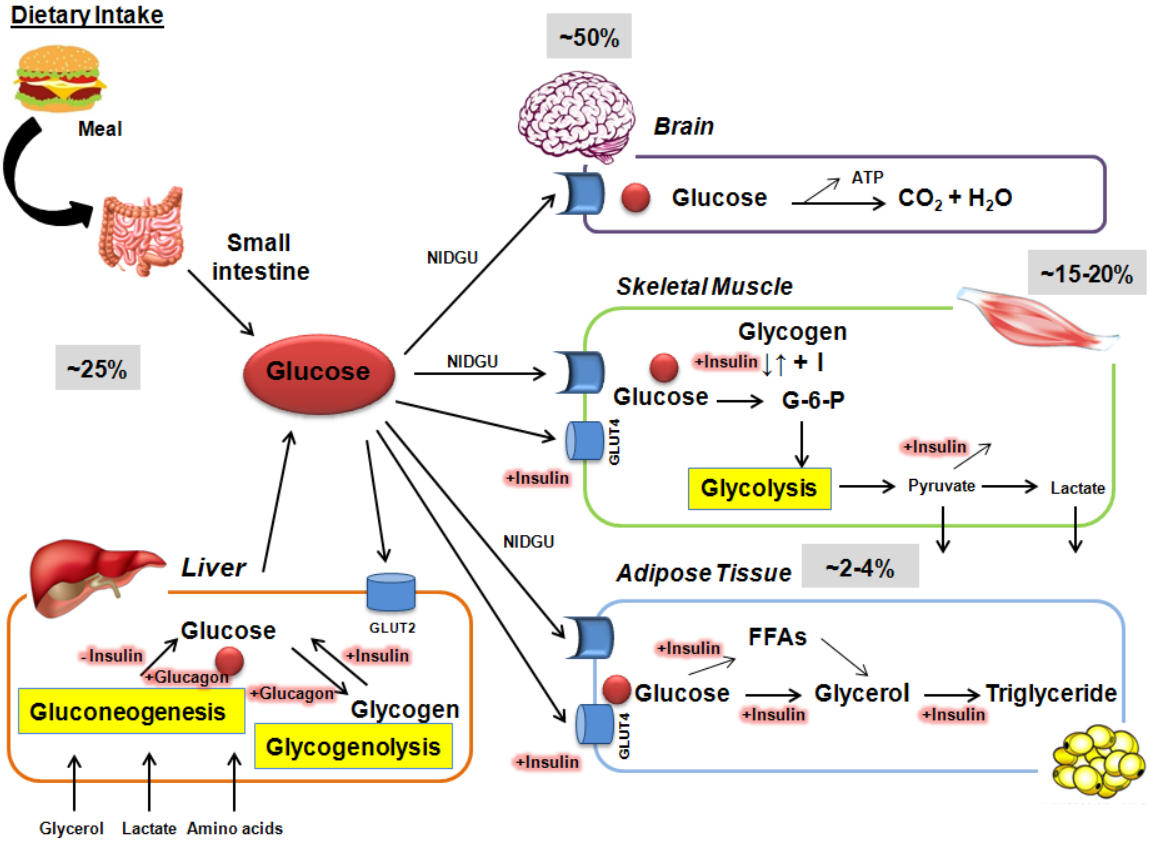

Figure 1.1: Overview of glucose disposal in target organs. Routes of glucose disposal after a meal ingestion and in a fasting state. The percentage of glucose consumption depends on the tissue involved (Grey square). From 100% of glucose production, the brain appears as the major glucose consumer (using 50%), followed by the liver and gastrointestinal tissue (~25%), the skeletal muscle (~15-20%), and adipose tissue (~2-4%). NDGU: non-insulin-dependent glucose uptake; G-6- P: glucose-6-phosphate; FFAs: free fatty acids; ATP: adenosine triphosphate; GLUT2: Glucose transporter 2; GLUT4: Glucose Transporter 4; +: stimulation; -: inhibition.

1.1. The endocrine pancreas and the mechanisms of insulin secretion

The endocrine pancreas is a major regulator of glucose homeostasis by releasing hormones directly implicated in metabolic pathways in the insulin sensitive organs, located in the periphery (skeletal muscle, adipose tissue and liver) (Aronoff, 2004; Gerich, 2003). The pancreas is constituted by four major cell types in the islets of Langerhans: the β cells, responsible for the production of insulin, the α cells for the production of glucagon, the δ cells for the production of somatostatin and the F (or PP) cells, responsible for the production of pancreatic polypeptides (**Figure 1.2**). Insulin and glucagon respond oppositely to changes in glycemia: while hypoglycemia conditions induce the release of glucagon by α-cells, β-cells release insulin when glucose levels increase (Nadal *et al*., 1999; Quesada *et al*., 2006).

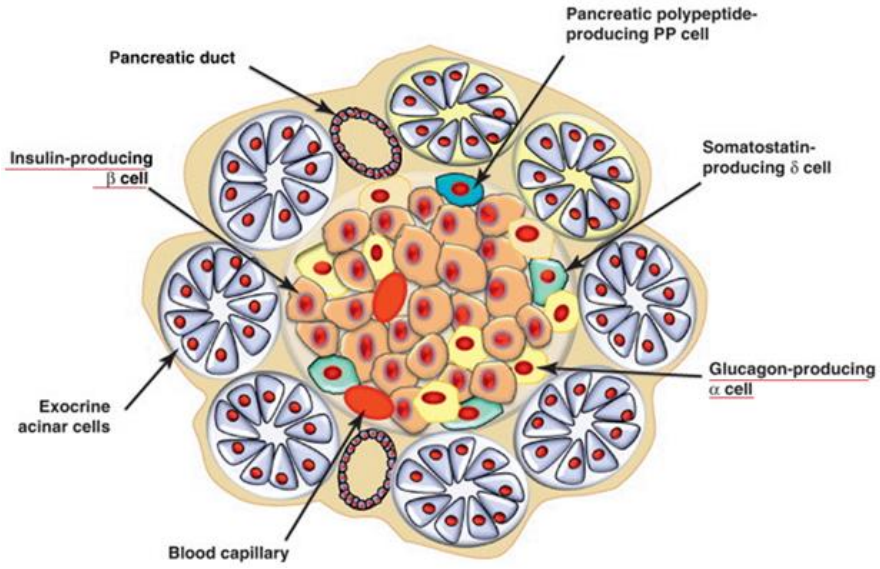

Figure 1.2: Schematic representation of islet and pancreas cell types. The endocrine pancreas consists of four major cell types α, β, δ and PP cells which are organized in compact islets secrete hormones into bloodstream, namely insulin and glucacon. The exogenous pancreas is composed of pancreatic acinar cells and duct cells that produce digestives enzymes. Adapted from (Efrat & Russ, 2012).

Insulin is an anabolic hormone with 51 amino acids (in humans) with a molecular weight of 5.8 kDa, organized in two polypeptide chains, an A chain of 21 amino acids and a B chain of 30 amino acids linked by two disulfide bridges (De Meyts, 2004). This hormone is synthesized in β-cells from a single amino acid chain precursor (proinsulin) that is packaged into vesicles in the Golgi apparatus. During the maturation of the secretory vesicles proinsulin is converted to insulin and to C-peptide that are released

into the bloodstream in equimolar amounts (De Meyts, 2004; Fu *et al*., 2013; Licinio-Paixão *et al*., 1986; Polonski, 1995). C-peptide possess a huge clinical importance in the measurements of insulin secretion in patients with diabetes, since its half-life is longer than insulin half-life, 20-30 minutes (min) *versus* 3-5 min respectively (Licinio-Paixão *et al.,* 1986; Polonsky, 1995).

Insulin secretion can be regulated by several nutrients, such as glucose, arginine and lipids, by hormones, such as incretins, and via neural signaling by the cholinergic pathway, -the *vagal* axis- and by the adrenergic pathways through α_2-adrenoceptors (Wilcox, 2005). In response to glucose, insulin is released by the pancreas through biphasic secretion (Bratanova-Tochkova *et al*., 2002; Polonsky *et a*l., 1988). This biphasic pulsatile pattern consists of an initial rapid phase (first phase) followed by a less intense but more sustained (second phase) release of the hormone (Bratanova-Tochkova *et al*., 2002; Polonsky *et a*l., 1988) (**Figure 1.3A**). In type 2 diabetic patients the biphasic patterns is impaired, the first phase of insulin release is missing while the second phase is present, however the second phase can be deficient in severe type 2 diabetes (T2D) (Del Prato *et al*., 2002; Guilausseau *et al*., 2008). Regarding the pulsatile pattern of insulin secretion this is maintained in T2D patients, with a similar number of pulses like in healthy subjects, however in post-prandial state the pulses are irregular, less frequent and with lower amplitude (Polonsky *et al.,* 1988).

The presence of circulating glucose leads to a quick uptake of glucose via glucose transporters 2 (GLUT2) by the β-cells that results in an increase in the cytoplasmatic ATP/adenosine diphosphate (ADP) ratio. This increase of ATP in cytosol leads to closure of ATP–dependent potassium (K_{ATP}) sensitive channels and subsequent depolarization, allowing the opening of voltage gated Ca^{2+} channels and Ca^{2+} influx into the cell causing exocytosis of insulin from granules (Ascroft, 2005 for review) (**Figure 1.3B**). In addition to this K_{ATP} channel –dependent pathway, which triggers insulin secretion, there is also a K_{ATP} channel–independent pathway that amplifies the effects of Ca^{2+} on exocytosis, being this pathway responsible for the second phase of insulin secretion (Henquin, 2000). The second phase of insulin secretion is thought to be augmented of by many factors such as the incretins hormones: glucagon-like peptide 1 (GLP-1) and glucose dependent insulinotropic polypeptide (GIP), and is termed the amplifying pathway (Bratanova-Tochkova *et al.,* 2002; Wilcox *et al*., 2005). Although glucose is the necessary stimulus for insulin secretion, this process is enhanced by

increased levels of both GLP-1 and GIP, released by gut cells (Aronoff, 2004; Taminato *et al*., 1977).

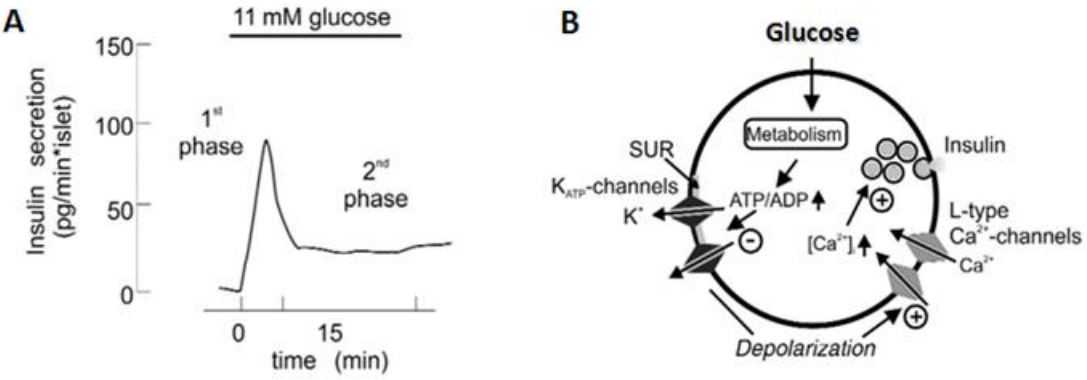

Figure 1.3: Insulin secretion in pancreatic β-cell. A) Characteristic biphasic secretion in response to glucose (11mM), that consists in a rapid first phase followed by a slower second phase. B) Schematic representation of insulin mechanism secretion upon glucose stimulation. SUR: sulfonylurea receptor. Adapted from Rorsman, (2000).

In situations where the blood glucose concentration decreases, a catabolic counter-regulatory hormone called glucagon is released by the pancreas stimulating hepatic glucose production through activation of glycogenolysis and gluconeogenesis in order to raise glucose levels (Jiang & Zhang, 2003). Glucagon activates G proteins coupled receptor, activating the hepatic adenyl cyclase leading to an increase in cyclic adenosine monophosphate (cAMP) levels, which activates protein kinase A (PKA) inducing an increased breakdown of glycogen and an increase in plasma glucose, in turn phosphorylates the enzymes involved in liver glycogenolysis (Burcelin *et al*., 1996). In addition, glucagon stimulates the release of free fatty acids (FFAs) that are taken by the liver and shunted towards β-oxidation and subsequent formation of ketoacidosis, instead of being guided to the synthesis of triglycerides (Carlson *et al*., 1993).

1.1.1. Insulin signaling

Insulin regulates glucose, lipid and energy homeostasis, predominantly via its action on the liver, skeletal muscle and adipose tissue, the insulin sensitive tissues. The action of insulin is initiated by the binding of the protein to the extracellular portion of the insulin receptor (InsR) (De Meyts & Whittaker, 2002). The InsR belongs to the family of cell

surface receptors that possess an intrinsic tyrosine kinase activity, called tyrosine kinase receptors. The InsR is composed by two extracellular α-subunits and two transmembrane β-subunits linked by disulfide bonds. When insulin is not present, the unbound extracellular α-subunits inhibit the intrinsic intracellular tyrosine kinase (White & Kahn, 1994). In contrast, when insulin binds to one of the α-subunits this inhibition is removed, and the adjacent intracellular β-subunit is autophosphorylated at its tyrosine residues of the regulatory domain (Lee *et al*., 1993). Subsequently, the autophosphorylation of the tyrosine residues allows the InsR to activate a panel of intracellular InsR substrate proteins (IRS) (Pessin & Saltiel, 2000; Sun *et al*., 1995; White, 1998). These IRS proteins act as InsR specific docking proteins that create recognition sites for additional effector molecules with Src homology (SCH) domains, such as the p85 regulatory subunit of the type 1A phosphatidylinositol 3-kinase (PI3K) (Pessin & Saltiel, 2000; Saltiel & Kahn, 2001). These IRS docking proteins serve multiple functions, as they allow the amplification of the InsR signal, the dissociation of the intracellular signaling cascade from the membrane bound InsR, to expand the number of pathways that can be regulated through one InsR, and the integration of multiple metabolic signals (Hotamisligil & Spiegelman, 1994; White & Kahn, 1994). The insulin signaling cascade encompasses several known downstream intermediates (Krüger *et al*., 2008) involved in three primary pathways: the PI3K – Akt/protein kinase B (PKB), the Cb1/CAP pathway, and the mitogen activated protein kinase (MAPK) cascade (Saltiel & Kahn, 2001) (**Figure 1.4**). The PI3K pathway appears to play the biggest role in the maintenance of the whole body glucose homeostasis. However, the MAPK and Cb1/CAP pathways are also of key importance for the maintenance of glucose homeostasis. The PI3K plays a significant role in many of the metabolic processes associated with insulin including: glucose uptake, growth factors, protein synthesis, and glycogen synthesis (White, 1998). Insulin stimulates glucose uptake via PI3K pathway through multiple steps including: activation of IRS by the InsR, binding of IRS to PI3K regulatory subunit p85, phosphorylation of phosphotidylinositol (3, 4,5) – triphosphate (PIP_3), activation of atypical protein kinase C (aPKC) and Akt through phosphoinositide-dependent kinase-1 (PDK1), and finally the translocation of glucose transporters 4 (GLUT4) to the plasma membrane to promote cellular glucose uptake (Saltiel & Pessin, 2002, Holman *et al*, 1994; Martin *et al*,.1998). Insulin also stimulates MAPK activity by activating guanosine-5'-triphosphate (GTP)-binding proteins that transduce the signal through a complex series of kinases tiers. There are four main

variants of the MAPK signaling cascade that arise as the signal navigates through these kinases tiers that include: extracellular signal –regulated kinase (ERK),c jun amino terminal kinase (JNK), p38 kinase and BMK signaling pathways (Shaul & Seger, 2007).

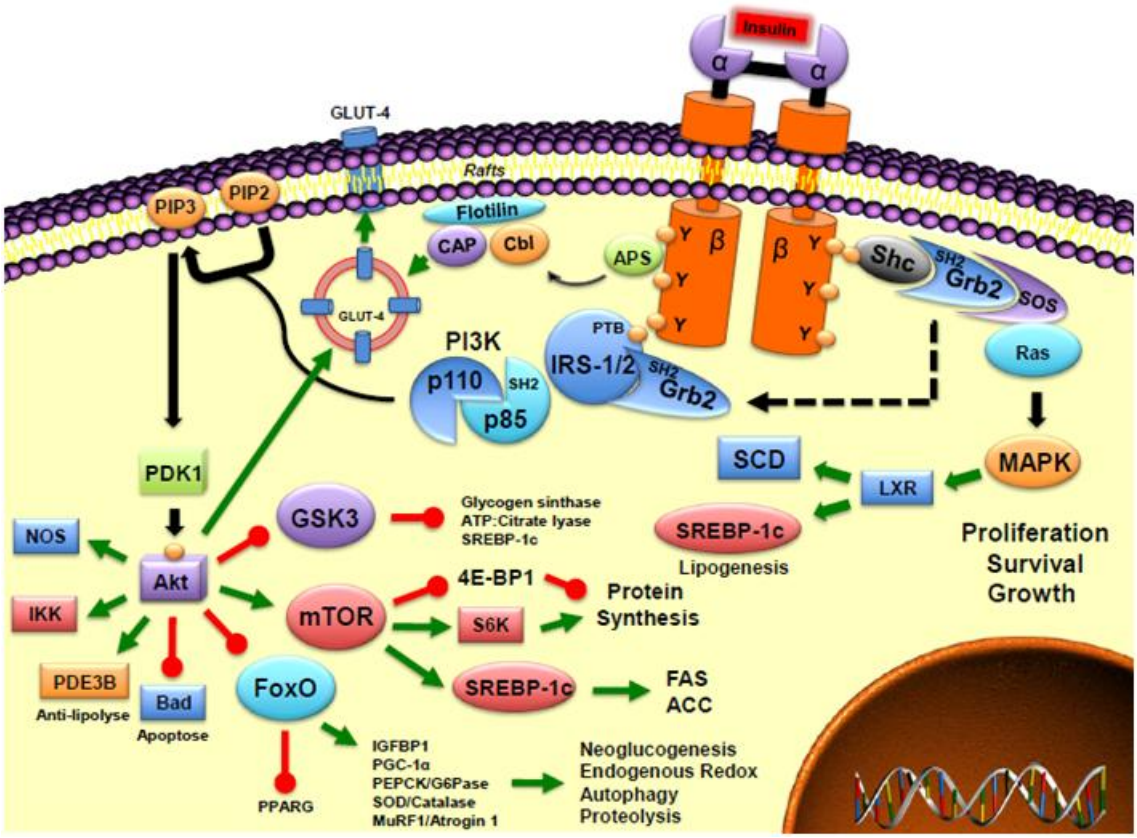

Figure 1.4: Insulin signaling pathways. Activation of insulin receptor (InsR) by insulin initiates a cascade of phosphorylation events that induce several different biological effects. The activation of InsR tyrosine kinases recruits and phosphorylates several substrates among theses, insulin receptor substract 1-4 (IRs1-4) and the src homology (SCH) and APS adaptor proteins, all of which provide specific docking sites for recruitment of other downstream signaling proteins, leading to the activation of both phosphatidil inositol-3-kinase (PI3K) → Akt signaling cascade and Ras→ MAPKs. The activation of (PI3K) → Akt signaling cascade mediates the effect of insulin on metabolism and pro-survival and Ras→ MAPKs mediates the effect on mitogenesis and cell growth. Assigned and adapted from (Veloso, 2015).

1.1.2. Glucose transporters

Glucose molecules cannot cross the plasma membrane due to the hydrophobic nature of the membrane therefore requiring specific transporters to enter into cells. There are two families of proteins involved in glucose transport through cell membranes: the family of sodium-driven sugar co-transporters (SGLTs) (Uldry *et al*., 2002) that are involved in the absorption and reabsorption of glucose from food, in the gastrointestinal tract, and from urine, in the kidney, respectively, and the GLUTs family involved in glucose

homeostasis (Mueckler, 1994; Zhao & Keating 2007). These transporters promote the facilitated diffusion of glucose, a process that is not energy dependent and that follows Michaelis-Menton kinetics, which inversely reflects the affinity to glucose (Cornish-Bowden, 2015). In humans, the GLUTs family comprises 14 isoforms that have high conserved sequence and share common structural characteristics, such as the 12 transmembrane domains, the N- glycosylation site on the first or fifth loop, and the sugar transporters signatures (Sun *et al*., 2012; Zhao & Keating 2007). Despite the well conserved structure among GLUT isoforms, individual transporters have distinct tissue distributions and biochemical properties, being divided in three subclasses (I-III) based on the phylogenetic analysis of their sequence and characteristic elements (Joost & Thorens 2001; Joost *et al.,* 2002; Mueckler & Thorens, 2013). The class I, characterized in terms of structure, function and tissue, comprises GLUT1 to 4 transporters, (including the gene duplication of GLUT3, which is GLUT14), has a huge importance in whole-body glucose homeostasis under physiological, and especially disease conditions. The class II contains the "odd" isoforms GLUT5, 7, 9 and 11 the isoforms GLUT6, 8, 10, 12, and the GLUT13, also known by proton driven myoinositol transporters (HMIT) belongs to class III (Joost & Thorens, 2001; Joost *et al*., 2002). Glucose transporter 1 and GLUT3 are the isoforms responsible for basal or constitutive glucose uptake, being GLUT1 the transporter that mediates glucose transfer across the blood brain barrier (Brockman, 2009); whereas GLUT3 is a neuron-specific glucose transporter with greater expression in human and rat brain (Bell *et al*., 1990; Haber *et al*., 1993; McCall et *al*., 1994). GLUT2 is expressed in the liver, brain, insulin-producing pancreatic β-cells, kidney and intestine absorptive epithelial cells (Fukumuto *et al*., 1988; Zhao & Keating, 2007 for review). In pancreatic β-cells GLUT2 plays a role in the glucose-sensing mechanism, while in the liver it is involved in the bi-directional transport of glucose under hormonal control (Fukumuto *et al.,* 1988; Orci *et al*., 1989; Johnson *et al*., 1990; Kellet & Brot-Laroche, 2005; Wright, 2013). GLUT4 is highly expressed in skeletal muscle and adipose tissue. This transporter display a characteristic that makes it unique, since it is the only transporter that has an intracellular localization in unstimulated states, being acutely translocated to the plasma membrane in response to insulin (Wood & Trayhurn, 2003) and other stimuli (Bryant *et al*., 2002; Czech & Buxton 1993). Briefly, under insulin stimulation, GLUT4 undergoes a rapid translocation from the intracellular location to the cell membrane increasing glucose transport activity (Gonzalez & McGraw, 2006) (**Figure 1.4**).

1.2. Whole-body and tissue specific insulin action

Insulin is the pivotal hormone with extensive effects on whole body metabolism since it regulates cellular energy supply and macronutrient balance, directing processes in the fed state, being essential for the intracellular transport of glucose into insulin-dependent tissues, such as skeletal muscle and adipose tissue (Burks & White, 2001). In the presence of abundant exogenous energy, adipose tissue fat breakdown is suppressed and its synthesis is promoted. In the muscle, glucose entry enables glycogen to be synthesized and stored, and carbohydrates, rather than fatty acids (or amino acids), to be use as an immediately available energy for muscle contraction. In brief, insulin promotes glycogen and lipid synthesis in the muscle, while suppressing lipolysis and gluconeogenesis from muscle amino acids (Wilcox, 2005) (see annexes IV, VI and VII). In the liver, insulin acts, not by directly stimulating glucose uptake, but by blocking glycogenolysis and gluconeogenesis, and glycogen synthesis (Aronoff, 2004). In pathological states, when the tissues are not able to adequately sense or respond to insulin we are in the presence of insulin resistance (IR). Therefore, IR can be defined as a state in which sensitive insulin tissues exhibit inadequate or reduced response to normal amounts of insulin, requiring higher concentration of insulin to maintain normoglycemia. The development of IR leads to a compensatory augmentation in insulin synthesis, which subsequently results in hyperinsulinemia (Cefalu, 2001). The clinical effects of IR are due to the disruption of insulin-mediated control of glucose and lipid homeostasis in the insulin target organs, leading to a decreased glucose uptake, increased hepatic-glucose production and increased whole-body lipolysis (Boden & Laako, 2004). This specific tissue IR is characterized by elevated hydrolysis of triglycerides from adipocytes and increased in FFAs, by reduced glucose uptake in skeletal muscle, and by impaired hepatic glycogen synthesis (Petersen & Shulman, 2006).

1.2.1. Insulin action in the liver

The liver is a central metabolic organ that controls several key aspects of glucose and lipid metabolism in response to nutritional and hormonal signals, being essential for the maintenance of systemic energy homeostasis (Van Den Berghe, 1991). It acts as a hub to metabolically connect various tissues, including skeletal muscle and adipose tissue.

Glucose, fatty acids and amino acids are absorbed in the gastrointestinal tract to the bloodstream and transported to the liver through the portal vein circulation system (Moore *et al*., 2012; Nordlie *et al*., 1999; Rui, 2014). This organ is able to both store and release glucose to minimize changes in glycemia in the rest of the body, between the fed and fasted states, thus contributing to whole body glucose homeostasis (Moore *et al*., 2012; Nordlie *et al*., 1999; Rui, 2014).

Overnight, in the post-absorptive state, circulating glucose is derived from two primary hepatic processes: 1) glycogenolysis, i.e., the release of glucose from stored glycogen, and 2) gluconeogenesis, i.e., the formation of new glucose from common metabolites in the body including amino acids, glycerol, lactate, pyruvate and intermediate metabolites of the TCA cycle (Nuttall *et al*., 2008) (see annexes V, II and IV). These processes occur after hepatic glucagon action and subsequent activation of PKA that phosphorylates several transcriptions factors inducing the expression of gluconeogenic genes, namely phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6-phosphatase (G6Pase). Consequently, gluconeogenesis occurs and in contrast, glycolysis is inhibited through the repression of glucokinase (GK) and glycolitic genes, such as pyruvate kinase and fructose-2-6-bisphosphate (Agius, 2007; Jiang & Zhang, 2003) (**Figure 1.5**). In addition, PKA activation also triggers glycogen phosphorylase (stimulating glycogenolysis, via activation of phosphorylase kinase), and inactivates glycogen synthase (inhibiting glycogenesis) (Agius, 2007). The resulting glucose-1-phosphate (G-1-P), besides being a potent inhibitor of glycogen synthase phosphatase and of glycogen synthesis is an important gluconeogenic intermediate. Glucose-1-phosphate is converted to glucose-6-phosphate (G-6-P), and then to glucose, through G6Pase (Jiang & Zhang, 2003). These actions together, result in an increase of hepatic glucose delivery into the blood, therefore re-establishing the physiological glucose level. During the post-absorptive state, the contribution of glycogenolysis and gluconeogenesis to overall glucose production is approximately equal. However, with continued fasting, glycogen stores become depleted, glucose production decreases and gluconeogenesis become the predominant and nearly exclusive process (Hellerstein *et al*., 1997; Nuttall *et al.,* 2008).

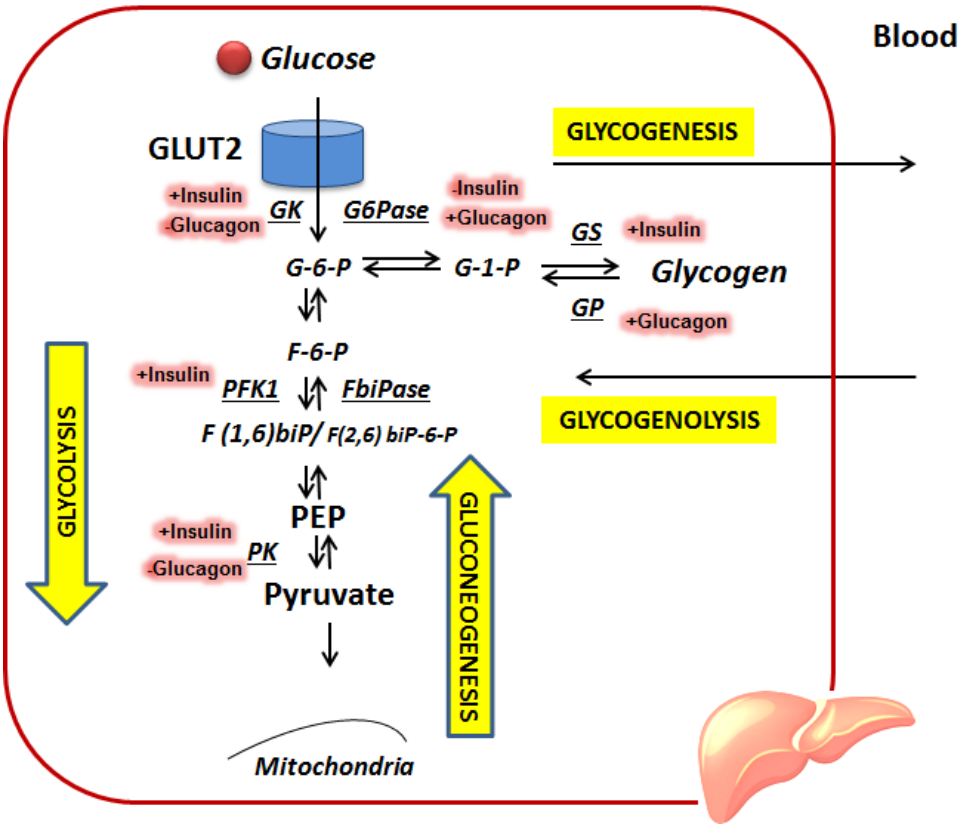

Figure 1.5: Glucose metabolism in the hepatocyte. Glucose is metabolized by different enzymes in different processes that are triggered or blocked by insulin or glucagon action. G-6-P: glucose-6-phosphate; F-6-P: fructose-6-phosphate; F(1,6)/(2,6) bip: fructose (1,6)/(2,6) biphosphate; PEP:phosphoenolpyruvate; GK: glucokinase; PFK1: phosphofructo-1-kinase; PK: pyruvate kinase; G6Pase: glucose-6-phospathase; FbiPase: fructose (1,6)/(2,6) biphosphatase; G-1-P: glucose -1-phosphate; GS: glycogen synthase; GP: glycogen phosphorylase; GLUT2: Glucose transporter 2; +:stimulation ; - inhibition.

After a meal, in the post-prandial state, the levels of glucose increase due to glucose absorption in the gastrointestinal tract; gluconeogenesis stops and the liver restores glycogen stores (Moore *et al*., 2012) At this time, insulin action in the liver blocks glycogenolysis and gluconeogenesis and stimulates glycogen synthesis (glycogenesis) by activation of the cascade IR/IRS1-2/PI3K/Akt promoting the phosphorylation and inactivation of glycogen synthase kinase 3 (GSK3), which in turn dephosphorylates and activates glycogen synthase (GS) (Aronoff *et al*., 2004).The crucial metabolic function of the liver is performed by hepatocytes which exhibit high capacity of glycogenesis, glycogenolysis, glycolysis and gluconeogenesis, enabling them to transiently store substantial amounts of glucose as glycogen, to synthesize glucose from lactate, glycerol and amino acids and to convert excess glucose into triglyceride (Nuttal *et al*., 2008). Hepatocytes take up glucose, independently of insulin, by the low affinity GLUT2,

which facilitates the entry of glucose in the presence of high concentrations of glucose and then rapidly phosphorylates glucose, via GK action, to obtain G-6-P, which cannot escape the cell. From G6Pase, the glucose flux is transformed into glycogen via uridine diphosphatase (UPD)-glucose (direct pathway of glycogen synthesis), the pentose phosphate shunt-or into glycolysis, yielding carbon -3 compounds such as pyruvate and lactate (Roden & Bernroider, 2003).

The GLUT2 and GK are expressed in the cell types in which glucose metabolism is dependent on extracellular glucose concentration, being known as glucose sensors (Leturque *et al*., 2009; Massa, 2011).

Impaired insulin sensitivity and dysregulated insulin action in the liver contribute significantly to the pathogenesis of T2D (Fritsche *et al.,* 2008). Type 2 Diabetes is associated with defective regulation of hepatic glucose metabolism and defective hepatic insulin clearance, involving elevated glucose production in euglycemic conditions and subnormal clearance of glucose by the liver after a meal, due to delayed suppression of hepatic glucose production and impaired conversion of glucose to glycogen (Basu *et al*., 2000; Krssak *et al*., 2004; Rizza, 2010). Patients with T2D have excessive rates of endogenous glucose production that fail to appropriately suppress after eating (Basu *et al.*, 2000; 2004). It was described that gluconeogenesis, and perhaps glycogenolysis, are increased early in the evolution of T2D (Basu *et al*., 2004; Basu *et al.,* 2005). In fact in the post-prandial state, the suppression of endogenous glucose production is incomplete both because of hepatic IR, impaired insulin and excessive glucagon secretion in T2D, thereby causing post-meal hyperglycemia. This leads to lower rates of hepatic glycogen synthesis primarily due to reduced uptake of extracellular glucose presumably because of inadequate activation of GK (Basu *et al*., 2000; 2001). Also, in T2D patients it was described that G6Pase, and phosphoenolpyruvate (PEP) carboxykinase, two rate limiting enzymes of hepatic glucose production are increased due to hepatic IR, since insulin is not able to suppress the expression of these enzymes, as below described (Haeusler *et al*., 2015; Konopelska *et al*., 2011). In contrast, some studies demonstrated that GK activity is decreased in T2D (Clore *et al*., 2000). Hepatic IR is essentially characterized by alterations in insulin signaling pathway in hepatocytes, which contribute for the onset of diabetes. Several defects have been described: the dysfunction in InsR proteins initially leads to post-prandial hyperglycemia, increased hepatic glucose production and dysregulated lipid

synthesis, and these are considered as major pathophysiological mechanisms for the development of IR and T2D (Dong, 2006;Taniguchi *et al*., 2005). Defects in InsR phosphorylation, accompanied by the suppression of IRS transcription and loss of IRS associated PI3K activity (Kerouz *et al*., 1997; Suzuki *et al*., 2004; Valverde *et al*., 2003), result in impairment of the downstream cellular insulin action in the liver. There are others factors that can also contribute for the development of hepatic IR, although in a smaller extent. Increased expression/function of protein-tyrosine phophatases, in addition to production of proinflamatory cytokines in adipose tissue, such as tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6), may also be responsible for InsR dysfunction and liver IR (Klover & Mooney, 2004). In addition, GLUT2 alterations can be involved in liver IR pathogenesis, since in humans GLUT2 deficiency (Fanconi-Bickel syndrome) is related with marked hypoglycemia in the fasting state (Brivet *et al*., 1983; Santer *et al*., 2002) and glycogen accumulation. The last one occurs due to a failure to adequately export of glucose generated by glycogen degradation. However, surprisingly the knockout mice for GLUT2 do not exhibit alterations both in hepatic glucose output and glucose response (Guillam *et al*., 1998).

1.2.2. Insulin action in the Skeletal Muscle

Skeletal muscle is the predominant site for disposal of ingested glucose in the postprandial state in lean individuals (DeFronzo *et al*., 1988, 1997; 2004; 2009). Following a meal, approximately one third of ingested glucose is taken up by the liver, being the rest taken up by peripheral tissues, primarily the skeletal muscle via an insulin dependent mechanism (DeFronzo *et al*., 1988, 1997; 2004; 2009). This organ uses both glucose and FFAs as a fuel sources for energy production (DeFronzo 1988; 1997; 2004; 2009; Saltiel & Kahn, 2001). During the fasting state, insulin concentration is low which reflects a decreased glucose uptake, being the major source of energy the FFAs that are elevated in bloodstream (Groop *et al*., 1989). After a meal, the increase in plasma glucose levels stimulates insulin secretion from pancreatic β-cells induces a rapid glucose uptake by the muscle, through the recruitment of GLUT4 transports and activation of key enzymes (pyruvate kinase and glycogen synthase) involved in glucose metabolism (DeFronzo, 1988). At the same time, the increase in insulin levels suppresses lipolysis, leading to a decline in plasma FFAs and subsequent decrease in the

rate of lipid oxidation (Blaak, 2005). Once inside the myocyte, glucose is immediately phosphorylated by hexokinase and then metabolized through or stored as glycogen (Saltiel & Kahn, 2001).

Defects in the skeletal muscle play a major role in glucose homeostasis in patients with T2D (Bjornholm & Zierath, 2005). In T2D and obesity, typical IR states, insulin-stimulated glucose disposal in skeletal muscle is impaired (DeFronzo *et al.,* 1988, 1997; 2004; 2009). The exact mechanism that leads to development of IR is not completely understood, however several articles have published in the last decade allowing a better understand concerning the defects important in this context. In IR states defects at different levels of glucose homeostasis, such as in the metabolism, transport and insulin signaling pathways occur in skeletal muscle (Abdual –Ghani & DeFronzo, 2010; Frojdo *et al.,* 2009, for review). It has been demonstrated that the decrease in insulin-stimulated glucose uptake observed in skeletal muscle is due to impaired insulin signaling, multiple post-receptor defects in proteins involved in insulin pathway, such as, InsR and PI3K/Akt, alterations in phosphorylation, impaired glucose transporter and reduced glucose oxidation and glycogen synthesis (Abdul-Ghani & DeFronzo, 2010, for review). Regarding InsR tyrosine phosphorylation several studies demonstrated a reduction in tyrosine kinase activity in non-obese and obese T2D patients (Cusi *et al*., 2000; DeFronzo *et al*., 1988; Nolan *et al*., 1994). This reduction cannot be explained by alterations in InsR number or InsR binding affinity, because it is known from previous studies that insulin binding to solubilized receptors in skeletal muscle of obese and lean T2D patients is normal (Caro *et al*., 1987; Klein, 1995). It has been described that the impairment of IR phosphorylation at IRS-1 level, seems to be the primary defect that leads to IR in skeletal muscle (Abdul-Ghani & DeFronzo, 2010, for review). Insulin receptor subtrate-1 (IRS-1) knockout mice demonstrated a decrease in insulin sensitivity and reduced growth, however, in this study, the authors suggested that the defects are partially compensated by the existence of an IRS-1 independent pathway for insulin signaling (Araki *et al*., 1994). In obese IR individuals and obese patients with T2D, insulin-stimulated IRS-1 phosphorylation in skeletal muscle was decreased in relation to control individuals, without changes in protein expression (Cusi *et al.,* 2000; Kim *et al*., 2003; Krook *et al.,* 2000).

Alterations in PI3K expression and/or activity are also related with diabetic skeletal muscle, in fact, a similar effect to increased phosphorylation/activity of IRS-1 without

increasing protein expression was observed in PI3K from T2D patients (Zierath & Wallberg-Henriksson, 2002). These results are in accordance with data from studies performed in Zucker fatty obese rats in which impairment of PI3K activity was also observed (Asano *et al*., 2007). Altogether, an impaired association between PI3K with IRS-1 is a characteristic abnormality in T2D, and this defect correlate closely with *in vivo* muscle IR (Abdul-Ghani & DeFrozo, 2010). The mechanism relating PDK1 regulation in IR and T2D, by which insulin induces activation of Akt is still in debate. In the literature there are some reports that demonstrated a significant reduction of insulin-stimulated phosphorylation of Serine 473 or threonine 308 in skeletal muscle of T2D patients (Cozzone *et al*., 2008; Krook *et al*., 1998; Meyer *et al*., 2002) whereas others did not show alterations in the phosphorylation or enzymatic activity of Akt between control subjects and T2D patients (Cusi *et al*., 2000; Kim *et al*., 1999; Meyer *et al.,* 2002). Another topic with some controversy is insulin binding to the InsR and the protein expression of InsR in the skeletal muscle, with some authors describing a normal InsR binding and expression (Krook *et al.,* , 2000) and others describing an impairment in Insulin-InsR binding in the skeletal muscle of T2D patients (Maegawa *et al*., 1991). Additionally, the existence of glucose transport defects in T2D is controversial, with some authors showing that glucose transport is severely impaired in T2D patients (Krook *et al.,* 2000; Shepherd & Kahn, 1999) while others showed that skeletal muscle from lean and obese T2D exhibit normal or increased levels of GLUT4 messenger RNA (mRNA) expression and normal levels of GLUT4 protein, demonstrating that transcriptional and translational regulation of GLUT4 is not impaired (Bonadonna, 1993; 1996). Since the number of GLUT4 transporters in the skeletal muscle of diabetic subjects is normal (Anderson *et al.,* 1993; Kahn *et al*., 1992) impaired GLUT4 translocation and decreased intrinsic activity of the glucose transporter may be responsible for the defect in muscle glucose transport. In fact, several reports in the literature described that GLUT4 regulation and translocation are causally linked to IR states and obesity (Kahn, 2000). However, the data with knockouts for GLUT4 are not consensual with some authors showing that the animals are normoglycemic in both fasting and fed state (Katz *et al*., 1995), while others showed that knockout mice for GLUT4 have reduced glucose uptake in response to insulin with consequent hyperglycemia, glucose intolerance and IR (Zisman *et al*.,2000). Additionally, conditional GLUT4 knockout mice in the adipose tissue, showed IR and glucose intolerance without changes in fat mass or size (Abel *et al*., 2001).

1.2.3. Insulin action in the Adipose Tissue

Adipose tissue is another insulin-sensitive tissue that possesses a critical function in maintaining glucose homeostasis. Adipose tissue contributes with 2-4% for whole body glucose uptake (Shrayyef & Gerich, 2010). This tissue modulates whole body glucose metabolism by two main mechanisms: by regulating the levels of circulating FFAs and by secreting adipokines, a large number of hormones and cytokines which can affect energy homeostasis and insulin sensitivity. Thereby adipose tissue has been looked at as a powerful endocrine organ (Dyck, 2009; Kershaw & Flier, 2004; Lin & Sun, 2010 for review). Current theories state that whole body IR is due to an increase in FFAs secretion and impaired secretion of factors such as adiponectin (Lin & Sun, 2010, for review).

In the adipose tissue insulin stimulated glucose uptake promotes lipogenesis while suppressing lipolysis, and hence preventing FFAs acid efflux into the bloodstream. Adipocytes are not dependent of glucose and consequently, in insulin deficient states, the energy may be supplied to adipose tissue by fat oxidation that releases FFAs into the circulation. The FFAs cleared into the bloodstream may be directly utilized by organs like the heart and the liver, where FFAs are converted to ketone bodies (Wilcox, 2005). The increased in FFAs resulted from increased lipolysis secondary to adipose tissue IR induces or aggravates IR in liver and skeletal muscle through direct (Randle cycle) or indirect (from triglycerides deposits) generation of metabolites, altering the insulin signaling pathway (Delarue & Magnan, 2007). In T2D, the plasma concentrations of FFAs are high, showing an impaired insulin action on lipolysis (Reaven *et al*., 1988; Swislocki *et al.,* 1987). Moreover, in T2D patients the exogenous insulin administration fails to reduce lipolysis and also to decrease FFAs plasma levels (Groop *et al*., 1989). Also, plasma FFAs concentrations were correlates with the degree of hyperglycemia, skeletal muscle IR, and the risk of developing T2D (Roden, 2001). The potential relationship between FFAs and skeletal muscle glucose metabolism is described by Randle et al. (1963) who suggest a competition between FFAs and glucose for mitochondrial oxidation that would led to decreased glucose utilization in presence of increased lipid concentrations. In human studies, it was demonstrated that FFAs directly inhibit glucose transport and phosphorylation in skeletal muscle (Roden *et al.*, 2004). In liver, fat oxidation inhibits glucose oxidation and subsequent hepatic glucose production, which is correlated with fasting hyperglycemia. Stimulation of

gluconeogenesis is the main effect of FFAs on hepatic glucose metabolism (Yki-Jarvinen, 2005).

The adipose tissue releases a large number of bioactive mediators named adipokines, like adiponectin, resistin, visfatin, tumor necrosis factor alfa (TNF-α), interleukin 6 (IL-6), leptin and others, hormones, FFAs and proinflammatory cytokines which modulate lipid homeostasis, blood pressure, lipid and glucose metabolism, inflammation, and atherosclerosis (Rabe *et al.,* 2008; Scherer, 2006; Shoelson *et al.,* 2006). Interleukin 6, TNF-α, plasminogen activator inhibitor 1 (PAI-1), angiotensinogen and leptin are associated with increased IR and adiponectin with increased insulin sensitivity (Devaraj *et al.,* 2004).

In adipose tissue of T2D patients, GLUT4 protein expression and its mRNA are downregulated (Garvey e*t al.,* 1991; Shepherd & Kahn, 1999). These results are in accordance with studies using knockout mice for GLUT4 that demonstrated the development of IR in these animals, in a similar way to skeletal muscle GLUT4 ablation (Abel *et al*., 2001; Zisman *et al*., 2000). Interestingly, the knockout for GLUT4 in skeletal muscle showed an increase in glucose uptake by the adipose tissue (Kim *et al*., 2001), suggesting a crosstalk between adipose tissue and skeletal muscle and the key role of adipose tissue in the IR context.

Regarding insulin signaling cascade in adipose tissue there are some characteristic defects that occur. Several reports have shown that IRS-1 protein expression and activity is diminished in the adipose tissue in T2D as well as PI3K activity (Rondinone *et al*., 1997). Contrarily with what happen in the skeletal muscle, in the adipose tissue the insulin receptor substrate 2 (IRS-2) is capable to compensate for changes in IRS-1 (Krook *et al*., 2000). Additionally, Akt activation is impaired in adipose tissue of T2D subjects, primarily via a reduction in insulin-stimulated serine phosphorylation (Carvalho *et al*., 2000).

Exposure of adipocytes to TNFα, or to elevated FFAs stimulates phosphorylation of serines residues of IRS-1. This phosphorylation reduces tyrosine phosphorylation of IRS-1 in response to insulin (Aguirre *et al*., 2002).

One of the effectors of insulin both in the periphery and centrally, in the brain, are Kv1.3 channels. It is consensual that Kv1.3 is an important regulator of peripheral insulin sensitivity, glucose metabolism and also body weight (Xu *et al*., 2003; 2004).

2. VOLTAGE GATED POTASSIUM CHANNELS

Ions channels are integrated membrane proteins that regulate flux of ionic species, such as potassium (K^+), sodium (Na^+) and calcium (Ca^{2+}), through biological membranes. These proteins, which are specific for ionic species, undergo conformational changes to regulate the flow of ions through the ion channel, with direction and magnitude determined by the electrochemical gradient for the particular species (Jiang *et al.,* 2002). More than 100 types of ion channels have been described so far, and new ones are still being added to the list.

Potassium channels have a wide range of kinetic properties, structure, pharmacology and regulation, making them the largest and most diverse group of ion channels. In general, ions channels were initially categorized by their electrophysiological signature and pharmacology; however the classification of channels is currently based on sequence homology. There are three major classes of potassium channels, named based on the number of transmembrane (TM) domains within pore forming subunits (2TM, 4TM and 6TM) (**Figure 1.6**).

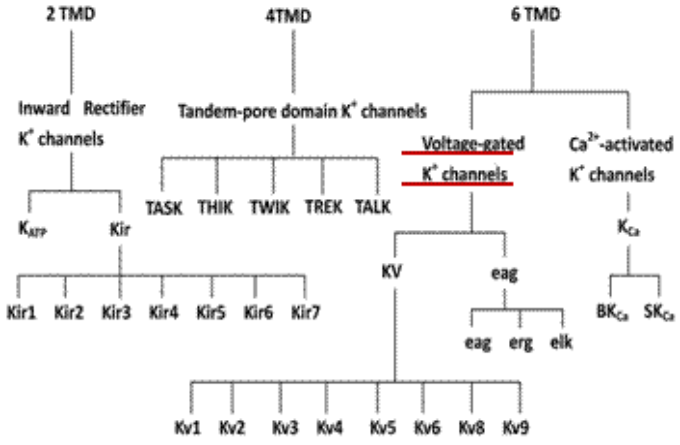

Figure 1.6: Classification of K^+ ion channels, based on the sequence homology. The three classes of K^+ channels are based on the structure and number of α subunits. TM: transmembrane domain; K_{ATP}, ATP-sensitive K^+ channel; Kir: inward-rectifier channel; TASK: tandem-pore domain K^+ channel; Kv: voltage-

gated K^+ channel; EAG: ether-á-go-go channel; K_{Ca}: Ca^{2+}-activated channel; BK_{Ca}, large conductance K_{Ca} channel; SK_{Ca}: small conductance: K_{Ca} channel. Adapted from (http://www.spandidos-publications.com/mmr/8/2/311).

Potassium channels primarily activated by voltage are termed voltage-gated K^+ channels (Kv), which represents the specificity for the K^+ and the mode of activation. These channels are involved in many important physiological functions, like the repolarization of the membrane after an action potential or the generation of depolarization in excitable cells (Hille, 2001). The first Kv1 sequence (also known as Shaker) was classified in *Drosophila melanogaster* and subsequent more subfamilies were identified, such as Shab, Shaw and Shal, corresponding to Kv2, Kv3 and Kv4, respectively, in humans (Hille, 2001; Papazian *et a*l., 1987). Currently this family is divided into 12 subfamilies which include Kv1 (KCNA), Kv2 (KCNB), Kv3 (KCNC), Kv4 (KCND), Kv7 (KCNQ, also named KQT), Kv10, Kv11 (KCNH, also named EAG) and Kv12. Kv5, Kv6, Kv8, and Kv9 channels which are not functional alone. These channels play a key role in the maintenance of resting membrane potential and in the control of action potentials by controlling the K^+ ion efflux from the cell and, therefore, modulate a large number of cellular processes (Hille, 2001; Yi *et al*., 2001). The typical structure consist of four α-subunits, each containing six transmembrane α-helical segments, S1–S6 with both N and C terminal in the intracellular part of the membrane, and a P-loop, which are arranged circumferentially around a central pore as homo-tetramers or hetero-tetramers (**Figure 1.**7) (Tian *et al*., 2014).

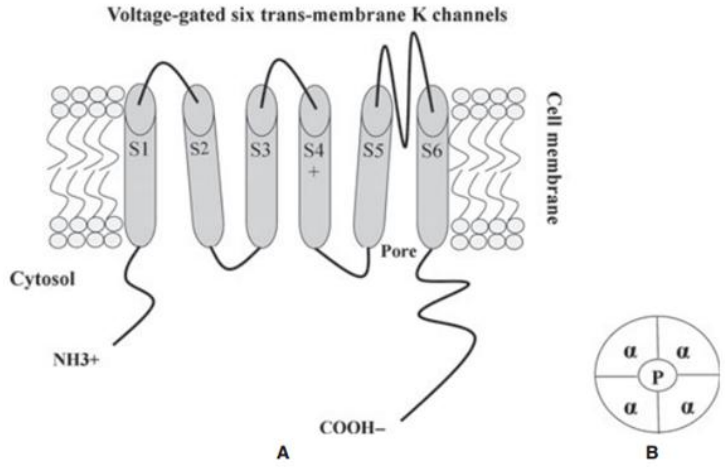

Figure 1.7: Schematic representation of the membrane topology and main features of the voltage activated K^+ (Kv) channel. A) Representive draw of the transmembrane segments with the pore regions formed by S5 and S6 segments, with both $NH3^+$ and $COOH^-$ terminals. B) Structure of the tetrameric assembly. Adapted from (Tian *et al.,* 2014).

The pore contains the selective filter (Zhou *et al*., 2001) through which K^+ and water molecules diffuse and a N and C terminal, the first one with a role in the inactivation gate and second one with a role in channel localization within membrane, with PDZ-binding motifs, respectively. The mechanisms by which the channels open and close (increasing or decreasing the K^+ channels current) are controlled by a voltage sensor, that causes the movement into the membrane with changes in the membrane potential and facilitate events (Aggarwal & MacKinnon 1996; Horn, 2004).

2.1. Kv1.3 channels

Kv1.3 is a delay-rectifier channel that belongs to the Shaker like family of Kv channels. In terms of biophysical properties it responds to voltage stimulation with a fast activation current (a fast Kv1.3 current is activated within few milliseconds τ_{activ} 3ms-20ms, at 40mV; Von= -50mV; V1/2=-30mV; k= 5-7mV (Coetzee *et al*., 1999; Gutman *et al*., 2005), whereas the inactivation is slower, taking up to some seconds (τinactiv= 250-600ms; V1/2=- 44,7mV) 7mV (Coetzee *et al*., 1999; Gutman *et al*., 2005). The inactivation rate is in between classically defined A-currents (τinactiv< 100ms) and delayed rectifiers (τinactiv>1s) (Conley & Brammar, 1998). The single channel conductance of Kv1.3 is 12 pS, and the voltage required for activation is -35 mV (Grissmer *et al*., 1994).

This channel is expressed in many tissues, including lymphocytes, central nervous system, kidney, osteoclasts, testis and insulin sensitive tissues (Tschritter *et al*., 2006; Xu et al., 2003). Classically, is viewed as an important dampener of neuronal excitability contributing to the resting membrane potential (Bean, 2007; Coleman *et al*. 1999; Doczi *et al.,* 2008; Grunnet *et al*., 2003; Jacob *et al*., 2000; Klumpp *et al.,* 1995; Mourre *et al*., 1999; Spencer *et al*., 1993; Veh *et al*. 1995). In central nervous system is involved in neurotransmitters release and neuronal excitability whereas in periphery has been implicated in the regulation of a wide of non-traditional functions, such as cellular proliferation, cell volume regulation, axonal targeting, insulin sensitivity, apoptosis, protein expression and scaffolding (Bean, 2007; Coleman *et al*. 1999; Doczi *et al.* 2008; Grunnet *et al*. 2003; Kaczmarek, 2006, for review; Klumpp *et al.* 1995; Mourre *et al*. 1999; Spencer *et al*. 1993; Veh *et al*. 1995; Jacob *et al*. 2000). Also, it was described that Kv1.3 channels participate in the pathways that regulate energy homeostasis and

body weight, since of this channel is expressed in postganglionic sympathetic neurons and influences sympathetic activity that regulates body weight and energy homeostasis (Doczi *et al*., 2008).

Kv1.3 channels are inhibited by 4-aminopyridine (4-AP) and tetraethylammonium (TEA), which are general K^+ channel blockers (Grissmer *et al.,* 1994). Also Psora-4 is other potent chemical inhibitor of Kv1.3, however with a less effect on the rest of Kv isoforms (Vennekamp *et al*., 2004). Highly specific toxins such as charybdotoxin and margatoxin (MgTx) (Garcia-Calvo *et al.,* 1993; Leonard *et al.,* 1992) as well as the anemone peptide Stichodactyla toxin (ShK) and their derivatives inhibit selectively the Kv1.3 channel. Kv1.3 channel is controlled not only by alterations in membrane potential but also by threonine, serine and tyrosine phosphorylation (Levitan, 1994 for review) that can be involved in the Kv channel modulation (Timpe & Fant, 1994). Insulin downregulates the activity of Kv1.3 channels (Bowlby *et al*., 1997).

2.1.1. Kv1.3 channels and glucose homeostasis

Kv1.3 is an important regulator of peripheral insulin sensitivity, glucose metabolism and also body weight (Xu *et al*., 2003; 2004). The knockout mice for Kv1.3 gene present a lower body weight and an increased basal metabolic rate, which protects from diet-induced obesity in relation to their littermates (Xu *et al*., 2003; 2004). Subsequently, Tucker et al. (2008) using knockout mice for Kv1.3 gene observed a reduced adiposity and total body weight due to increased locomotor activity and increased total energy expenditure, although without changes in blood glucose levels and insulin levels. The mechanism by which Kv1.3 regulates body weight is not understood yet, however this channel has been studied as a therapeutic tool in the treatment of obesity and IR (Xu *et al.,* 2003). In fact, animals submitted to a high-fat (HF) and fructose diet in the presence of a potent blocker of the voltage gated Kv1.3 channels, ShK-186, did not develop the effects associated with obesity such as, weight gain, adiposity and fatty liver (Upadhyay *et al*., 2013). In addition, these animals exhibited decreased blood levels of cholesterol, glucose, glycated haemoglobin (HbA1c), insulin, leptin and enhanced peripheral insulin sensitivity (Upadhyay *et al*., 2013). In the last decades a functional link between Kv1.3 channels and the InsR has been described, due to the presence of phosphorylation sites for tyrosine kinase in

Kv1.3 channels that regulate the activity of Kv1.3 in the olfactory bulb (OB) neurons (Fadool & Levitan, 1998). In fact, in humans a single nucleotide polymorphism, which promotes an increase in expression or activity of Kv1.3, is associated with impaired glucose tolerance (Tschritter *et al*., 2006). When Kv1.3 current are inhibited by MgTx or by gene deletion of Kv1.3 channel protein, the peripheral insulin sensitivity increases together with the increase in GLUT4 translocation to membrane in skeletal muscle and white adipose tissue through a PI3K-independent pathway via a Ca^{2+}-dependent mechanism (Li *et al*., 2006).

3. DISEASES ASSOCIATED WITH ALTERED GLUCOSE METABOLISM AND INSULIN ACTION

Alterations in glucose metabolism and insulin action in insulin-target organs result in major complications, such as, hyperglycemia, hyperinsulinemia, and hypertriglyceridemia, which are common features of T2D and metabolic syndrome (MS).

3.1. Epidemiology of metabolic diseases

The rapid increase of metabolic diseases, such as, MS and T2D, together with obesity gives them the status of a major epidemic of the XXI century and one of the major public-health problem of the actuality (Zimmet *et al*., 2001). It has been estimated that in 2015, 415 million people globally were affected by diabetes, and the number is expected to increase to 642 million in 2040 (IDF, 2015). Metabolic syndrome prevalence is rising, however it is difficult to know the exact numbers of the population affected because they depend on the population studied, on the region and, very importantly, on the definition of MS used, see below (Desroches & Lamarche, 2007). For example, in the United States the estimated prevalence of MS is nearly 35% of all adults and 50% in those aged 60 years or older (Aguilar *et al.* 2015; Ford, 2005).

It is not novel that abdominal obesity is a pathogenic feature that predisposes to development of MS and T2D, and together with IR represents a feature that triggers metabolic dysfunction, especially in early stages of these diseases (Bartnik *et al*., 2007; Dixon, 2010; Grundy *et al.,* 2005). The IR and the resultant hyperinsulinemia are

pivotal, and these pathological signs are thought to, apart from causing abnormal glucose metabolism, contribute to the pathogenesis of hypertension (HT), dyslipidemia and hyperuricemia in a complex manner (Bartnik *et al*., 2007; Dixon, 2010; Grundy *et al.,* 2005).

Metabolic diseases also represent an economic problem, due to the high-cost of treatment of these pathological conditions. Despite the growing evidence of the economic impact, the global response to this problem remains inadequate, being imperative the buildup of a concerned strategy to consolidate applied research in the field, in order to have an impact on healthcare and the economic system.

3.2. Metabolic Syndrome

Metabolic syndrome is defined by a cluster of interconnected physiological, biochemical and metabolic factors that directly increase the risk of coronary heart disease, other forms of cardiovascular atherosclerotic diseases, and T2D (Grundy *et al*., 2005; Wilson *et al.*, 2005). Its main components are atherogenic dyslipidemia, impaired glucose tolerance, HT, abdominal obesity and IR, with these last ones being considered central manifestations of the syndrome (IDF, 2006; Grundy *et al*., 2005). There are many international organization and expert groups, such as, the world as the World Health Organization (WHO), the European Group for the study of Insulin Resistance (EGIR), the National Cholesterol Education Program Adult Treatment Panel III (NCEP: ATPIII), the American Association of Clinical Endocrinology (AACE), the International Diabetes Federation (IDF) and others that attempted to develop clinical criteria for the MS diagnosis. Currently, the most widely used are those of the NCEP: ATP III and IDF focusing specifically on waist circumference, which is a surrogate measure of central obesity. In contrast, the AACE, WHO and the EGIR definitions are all largely focused on IR (Kassi *et al*., 2011).

The IDF has attempted to refine the MS definition in order to allow its use by different clinical and research groups, thereby enabling comparisons between results from different studies. According to IDF, the criteria for MS diagnostic are: central obesity (defined as waist circumference with ethnicity specific values) plus any two of the following four factors: raised triglycerides (≥ 150 mg/dl (1.7 mmol/L) or specific

treatment for this lipid abnormality), reduced high density protein (HDL) cholesterol < 40 mg/dl (1.03 mmol/L) in males and < 50 mg/dl (1.29 mmol/L) in females or specific treatment for this lipid abnormality, raised systolic blood pressure ≥ 130 or diastolic blood pressure ≥ 85 mmHg or treatment of previously diagnosed HT and raised fasting plasma glucose (FPG) ≥ 100 mg/dl (5.6 mmol/L), or previously diagnosed T2D . If above 5.6 mmol/L or 100 mg/dl, oral glucose tolerance test (OGTT) is strongly recommended but is not necessary to define presence of the MS (IDF, 2006) (**Table 1.1**).

The MS concept was suggested for the first time by Gerald Reaven, who described MS as "a cluster of cardiovascular risk factors" and named it "Syndrome X", thereby introducing the IR concept (Reaven, 1988). Twenty years later, the IR syndrome has gradually to become MS. Nowadays, the clinical definition of MS remains a topic that continues surrounded by controversy causing substantial misunderstanding (Alberti & Zimmet, 1998; Balkau & Charles, 1999; Einhorn *et al*., 2003; Grundy *et al*., 2004; Kassi *et al*., 2011; Parikh & Mohan, 2012). According to IDF (**Table 1.1)**, the presence of MS potentiates the cardiovascular risk; however this is not consensual as some clinical studies failed to demonstrate an aggravation of cardiovascular risk when single factors were diagnosed in separate (Kassi *et al*., 2011; Parikh & Mohan, 2012).

Table 1.1: The criteria for metabolic syndrome (MS) diagnose according to the International Diabetes Federation (IDF). For a person to be defined as having MS it should have: central obesity, defined as waist circumference) *with ethnicity values) plus any of the following four factors present in following table. Adapted from (IDF, 2006).

RAISED TRIGLYCERIDES	≥ 150 mg/dL (1.7 mmol/L) or specific treatment for this lipid abnormality
REDUCED HDL CHOLESTEROL	< 40 mg/dL (1.03 mmol/L) in males < 50 mg/dL (1.29 mmol/L) in females or specific treatment for this lipid abnormality
RAISED BLOOD PRESSURE	systolic BP ≥ 130 mmHg or diastolic blood pressure ≥ 85 mmHg or treatment of previously diagnosed HT
RAISED FASTING PLASMA GLUCOSE	(FPG) ≥ 100 mg/dl (5.6 mmol/L), or previously diagnosed T2D. If above 5.6 mmol/L or 100 mg/dl, OGTT is strongly recommended but is not necessary to define presence of the syndrome.

* If BMI is >30kg/m², central obesity can be assumed and waist circumference does not need to be measured. BMI: Body Mass Index; HDL: high density lipoprotein; OGTT: oral glucose tolerance test; FPG: fasting plasma glucose; HT: hypertension; T2D: type 2 diabetes.

It has been shown that the presence of MS confers a 5 fold increase in the risk of T2D over the next 5 to 10 years (Alberti *et al*., 2009). From the several components that are present in MS, the presence of IR significantly increases the risk of developing T2D (although approximately 25% of IR patients exhibit normal glucose tolerance) (Reaven *et al*., 2004), whereas atherogenic dyslipidemia (increased low-density protein (LDL), decreased HDL, and high blood triglycerides concentrations) are frequently associated with cardiovascular disease (Giugliano *et al*., 2006). Central obesity appears as the common component, which is associated with an increased risk for both diseases, metabolic and cardiovascular (Padwal & Sharma, 2010). Several studies demonstrated the association of MS and T2D, not only with increased risk of developing the latter, but also as predictive of new cases of T2D (Eckel *et al*., 2005; Hanson *et al*., 2002; Grundy *et al*., 2005). The underlying cause of MS keeps challenging the experts, but IR is considered a significant factor that leads to metabolic disturbances and subsequently to T2D (Hu *et al*., 2004) In fact, IR is a precursor of a variety of metabolic disturbances, including visceral obesity and T2D being also a risk factor for cardiovascular diseases including HT, dyslipidemia, disordered fibrinolysis, atherosclerosis and endothelial dysfunction (Pradhan *et al*., 2001) (**Figure 1.8**).

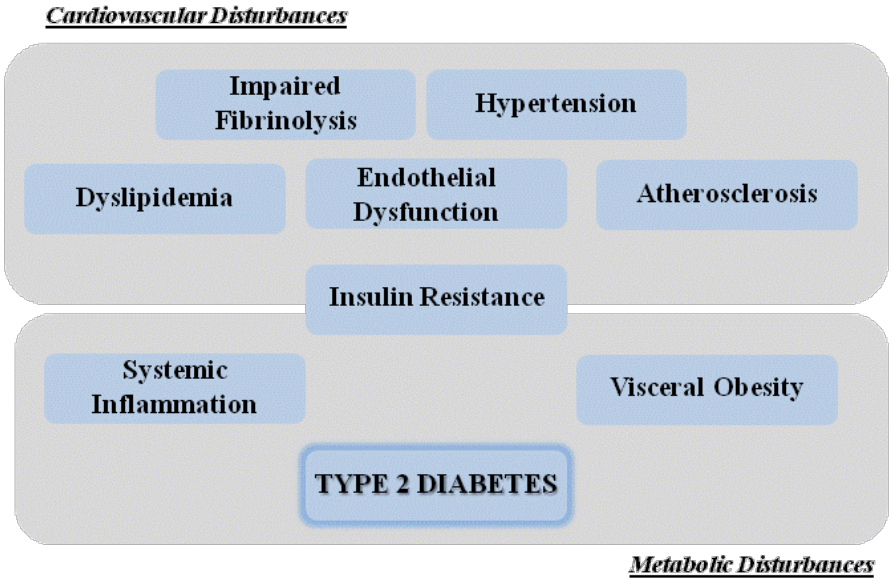

Figure 1.8: Diagram of insulin resistance as a core feature for the development of type 2 diabetes and a risk factor for both cardiovascular and metabolic complications.

3.3. Type 2 Diabetes

Diabetes mellitus, in a simplistic way, could be defined as a metabolic disorder or a chronic condition where the glycemia levels are high. It may be also defined as a chronic disease of carbohydrate metabolism due to the lack of insulin, or lack of insulin action, resulting in hyperglycemia and glycosuria (ADA, 2016). According with the WHO estimated that 442 million adults were living with diabetes in 2014, compared to 108 million in 1980. The global prevalence has nearly double since 1980, rising from 4.7% to 8.5% in the adult population (WHO, 2016). According with the IDF, in Europe, there are about 60 million people with diabetes, or about 10.3% of men and 9.6% of women aged 25 years and over. In Portugal, there were over 1 million cases of diabetes in 2015 (IDF, 2015) and if we take in consideration PREVADIAD, the first study that described the prevalence of diabetes in Portugal, the incidence of this disease is high (Gardete-Correia *et al*., 2010). If the pre-diabetic cases are taken in consideration, approximately one-third (34.9%) of the Portuguese population is affected (Gardete-Correia *et al*., 2010).

Diabetes can be classified into several categories, being type 1 diabetes and T2D, the most common. The first one occurs primarily due to autoimmune mediated destruction of pancreatic β-cell islets, resulting in absolute insulin deficiency (ADA, 2016). Its prevalence is low in comparison with T2D, which accounts for the majority of total diabetes prevalence (>85%) (Das & Elbein, 2006).

T2D is a progressive and complex heterogeneous group of metabolic conditions characterized by a combination of impaired insulin secretion by the pancreatic β-cells and IR in the target organs, leading to hyperglycemia (ADA, 2016). In an early stage of T2D, insulin hypersecretion by the pancreas occurs due to IR in several tissues which precede the development of hyperglycemia (**Figure 1.9)**. Briefly, IR leads to a compensatory increase in insulin secretion by the pancreatic β-cells, allowing the normoglycemiato be maintained due to pancreatic β-cell hypersecretory compensation (DeFronzo, 2004). The mechanism behind this β-cell compensation involves the expansion of cell mass, enhanced insulin biosynthesis and increased nutrient secretion coupling (Prentki & Nolan, 2006). In a second phase of the disease progression, pancreatic β-cells fail to compensate the IR during glucose uptake (i.e. after a meal), since they cannot produce sufficient insulin, leading to impaired glucose tolerance

(IGT). Overtime, the β-cell function deteriorates and when insulin secretion is no longer able to compensate the IR, hyperglycemia manifests and ensues T2D (DeFronzo, 1992; 2004) (**Figure 1.9**).

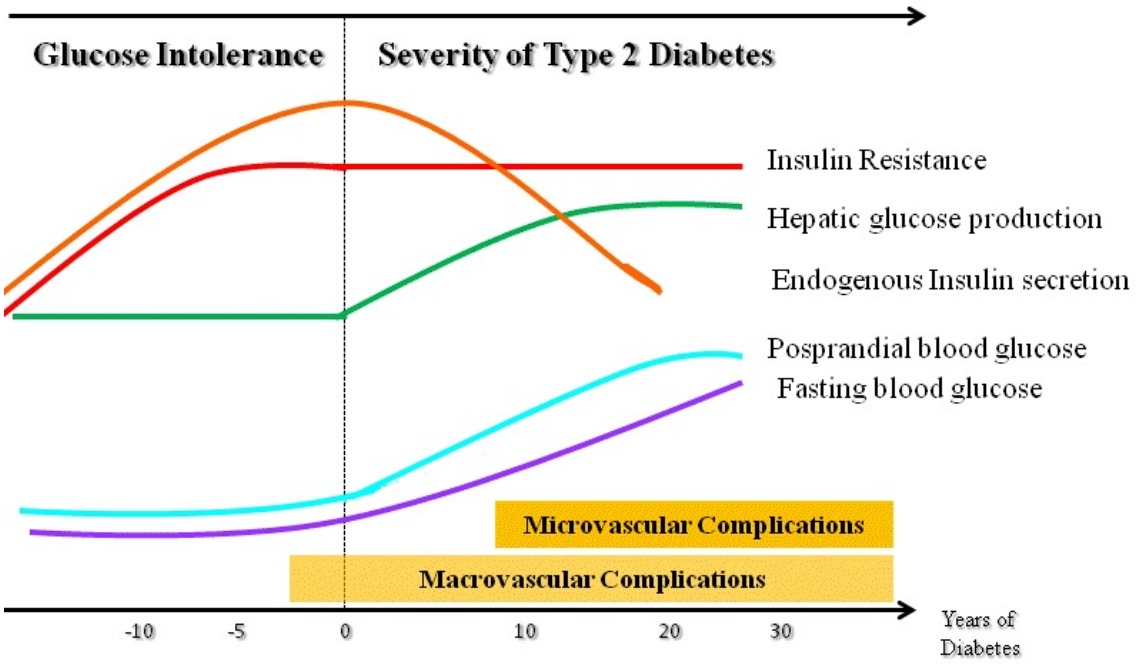

Figure 1.9: Metabolic alterations during progression of impaired glucose tolerance and subsequent type 2 diabetes onset. Firstly, pancreatic β-cells are able to compensate the hyperglycemia by increasing insulin levels, leading to an hyperinsulinemia that maintain glucose levels normalized for a period of time, however this period is characterized by an impaired glucose tolerance with a mild postprandial hyperglycemia.When the pancreas is no longer able to produce sufficient insulin to maintain normoglycemia, hyperglycemia appears and its exacerbation leads to the the development of type 2 diabetes. Adapted from (Ramlo-Haslted & Edelman, 2000).

There are multiple factors that contribute to pancreatic overstimulation leading to progressive β-cell dysfunction, such as genetic determinants, chronic inflammation, glucotoxicity and lipotoxicity (deleterious effects of highly levels of glucose and FFAs on β-cell, respectively (Cheng & Fantus, 2005).

Before the development of T2D, a prediabetic stage occurs being characterized by a state of abnormal glucose homeostasis, where IR, hyperinsulinemia and impaired glucose tolerance are present. Additionally, alterations in the levels of fasting glycemia can be present. For healthy individuals normal fasting (12 hours fast) glycemia levels are less than 100 mg/dl, whereas pre-diabetic fasting glycemia ranges from 100-125 mg/dl. In relation to the OGTT (where the levels of glucose 2 h post-administration

should be less than 140 mg/dl for healthy individuals, in pre-diabetic patients glucose levels range from 140 to 199 mg/ dl (Cefalu, 2001). When hyperglycemia exacerbates, by additional peripheral IR, pancreatic β-cell dysfunction, and/or increased hepatic gluconeogenesis, the consequence is T2D (ADA, 2004; Lillioja *et al*., 1993). According to the American Diabetes Association (ADA), diabetes may be diagnosed based on HbA1c (that is produced by non-enzimatic glycation of haemoglobin) or plasma glucose criteria, either the FPG or the 2 h plasma glucose values after a 75 g OGTT (ADA, 2016). The HbA1c blood test provides information concerning the average levels of glucose, over the past 3 months.

4. SYMPATHETIC NERVOUS SYSTEM AND INSULIN RESISTANCE IN METABOLIC DISTURBANCES

The sympathetic nervous system is an important component of the autonomic nervous system playing a major role in the maintenance of body homeostasis due its involvement in the control of cardiovascular system and metabolism (Thorp & Schlaich, 2015 for review). In high-energy expenditure situations, sympathetic activation leads to the release of norepinephrine from the nerve endings and stimulation of adrenergic receptors. The responses are organ-specific and depend on the adrenoreceptor isoforms expressed in the tissues. Until now at least nine subtypes of adrenoreceptors have been cloned, including six alpha (α) (α1A, α1B, α1D, α2A, α2B and α2C and three beta (β) (β1, β2 and β3) subtypes (for a review, see Lambert *et al*. 2010; Lynch & Ryall, 2008). Acute sympatho-excitation (**Figure 1.10**) leads to activation of hepatic sympathetic nerves, which stimulate glycogenolysis, in the fed state, and gluconeogenesis in fasting conditions. In the pancreas, sympathetic stimulation leads to increased glucagon release into the portal vein and to a moderate inhibition of insulin secretion. Activation of sympathetic fibers that innervate adipose tissue leads to lipolysis and release of FFAs into the circulation. In response to sympathetic stimulation of the kidney, renin is released and, at higher firing rates, sodium retention and local vasoconstriction also occur. In the adrenal glands, sympathetic stimulation causes release of epinephrine into the bloodstream. These effects, if sustained in the long-term, may contribute to the development of IR since they adversely affect metabolic control. Therefore, is not

surprising that sympathetic overactivity has been associated with several diseases, such as cardiovascular diseases (Graham *et al*., 2004), kidney disease (Converse *et al*., 1992), and metabolic disturbances, including T2D (Grassi *et al*., 2005; 2007; Huggett *et al*., 2003; Kobayashi *et al.,* 2010).

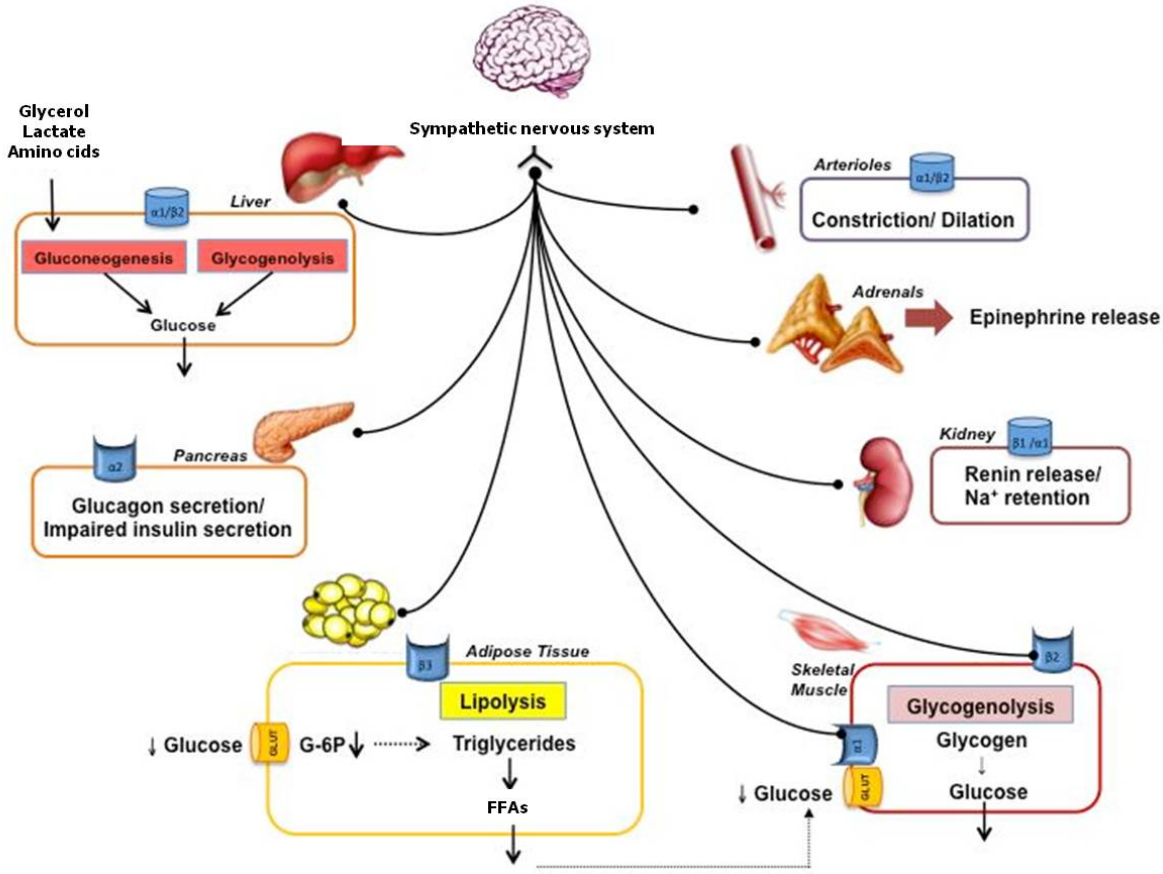

Figure 1.10: The effect of sympathetic nervous system activation and action in target organs through noradrenaline release. Physiological response is regionalized and is dependent upon the receptor present changing in fed or fasting state. In the liver, hepatic sympathetic nerves stimulates glycogenolysis, in fed state, and gluconeogenesis in fasting conditions. In the pancreas, sympathetic stimulation leads to increased glucagon release into the portal vein and to a moderate inhibition of insulin secretion. Activation of sympathetic fibers that innervate adipose tissue leads to lipolysis and release of FFAs into the circulation. In response to sympathetic stimulation of the kidney, renin is released and, at higher firing rates, sodium retention and local vasoconstriction also occur. In the adrenal glands, sympathetic stimulation causes release of epinephrine into the bloodstream. FFAs: free fatty acids; GLUT: Glucose transporters; G-6-P: glucose-6-phosphate; β1: beta-1 adrenergic receptor; β3: beta-3 adrenergic receptor; α1: Alfa -1-adrenergic receptor; Na^{+}: Sodium ion. Adapeted from (Conde *et al*., 2016).

It is known that chronic overactivation of sympathetic nervous system augments the risk for the development of MS since is involved in mechanisms that lead to obesity, hyperglycemia, IR and HT. While the exact mechanisms are yet to be fully elucidated,

several lines of evidence suggest that sympathetic nervous system overactivity is a key factor both in initiating and the maintaining metabolic abnormalities commonly seen in the metabolic disturbances. Several factors have been pointed out as possible causes of the increased sympathetic nerve activity seen in metabolic disturbances, namely hyperinsulinemia, hyperleptinemia, increased FFAs, inflammation, and obesity among others (for review see Lambert *et al.*, 2010; and Thorp & Schlaich, 2015) **(Figure 1.11).**

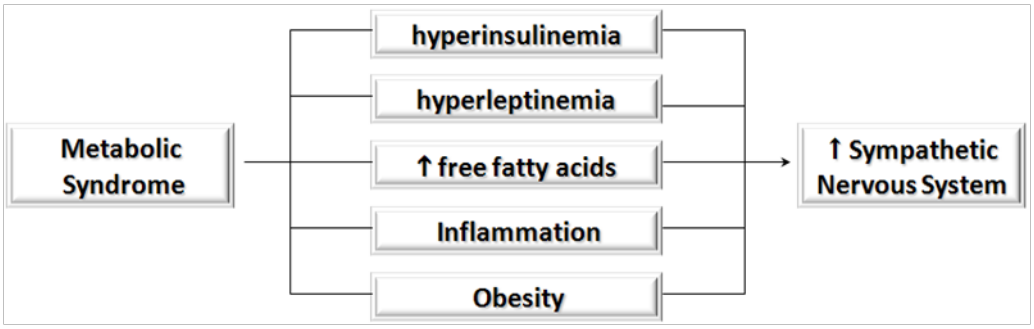

Figure 1.11: Activation of sympathetic nervous as a consequence of metabolic syndrome. Several factors present in subject with metabolic syndrome activate the sympathetic activation.

4.1. Sympathetic overactivity as a cause of insulin resistance

Despite increasing knowledge in this area of research, the precise mechanism and the evolutive pathochrony linking sympathetic overactivation, increased insulin secretion and peripheral IR is complex. Several theories have been postulated to link features of the MS with changes in sympathetic activation (**Figure 1.12**). Landsberg and Reaven´s work supports that overeating and obesity lead first to peripheral IR followed by compensatory hyperinsulinemia and subsequent sympathetic activation (Landsberg & Young 1978; Reaven *et al.* 2004). Alternatively, other groups have postulated that sympathetic overactivation is the trigger that initiates IR by compromising glucose disposal and lipid kinetics (Laakso *et al*. 1990; Jamerson *et al*. 1993). In fact, the increase in sympathetic nervous system activation causes acute IR in the forearm of healthy individuals (Jamerson *et al.,* 1993), an effect justified by the capacity of sympathetic nervous of acting as a vasoconstrictor, and by the consequent antagonism of insulin-mediated glucose uptake via a secondary effect on blood flow in skeletal muscle (Lasko *et al.,* 1990; Jamerson *et al*., 1993). This latter paradigm postulates that hyperinsulinemia is a compensatory mechanism for decreased glucose uptake at the

skeletal muscle caused by sympathetic overactivation (Julius *et al.,* 1992) and is supported by evidence derived from prospective trials, demonstrating that increased sympathetic activation precedes and predicts obesity and IR development (Flaa *et al.,* 2008; Masuo *et al.* 1997; 2000). This hypothesis was supported by other authors that showed that hyperinsulinemia is a consequence of sympathetic activation, principally when obesity is present (Mancia *et al*., 2007), due to the reduction of blood flow in skeletal muscle. At this line of thinking, it was observed that administration of peripherally acting vasoactive agents improve insulin sensitive in obese hypertensive patients (Pollare *et al.,* 1988; 1989).

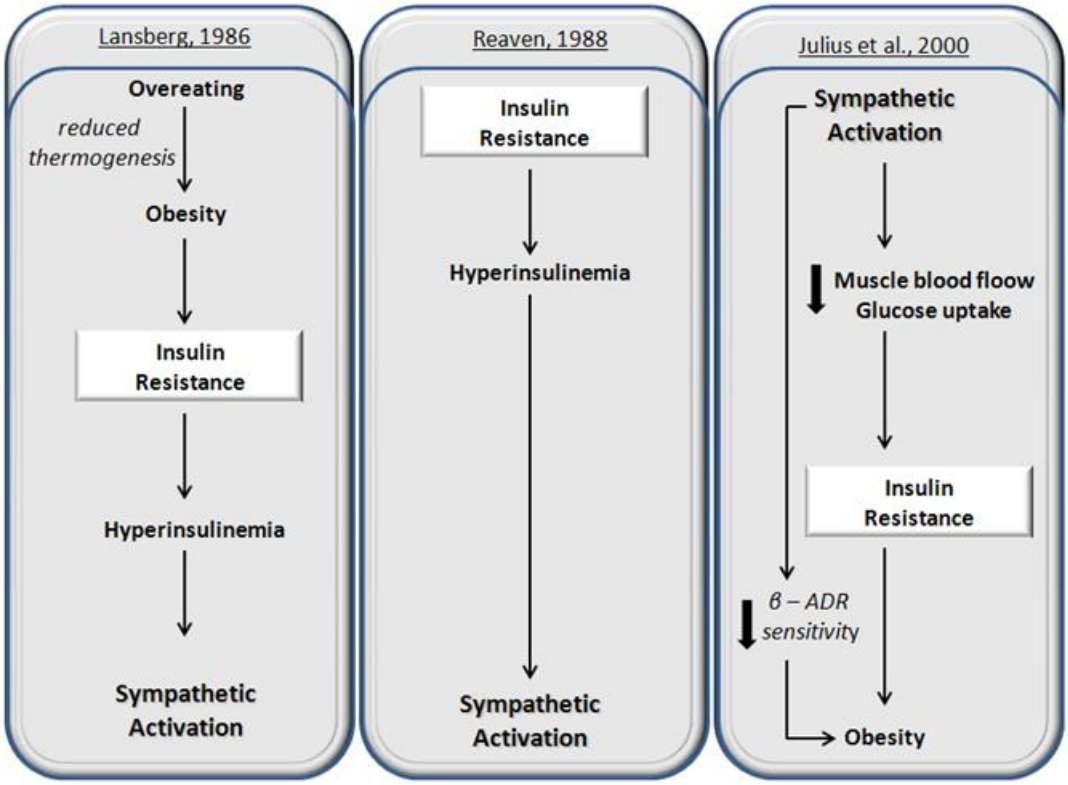

Figure 1.12: Theories linking hyperinsulinemia and sympathetic nervous system activation. From left to right: Landsberg, 1986 proposed that sympathetic activation represents an insulin-mediated adaptive response to overacting with promotes thermogenesis and acts as buffer against weight gain; Reaven, 1988 suggested that insulin resistance is the principally abnormality that leads to hyperinsulinemia, sympathetic activation, and hypertension; and Julius *et al*., 2000 proposed that increased sympathetic activity is the primary defect leading to insulin resistance and weight gain.β –ADR: Beta-adrenoreceptors.Adapted from (Lambert *et al*., 2010).

Also, α-adrenergic vasoconstriction resulting from chronic sympathetic activity can blunt post-prandial increases in skeletal blood flow impairing glucose uptake and stimulating additional insulin production by the pancreas leading to IR (Julius & Valentini, 1998). However, it is not well understood if skeletal muscle blood flow is reduced in human obesity and hence how it contributes to IR. In fact, the literature is controversial, with some works showing that obese hypertensive patients have an increase in the bloodstream in skeletal muscle (Raison *et a*l., 1988) whereas the studies performed by Ribeiro et al. (2001) and Kuniyoshi et al. (2003) documented an increase in skeletal muscle and forearm vascular resistance coupled with reduced forearm blood in obese women. Furthermore, Ribeiro et al. also recognized an association between the degree of sympathetic activation and reduction in forearm blood, since the diminution in forearm blood was proportional to the elevation in muscle sympathetic nerve activity (MSNA) (Ribeiro *et al*., 2001). Altogether the data demonstrate that elevated sympathetic nerve activity promotes IR through peripheral vasoconstriction.

Insulin resistance states are characterized by sympathetic predominance in a resting/basal state and reduced sympathetic responsiveness after physiological sympathetic stimuli. In fact, sympathetic nervous system responses to carbohydrate ingestion are blunted in insulin resistant states (Straznicky *et al*. 2015), and β-adrenoreceptor -mediated lipolysis and lipid oxidation in adipose tissue are severely impaired in obesity (Guo *et al*., 2014). Decreased responsiveness to sympathetic activation could be caused by polymorphisms in genes that are involved in catecholamines (CAs) signal transduction and have effects on fat cell lipolysis (Arner, 2001). Interestingly, sympathetic activation has also been associated with triggering of the hypothalamic-pituitary axis and to increased inflammatory cytokine production (Björntorp, 1995). Cortisol is associated with glucose intolerance and may be one of the pathophysiological mechanisms involved in IR modulated by sympathetic overdrive, although the presence of hypercortisolism in insulin resistant individuals is not ubiquitous. Chronic overactivation of the sympathetic nervous system also induces a proinflammatory state mediated by IL-6 production by adipose tissue, which results in an acute phase response by the liver, indicating that increased levels of inflammatory markers seen in insulin resistant states may also, at least in part, be mediated by the sympathetic nervous system. Pro-inflammatory cytokines also cause IR in adipose tissue, skeletal muscle and liver by inhibiting insulin signal transduction (deLuca &

Olefsky 2008). Noticeably, maintenance of all or part of the aforementioned adaptor responses induced by chronic activation of the sympathetic nervous system culminates into impaired insulin action.

4.2. Hyperinsulinemia contributes to sympatho-excitation

Among the several factors proposed to be responsible for increased sympathetic nerve activity in metabolic diseases lies hyperinsulinemia (Reaven, 1988; Landsberg 2005; Lambert et al., 2010; 2015). Increased insulin levels contribute to aggravate pathological features of metabolic disturbances by enhancing atherogenesis, increasing blood pressure and endothelial dysfunction, increasing adipose tissue mass and systemic inflammation and contributing to obesity and the development of T2D (Arcaro *et al*. 2002, Landsberg, 2005, Pedersen *et al.* 2015; Reaven, 1988).

The idea that insulin stimulates sympathetic nervous system activity emerged in the early 80's, with the finding that insulin infusion increased norepinephrine levels in healthy men (Rowe *et al*., 1981). After that, several studies were published, both in animals and in humans supporting the hypothesis that insulin increases sympathetic activation. Insulin-induced sympathetic activation has been suggested to be a cause of increased arterial pressure, since it increased norepinephrine levels in dogs (Liang *et al*., 1982), rats (Tomiyama *et al.,* 1992) and in humans (Anderson *et al*., 1991; Berne *et al*., 1992; Lembo *et al*., 1992; Rowe *et al*., 1981) during euglycemic clamps.

Also, euglycemic hyperinsulinemia in rats, promoted increases in the release of norepinephrine in nerve endings accompanied by changes in arterial pressure and heart rate (Edwards & Tipton, 1985). Even in concentrations within the physiological range insulin, exhibited marked, and potentially long-lasting, sympathoexcitatory effects (Vollenweider *et al*., 1994; Scherrer & Sartori, 1997). Sympathoexcitatory effects of insulin were later confirmed by direct recording of MSNA, where, in humans, insulin has shown to increase MSNA (Anderson *et al*., 1991; Scherrer *et al*., 1993; Vollewweider *et al.,* 1993), as well as norepinephrine levels (Anderson *et al*., 1991; Berne *et al.,* 1992; Lambert *et al*., 2010 for review) in euglycemic conditions. The MSNA response observed in response to insulin administration is both gradual (Anderson *et al*., 1991; Banks, 2004; Scherrer *et al*., 1993; Vollenweider *et al*.,

1993;1994) and sustained because MSNA remains increased even after plasma insulin levels return to baseline (Anderson *et al.,* 1991; Banks, 2004; Scherrer *et al.,* 1993; Vollenweider *et al.*, 1993;1994,). However, the discovery that insulin infusion did not increase sympathetic nerve activity in the skin in humans (Berne *et al*., 1992) and also that graded increases in plasma insulin failed to significantly increase renal and adrenal sympathetic activity in rats while leading to increased lumbar sympathetic nervous system activity, suggested that hyperinsulinemia produces regionally non-uniform increases in sympathetic nervous system activity (Morgan *et al*., 1993; Munztel *et al.,* 1994). Also, while some authors claim that the relationship between insulin concentrations and sympathetic nerve activity is dose-dependent (Anderson *et al,* 1991; Berne *et al.,* 1992), others have shown that this relationship is not apparent (Vollenweider *et al.,* 1993; 1994) attributing this effect to a saturation of the receptors needed for insulin to cross the blood brain barrier (Banks *et al.,* 1997; Dampney, 2011). The central nervous system has been proposed to be involved, at least in part, in the mechanism that drives sympathoexcitation during hyperinsulinemia (Rowe *et al*., 1981; Landsberg & Krieger, 1989; for review Dampney, 2011). In mice, parenteric administration of the necrosis-inducer gold thioglucose in the ventromedial portion of the hypothalamus abolished changes in cardiac norepinephrine turnover (Young & Landsberg, 1980). Also, in normotensive Sprague-Dawley rats insulin administration into the third cerebral ventricle increased lumbar sympathetic nerve activity (Muntzel *et al*., 1993a). More recently, in Sprague-Dawley rats, insulin administration in the arcuate nucleus and paraventricular nucleus produced an increase in spinal sympathetic outflow, mediated by dorsal hypothalamus and rostral ventrolateral medulla (Cassaglia *et al*, 2011; Dampney, 2011). In agreement with this insulin- central nervous system mediated effects, the injection of anti-insulin affibody at the arcuate nucleus prevented the sympathetic excitation induced by insulin (Luckett *et al.* 2013). Altogether these data demonstrated that insulin acts to increase sympathetic nervous system through a central mechanism. However, this effect cannot be exclusively assigned to the central nervous system, since the injection of insulin into the carotid artery of anaesthetized dogs produces an increase in blood pressure and sympathetic activity higher than the systemic insulin administration, being the affect abolished by ganglionic blockade (Pereda *et al*., 1962). Additionally, the fact that insulin transport through the blood brain-barrier is decreased (Kaiyala *et al*. 2000) or unchanged (Israel *et al*. 1993) both in animal models of diet-induced obesity and in insulin resistant patients (Heni *et al.* 2014;

Kern *et al*., 2006), suggests the existence of an insulin-sensitive sympatho-modulator in the periphery. In 2004, in a study performed by Bin-Jaliah et al., it was observed that insulin infusion in the rat, produces hypoglycemia, increases minutes ventilation and the rate of O_2 consumption, an effect that is mediated by the carotid body (CB), a peripheral chemoreceptor, since carotid sinus nerve (CSN) denervation blunted it (Bin-Jaliah *et al*.,2004).

5. THE CAROTID BODY

The CB is a peripheral chemoreceptor, located bilaterally in the bifurcation of the common carotid artery (CCA) into the internal and external branches, an area of high blood flow (**Figure 1.13**). The CB receives blood supply from a small branch of the external carotid artery being the organ with the largest blood flow of the entire organism, due to its small weight. Whether measured by gravidimetric or radioactive microsphere methods, the CB blood flow oscillates between 1.5 and 2 l/100 g/min (Gonzalez *et al*., 2010 for review). Accordingly to this high blood flow, the density of capillaries in the CB represents 25–33% of the surface of histological sections obtained from organs perfused at normal (80–100 mmHg) pressure.

The CB projects afferents to the CSN, which projects to petrosal ganglion and joins the glossopharyngeal nerve before it enters the cranium, more specifically in the nucleus solitary tract (NST) at medulla oblongata (**Figure 1.13**). The CSN joins with filament of *vagus* nerve to provide both sympathetic and parasympathetic influence over the CB (Gonzalez *et al*., 1994).

Classically, the CB senses changes in arterial blood such as low O_2 (hypoxia), high CO_2 (hypercapnia) and low pH (acidosis). Hypoxia and acidosis/hypercapnia activate the CB inducing an increase in the frequency of discharge in the nerve endings of the CSN. The CSN activity is integrated in NST to induce a fan of respiratory reflexes aimed to normalize the altered blood gases via hyperventilation (Gonzalez *et al*., 1994) and to regulate blood pressure and cardiac performance via an increase in sympathetic nervous system activity (Marshall, 1994). Sympathetic activity modulates (and most often increases) CB sensory activity primarily by altering CB blood flow. Parasympathetic

efferent nerve activity, on the other hand, has been proposed to inhibit CB chemosensory activity through a paracrine ATP effect on P_2X-receptors located on efferent nerve endings, resulting in the subsequent nitric oxide-mediated inhibition of type I cells of CB (Campanucci & Nurse, 2007).

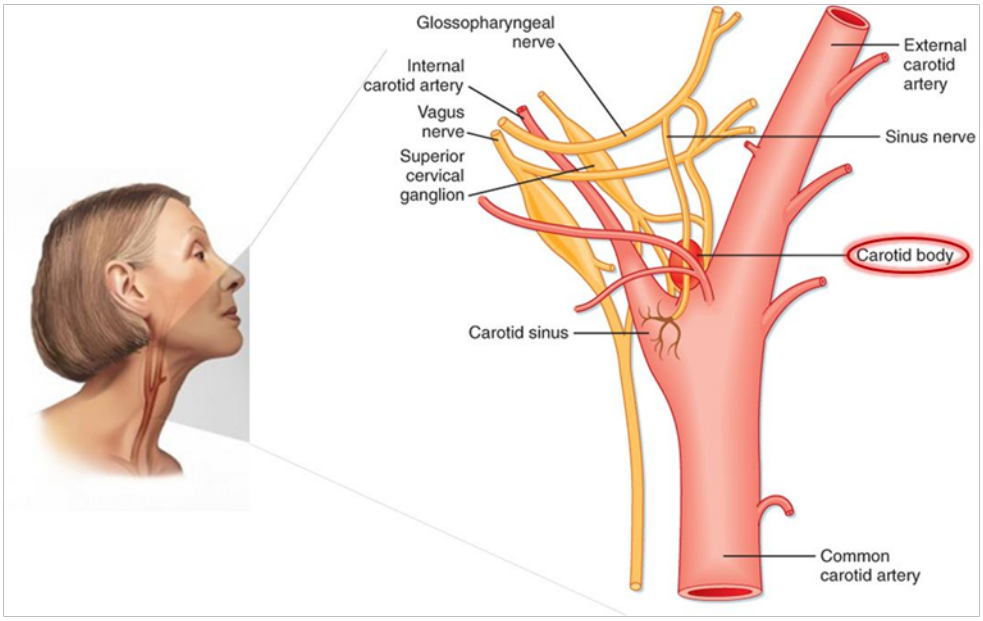

Figure 1.13. The Carotid body (CB) localization and innervation. Association of the CB to adjacent structures, arteries and nerves. Adapted from (Koeppen & Stanton, 2008).

The CB is composed of lobules or clusters of chemoreceptor cells, also known as glomus or type I cells with electrical excitable properties surrounded by glia-like type II or sustentacular cells, involved by a superficial connective tissue (**Figure 1.14**). The type I cells are the main cellular constituent of the CB and are generally accepted as its chemosensory unit. These cells, which are derived of the neural crest, contain secretory vesicles with several neurotransmitters, like CAs (dopamine (DA) and norepinephrine), serotonin, acetylcholine (Ach), neuropeptides (substance P and enkephalins), adenosine and ATP (Gonzalez *et al*., 1994; Zhang *et al*., 2000; Rong *et al*., 2003; Buttigieg & Nurse, 2004; Conde & Monteiro, 2004; Conde *et al*., 2012a). In addition, type I cells also contain voltage Na^+ and Ca^{2+} channels, as well as K^+ channels, and other ion channel types, as the ligand gated transient receptor potential and the background K^+ channels (Lopez-Barneo *et al*., 1988, Buckler, 2007). Regarding type II cells, they have been previously considered to have merely supportive role, however nowadays it has been proposed that the CB possess adult neural stem cells (or a subpopulation of them)

(**Figure 1.14).** It has been shown that these type II cells contribute to neurogenesis *in vivo* in response to prolonged hypoxia by acting in paracrine signaling and contribute to the growth of the organ at these conditions (Pardal *et al*., 2007; 2010; Piskuric & Nurse, 2013; Platero-Luengo *et al*., 2014).

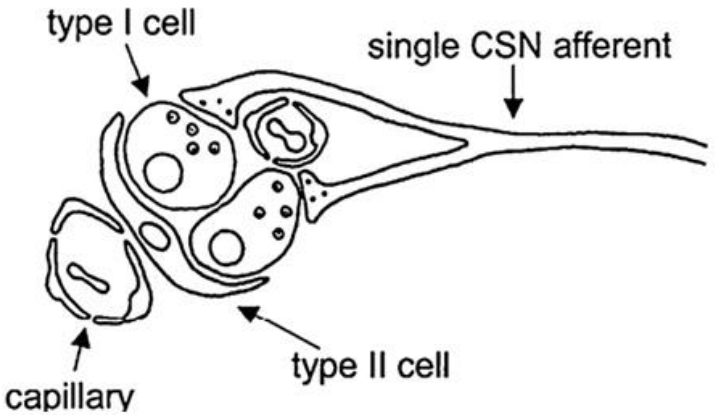

Figure 1.14: Basic cellular arrangement of the carotid body. Clusters of type I cells with neurotransmitters vesicles, surround by type II cells, receiving a rich supply of arterial blood. Type I cells are in synaptic contact with afferent chemosensory nerve fibers wich terminates in *cranium*. CSN: carotid sinus nerve. Adapted from (Peers *et al*., 2010)

5.1. Carotid body responses to chemical/physiological stimuli: coupling stimulation to secretion

Hypoxia is the classical stimulus for the CB chemoreceptors but besides it, the CB senses and responds to a multitude of stimuli, as hypercapnia/acidosis, hypoperfusion, hyperkalemia, hyperthermia and osmolarity (Gonzalez *et al*., 2010; Kumar & Bin-Jaliah, 2007; Kumar & Prabhakar, 2012; Peers *et al*., 2010). Despite the growing knowledge on the several stimuli that activate the CB, no consensus has been achieved on how the CB senses it most important stimulus, low arterial O_2, and translates it into increased chemosensory activity and to a systemic cardiorespiratory response. Last years, several hypothesis have been proposed to account for the ability to type I cells to respond to O_2, such as: 1) the membrane hypothesis which suggest a direct inhibition of oxygen sensitive K^+ channels; 2) the mitochondrial hypothesis, which suggest that the sensor is the inhibition of specific complexes in the electron transport chain and finally 3) the byosintetic hypothesis is which the sensor is the altered balance of reactive oxygen species (ROS) or 4) the energetic hypothesis in which the sensor is a change in

the energy status of the cell sense by 5´AMP-activated protein kinase (AMPK) (Peers *et al*., 2010; Kumar & Prabhakar, 2012, for review). However none of them are currently accepted, but it is believed that due to the quickness and stamina of the CB to respond to a fall in O_2, the mechanism behind this could be a mix between several parts of all theories. Despite the controversy on the nature of the O_2 sensor, it is generally accepted that the stimulus-secretion coupling in type I cells during CB activation by hypoxia occurs according to the following steps (Gonzalez *et al*,. 1992; 1994): 1) O_2-sensing at an O_2-sensor; 2) activation of coupling mechanisms with K^+ channels; 3) change in kinetics of these K^+ channels resulting in a decrease in their opening probability; 4) cell depolarization; 5) activation of voltage operated channels; 6) Ca^{2+} entry and increase in intracellular free Ca^{2+}; 7) activation of exocytosis and neurotransmitter release (**Figure 1.15**).

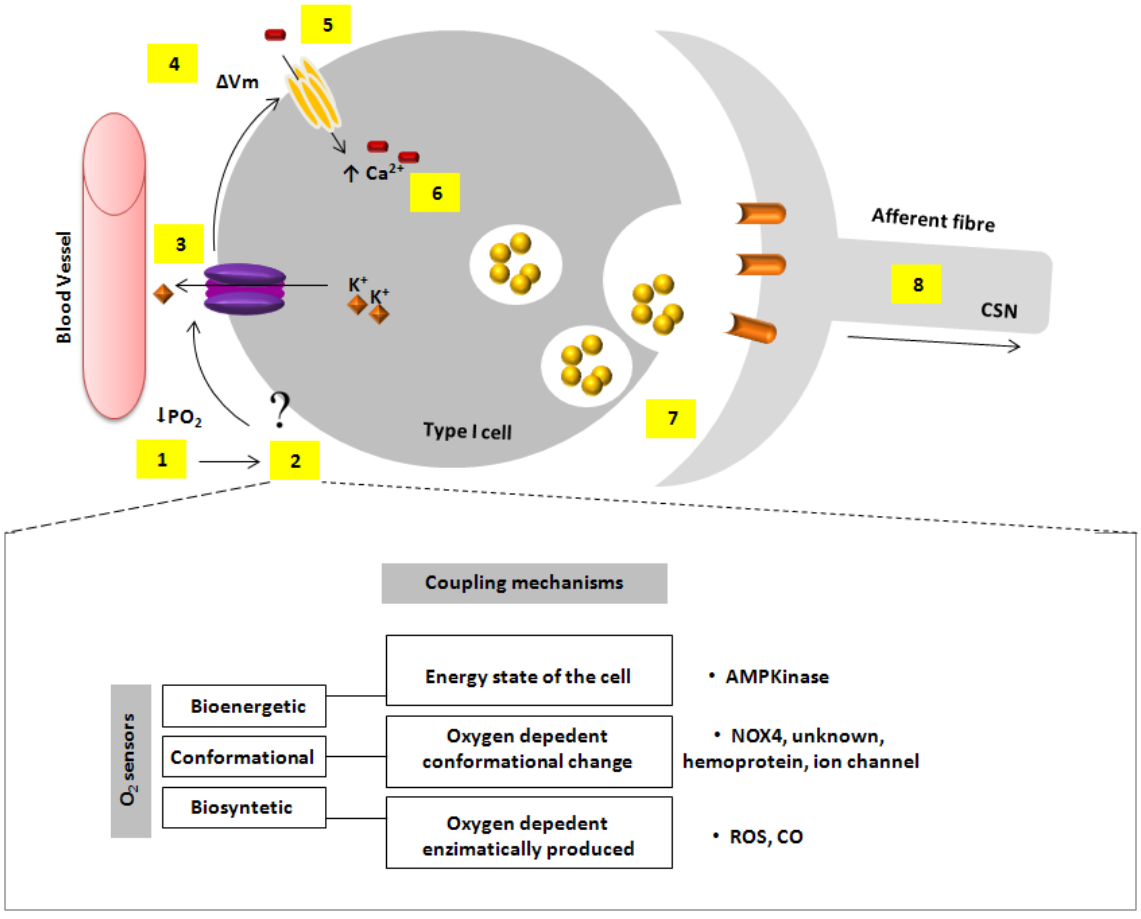

Figure 1.15: Oxygen transduction cascade and putative nature of O_2 sensor(s) in carotid body (CB) cells. 1) O_2 sensing at an O_2 sensor; 2) Activation of coupling mechanisms with K^+ channels; 3) change in kinetics of these K^+ channels result in a decrease in their opening probability; 4) cell depolarization; 5) activation of Ca^{2+}voltage operated channels; 6) Ca^{2+} entry and increase intracellular free Ca^{2+}; 7) exocytosis and neurotransmitter release; and 8) increase in the frequency of action potentials of carotid

sinus nerve , which send information to central nervous system. ROS: reactive oxygen species; CSN: carotid sinus nerve; ΔVm: change in membrane voltage; CO: carbon monoxide.

5.1.1. Oxygen sensing: role of potassium channels

It is clear that depolarization induced by closure of selective K^+ channels, located in the plasma membrane of type I cells, is a critical step in the hypoxia transduction process. The membrane hypothesis for chemotransduction at the CB suggest the ion channels as are the key component of the oxygen-sensing capabilities of type I cells. In 1988, Lopez-Barneo et al. (1988) reported that rabbit CB type I cells express oxygen-sensitive K^+ channels by demonstrating that voltage activated K^+ channels current decreases in the presence of low O_2 levels. Nevertheless, since that earlier finding a lot of controversy emerged on the specific molecular nature of the K^+ channels involved in the hypoxic response, with a lot of differences being found between and within species, as well as, between different animals age making difficult to define the identity of the channels responsible for initiating depolarization in type I cells (Buckler *et al*., 2007; Lopez-Lopez *et al*., 2007; Peng *et al.,* 2003) (**Table 1.2**). Specifically in rat, there are development changes in the expression of O_2 sensitive K^+ channels which might account for, or contribute to, post-natal maturation of O_2 sensitivity (Wasicko *et al*., 2006). Despite the differences in animal specie that account for some of this diversity several evidences indicate that more than one O_2-sensitive K^+ channel can be found within cells of the same specie. A number of different K^+ channels have been implicated in hypoxia sensing in the CB, including voltage dependent and voltage independent K^+ channels (Lopez-Lopez *et al*., 2007; Patel & Honore, 2001;Peers & Kemp, 2001). In rat type I cells, there are two families of K^+ channels that are reported: the high conductance, Ca^{2+} sensitive K^+ channels (BKCa) (Peers, 1990; Waytt & Peers, 1995) and TASK-like members of the tandem-P domain "leak" K^+ channel (Buckler, 1997; Buckler *et al.,* 2000; Peers & Wyatt, 2007). More recently, Kim et al. (2009) have postulated that the predominant oxygen sensitive, TASK-like current in the rat is carried by a heteromultimer of TASK-1 and TASK-3. These results can help to explain the previous findings obtained by Buckler (2007) in where is indicated that the oxygen sensitive TASK-like current had both TASK-1 like and TASK-3 like properties (Buckler, 2007). Reflecting the difference in species, it has been shown in the rabbit that

Kv4 channels exhibit oxygen sensitivity, while in the mouse Kv3 channels also exhibit oxygen sensitivity (Lopez-Lopez *et al*., 2007; Sanchez *et al.*, 2002) (**Table 1.2).**

Table 1.2. Different K^+ channels identified in carotid body (CB) type I cells in different species. Adapted from (Peers *et al*., 2010).

Specie	*Channel*
Rat	Task-like "leak" (TASK-1/-3) MaxiK (Bkca)
Rabbit	Voltage-gated, inactivating Kv4.1/4.3 HERG-like
Mouse	Voltage-gated, inactivating Kv3.1-3.3 Voltage-gated, possibly Kv1.2 MaxiK (Bkca)
Cat	Voltage-gated, charybdotoxin insensitive

The oxygen sensitivity of Kv4 and Kv3 in the rabbit and the mice, respectively, do not exclude the possibility of having other types of channels with oxygen sensitivity in these species. Additionally, it has been shown that hypoxia modulates a voltage dependent, transient outward K^+ current in rabbit (Lopez-Lopez *et al*., 1993; Sanchez *et al.,* 2002) and selectively affected the fast deactivation component in mouse (Perez-Garcia *et al*., 2004) whereas in the cat affected a non-inactivating K^+ current (Chou *et al.,* 1996).

5.2. Carotid body responses to chemical/physiological stimuli: coupling secretion to carotid sinus neural activity

Among the neurotransmitters contained in type I cells are CAs (DA and norepinephrine) serotonin, ACh, GABA, neuropeptides (substance P and enkephalins), adenosine and ATP (Buttigieg & Nurse, 2004; Conde & Monteiro, 2004; Gonzalez *et al*., 1994; Zhang *et al*., 2000). All these substances, as well as their agonists and antagonists, are capable of modifying, CSN activity.

Within the proposed hypotheses that account for stimulus-generated neural activity from the CB, the cholinergic hypothesis was the first one. Several authors described the

presence (Eyzaguirre *et al*., 1965; Hellstrom, 1977) and subsequent release of ACh from CB (Shirahata *et al.,* 1996), as also the enzymatic machinery involved in the generation and inactivation of this neurotransmitter in CB cells (Fitzgerald, 2000; Nurse & Zhang, 1999; Wang *et al.*, 1991; Nurse & Zhang, 1999).

These results are surrounded by controversy as Almaraz's et al. (1983) and Gauda (Gauda, 2002; Gauda *et al*., 2004) did not observe any ACh synthesis, acetylcholinesterase activity or expression of the vesicular ACh transporter. More recently, it was shown that the application of nicotinic ACh antagonists, into co-cultures of type I cells and "juxtaposed" petrosal ganglions (Zhang *et al*., 2000) and in the CB-CSN preparation (Reyes *et al*., 2007a; 2007b), only partially inhibited the hypoxia-evoked excitatory postsynaptic responses, questioning the cholinergic hypothesis (Zapata, 2007 for review). Previously, Nurse and co-workers already suggested that the excitatory effects of ACh on CB involved the release of ATP (Zhang *et al*., 2000), since they observed that application of suramin (P_2X ATP receptor antagonist) and mecamylamine (nicotinic ACh receptor antagonist) suppressed hypoxic-evoked electrical activity in post-synaptic petrosal ganglion preparations. This cholinergic-purinergic hypothesis is far from being consensual, as the application of the same nicotinic and P_2X blockers did not completely suppress CSN chemosensory activity *in vivo* in the cat (Reyes *et al*., 2007a).

Dopamine was also considered as a leading candidate for neurotransmission at synapses between type I cells and CSN nerve endings in early studies. Catecholamines synthesis, particularly of DA, increases in type I cells in response to both acute (Fidone *et al*., 1982, Vicario *et al*., 2000) and chronic hypoxia (Conde *et al*., 2012a; Pequinot *et al*., 1987), suggesting an increase of its utilization in response to physiological stimuli. Also, all the stimuli that activate the CB (hypoxia, hypercapnia, cyanide $(CN)^-$, high extracellular K^+, induces the release of DA in several mammalian species, being the release proportional to stimulus intensity, to the increase in CSN activity and dependent on extracellular Ca^{2+} (Fidone *et al*., 1982; Obeso *et al.,* 1985, 1986, 1992, 1999; Rigual *et al*., 1986; 1991; 2002; Rocher *et al*., 1991; Vicario *et al*., 2000) (**Figure 1.16**). Dopamine is the most well characterized neurotransmitter in the CB, its role in chemotransduction is still controversial (see Gonzalez *et al*., 1994; Nurse & Piskuric, 2013). In fact, while initial studies on the effect of DA on ventilation have suggested an excitatory effect of this amine (Black *et al*., 1972; Jacobs & Comroe, 1968), later

publications indicated that intracarotid injections or infusions of DA mostly depress ventilation in cats, dogs, rats, goats and new born lambs (for a review see Zapata, 1997), with the effect disappearing after CSN section. The contradictory role of dopamine may be related with a dose-dependent effect, since low doses of dopamine depress CSN activity whereas high doses have an excitatory outcome (Zapata, 1997).

More recently an important role in chemotransduction has been assigned to ATP, essentially as an excitatory neurotransmitter (**Figure 1.16**). In 1983, McQueen and Ribeiro have shown, for the first time that ATP increased CSN activity in a dose-dependent manner. After that pioneer study, Zhang et al. (2000) showed that the application of blockers of P_2X receptors decreased the hypoxia-evoked excitatory postsynaptic responses, an effect that was also observed in P_2X_2 receptor knockout mice, that had a markedly attenuation in ventilatory response to hypoxia (Rong *et al*., 2003), and by the finding that ATP is released from rat CB in hypoxia (Buttigieg & Nurse, 2004; Conde *et al*., 2007; 2012b). Thus, in response to hypoxia, the type I cells depolarize and release ATP, which acts postsynaptically in $P_2X_{2/3}$ receptors on afferent nerve terminals.

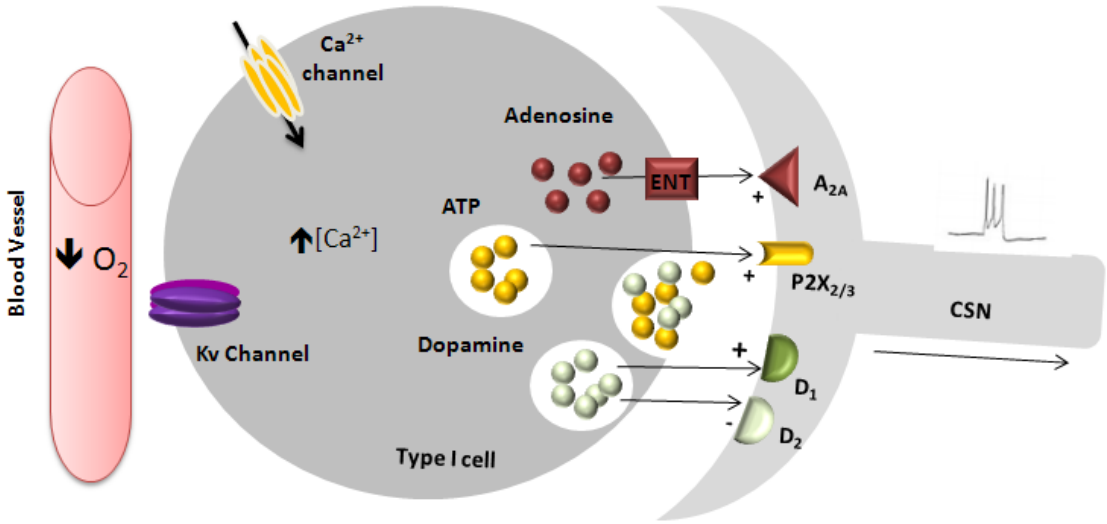

Figure 1.16: Simplified schematic diagram of the hypoxic transduction cascade and the well characterized neurotransmitters release from the carotid body (CB) in response to hypoxia. ATP, adenosine and dopamine are release from in type I cells and act on their receptors present in tthe carotid sinus nerve (CSN) to modulate its activity. Apart from ATP, adenosine and dopamine type I cells contain more neurotransmitters, like acetylcholine (ACh), 5-hydroxytryptamine (5-HT); histamine, GABA among othets. A_{2A}: Adenosine receptors; $D_{1\text{-}2}$: Dopamine receptors; $P_2X_{2/3}$ purinergic receptors; ENT: equilibrative nucleoside transporter +: stimulation; -: inhibition.

Adenosine is both a catabolic product and a precursor of ATP and has been defined as an excitatory neurotransmitter in the CB (**Figure 1.16**). Exogenously applied adenosine is known to increase CSN chemosensory discharges both *in vivo* (McQueen & Ribeiro, 1981; 1983) and *in vitro* (Runold *et al*., 1990). Adenosine, and its analogs, stimulate ventilation in a dose-dependent manner in several species, including humans and rats (see Conde *et al*., 2009), an effect that is mediated by A_2 receptors and abolished by CSN section (Monteiro & Ribeiro, 1987). The implication of these findings on CSN firing rate and ventilation control became obvious by the demonstration that mild hypoxia augments adenosine release from the rat CB (Conde & Monteiro, 2004). Adenosine mediates 60% of the low PO_2-induced CSN activity in the rat CB-CSN through its action on A_{2B} pre-synaptic and A_{2A}, postsynaptic receptors (Conde *et al*., 2006). More recently, it was demonstrated that both adenosine and ATP are the main players of the hypoxic chemotransmission in the CB sensory synapse, the contribution of each neurotransmitter depending on the intensity of hypoxia (Conde *et al*., 2012b). Therefore the excitatory effects of adenosine on the CB via A_{2A} and A_{2B} receptors, together with the activation of P_2X ATP receptors, is primordial in the genesis of CSN chemoresponses to hypoxia. Additionally, it has been shown that adenosine is involved not only in the response to acute hypoxia but also contributes to fix CSN basal activity during chronic intermittent hypoxia, being involved in hypoxic CB chemotransduction in chronic intermittent hypoxia and in chronic sustained hypoxia (Conde *et al*. 2012c; Sacramento *et al*. 2015).

Nowadays we possess plenty of information about the identity of the neurotransmitters involved in the hypoxic response in the CB, however several pieces of the puzzle remain to be discovered regarding the neurotransmitters involved in the response to other stimuli, like hypercapnia/acidosis among others. It can be postulated that different stimuli will activate different sensors, originating different intracellular responses and culminating in the release of different neurotransmitters, or in different concentrations. In fact, it has already been shown that in the rabbit and in rat type I cells, the release of CAs and of ATP/adenosine and the mechanism of stimulus-secretion depends on the type of stimuli like: high K^+ of stimulation (Nurse, 2014; Rocher *et al*., 2005); hypoxia, hypercapnic acidosis; or dinitrophenol, and on the intensity of stimulation (Conde *et al*., 2012b; Nurse, 2014; Rocher *et al*., 2005).

5.3. Chemoreflex initiated by carotid body

The CB is mainly known for its role in the control of ventilation, with the respiratory response to CB stimulation being the most expected. Increases in respiratory rate, tidal volume (VT), airway secretions and airway resistance are associated with the ventilatory responses to several physiological stimuli such as hypoxia, hypercapnia/acidosis (Fitzgerald *et al.*, 2009) or even hypoglycemia (Bin-Jaliah *et al*., 2004) (**Figure 1.17**). This organ is pivotal in the ventilatory changes associated with exercise, pregnancy and adaptation to high altitude (Kumar & Prabhakar, 2012). For example, in humans in which the CBs have been surgically removed, a common practice in the 1950´s and 60´s to treat severe asthma, the hyperventilatory response to hypoxia was absent and the ventilatory response to exercise was reduced by 30% (Kumar & Prabhakar, 2012 for review). Similar results have been found in animals: the response to hypoxia was abolished by CSN section (for a review see Gonzalez et al. 1994). Additionally, an important role has been attributed to CB chemoreceptors in maintaining resting ventilation. The CB resection or CSN denervation produces alterations in the resting respiratory parameters, like a decrease in minute ventilation (VE) and a moderate increase of PCO_2 (8 - 10 mmHg) (Bisgard *et al*., 1976; Bisgard & Vogel, 1971; Eugenin *et al*., 1989; Feustel *et al*., 1981). At normal arterial blood gas pressures and pH, type I cells possess a basal activity that can be measured as basal release of neurotransmitters or as basal CSN electrical activity (Gonzalez *et al*., 1994 for review).

In contrast with the respiratory responses, the cardiovascular responses to CB stimulation have been involeved in a lot of debate. They have been extensively studied in the 70's and the 80's although, as pointed out by Marshall (1994), multiple factors have contributed to an uneven interpretation of results, such as hyperventilation, hypocapnia, pulmonary stretch/vagal activation, central respiratory drive, baroreceptor involvement, circulating CAs and the preparation studied (species, awake/anesthetized animal, among others). Cardiovascular responses can be smaller and slower than respiratory responses, they can be modified by respiratory responses or even be secondary to respiratory responses, as it happens with arterial HT associated with obstructive sleep Apnea (OSA), however they are not less important.

Fitzgerald's group recently published results that contribute to clarify the role of the CB in the autonomic control of cardiovascular system and in the autonomic regulation of organ vascular resistances (Fitzgerald *et al*. 2013a; 2013b). They showed in anesthetized, paralyzed and artificially ventilated cats that hypoxic hypoxia (10% O_2 in N_2), that stimulates both CBs and aortic bodies, and carbon monoxide hypoxia (30% O_2 in N_2 with CO addition), that stimulates only the aortic bodies, induced an increase in cardiac output, cardiac contractility, systolic/diastolic pressure, aortic blood pressure, total peripheral resistance and pulmonary arterial pressure (Fitzgerald *et al*., 2013a). They have showed that CBs have a higher effect in the majority of these variables, except for blood pressure – whose major control seems to be assured by the aortic bodies (Fitzgerald *et al*., 2013a). Also, the same authors performed a parallel study to evaluate the contribution of arterial chemoreceptors to organ vascular resistance, having shown that CB stimulation increased sympathetic vasoconstrictor outflow to several organs, increasing the majority of vascular resistances, namely to the brain, spleen, muscle, splanchnic area and pancreas among others (Fitzgerald *et al*., 2013b) (**Figure 1.17**). The same group showed that CB stimulation decreased pulmonary vascular resistance, bronchial vascular resistance, ocular vascular resistance, and venous capacitance (Fitzgerald *et al*., 2013b). One surprising result obtained by Fitzgerald et al. (2013b) was the absence of effect of CB stimulation on the renal vasculature, since CB stimulation increases renal sympathetic activity (Marshall *et al*., 1994 for review) affecting kidney performance, renin and vasopressin secretion (Iovino *et al*., 2012) also leading to an increase in renal water and sodium excretion (Karim *et al*., 1987; Honig, 1989). In fact several authors did report that stimulation of CB results in a significant decrease in renal blood flow and a decreased filtration rate, effects that are abolished by CB denervation (Behm *et al*., 1993; Karim *et al*. 1987). Carotid body stimulation may also interfere with hemodynamic parameters through direct activation of the adrenals, via increased sympathetic drive, causing a release of corticosteroids and CAs from the adrenal glands (**Figure 1.17).**

The first study that investigated the reflexes from CBs upon the adrenals dates from the 60's and it was observed that CB stimulation releases corticotrophin from the anterior lobe of the pituitary gland, and thus corticosteroids from the adrenal cortex (Anichkov *et al*., 1960). Twenty years later, Critchley et al. (1982) showed that hypoxic-CB stimulation induced a release of CAs from the adrenal medulla, an effect abolished by

denervation of the adrenal gland the immediate release of CAs (Critchley *et al*., 1982). As a whole, although the CB is mainly known for its role in the control of ventilation, it also has key roles in the control of cardiovascular, endocrine and renal systems.

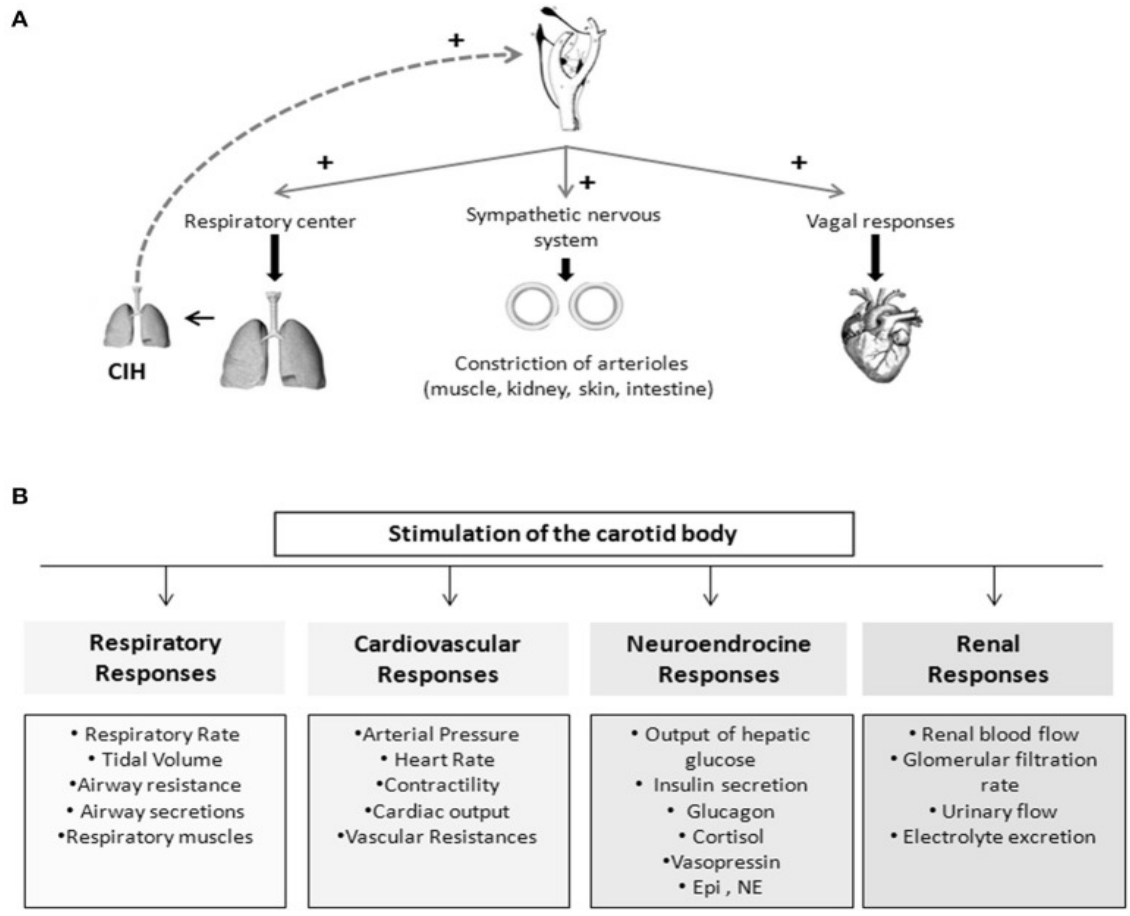

Figure 1.17: Schematic representation of the chemoreflexes elicited by the carotid bodies (CBs). A) Representation of important mechanism involved in the reflex-responses elicited by the carotid body. B) Stimulation of the CB is capable of produce cardiovascular, respiratory, endocrine, and renal responses (Conde *et al*., 2014). CIH: Chronic intermittent hypoxia; Epi: Epinephrine; NE: Norepinephrine.

5.4. Carotid body and sympathetic mediated diseases

Stimulation of the CB modulates systemic sympathetic tone, being CB activity critical for the development and progression of several sympathetic mediated-diseases. In last decades, several works demonstrated that increased CB activity is linked to sympathetic overactivation present in essential HT (Abdala *et al*., 2012; Paton *et al*., 2013) associated with OSA (Del Rio *et al.*, 2010; Marcus *et al*., 2010; Prabhakar & Peng, 2004) and chronic heart failure (Del Rio *et al.* 2013; Schultz *et al*., 2013). The first

work demonstrating that CB chemoreceptors are involved in the progression of chronic intermittent hypoxia-induced HT dates from 1992 (Fletcher *et al*. 1992). In this work, it was showed that bilateral CB denervation prevented the development of HT in rats exposed to chronic intermittent hypoxia (that mimics OSA in Humans) during 35 days (Fletcher *et al*. 1992). Moreover, subsequent works demonstrated that chronic intermittent hypoxia resulting from sleep-disordered breathing leads to an overactivation of the CB, manifested by an increased hypoxic sensory response (Peng *et al*., 2004; Rey *et al*. 2004). Furthermore, it was described in spontaneous hypertensive animals, as well as in hypertensive patients, an enhanced chemoreceptor reflex that contributes to the excess sympathetic activity present in this pathology (Fukuda *et al*. 1987; Przybylski *et al*. 1982; Somers *et al.,* 1988; Trzebski *et al*. 1982;). Recently, some studies in spontaneously hypertensive rats confirmed the role of the CB in the pathogenesis of essential HT, since these animals exhibit a decrease in the development and maintenance of HT, a reduction in sympathetic vasomotor tone and in the renal sympathetic activity when submitted to bilateral CSN denervation (Abdala *et al*., 2012; McBryde *et al*. 2013). In contrast, the unilateral CSN resection in spontaneously hypertensive rats was ineffective in decreasing arterial pressure and bilateral CSN resection was more effective in reducing arterial pressure than the renal denervation (McBryde *et al*., 2013). These results were also observed in humans, since the functional abolishment of CB activity with 100% O_2 induced a reduction in both arterial pressure and sympathetic activity in hypertensive patients (Siński *et al*. 2012). The latter study is in contrast with a recent study performed in hypertensive patients with unilateral resection of CB due to CB tumors, where this procedure decreased blood pressure (Fundim *et al*, 2015). However, over the long term, the effect on pulse pressure and systolic blood pressure were smaller and without statistical significance (Fudim *et al*. 2015). Fudim´s results are in agreement with the work presented by Julian Paton at the International Congress of the Autonomic Neuroscience in 2015 (Paton, 2015), where he showed that CB unilateral ablation decreases short-term arterial pressure, although 12 months after CB ablation the effect was attenuated, suggesting a compensatory effect of the remaining CB.

Regarding chronic heart failure, several studies demonstrated that CB chemoreceptors are also responsible for increases in sympathetic activation observed in this pathology (Ponikowski *et al.,* 2001; Sun *et al*, 1999). In rat and rabbit animal models of chronic

heart failure, CB ablation, performed by cryogenic destruction, reduced hyperventilation, oscillatory breathing and the tonic sympathetic outflow, resulting in an improvement in cardiac function and prolonged survival (Del Rio *et al*., 2013; Marcus *et al*., 2014). These results were supported by recent data obtained in patients with chronic heart failure. In a case report, Niewiński et al. demonstrated that unilateral CB removal resulted in a decrease in peripheral chemosensitivity, which was accompanied by improvements in autonomic function, cardiac function, exercise capacity and resting ventilation (Niewiński *et al*. 2013). In a subsequent work, the same team demonstrated a reduction in the ventilatory and blood pressure responses to hypoxia, in chronic heart failure patients with bilateral CB removal, suggesting a decrease in sympathetic tone (Niewiński *et al*., 2014). Also in the same study, CB removal did not modify heart rate suggesting a possible involvement of other peripheral chemoreceptors, like the aortic bodies, that could be involved in this response to hypoxia (Niewiński *et al*., 2014). Altogether, the overall results confirm the action of CB in regulation of blood pressure and cardiac performance via sympathetic nervous system activation.

5.5. Glucose sensing in the carotid body

One of the hypotheses that came out to explain the role of the CB in glucose homeostasis was the potential role of the CB as a glucose sensor. In fact, apart from the well established protagonism of the central nervous system in the regulation of glucose levels, the most important organs that are involved in the control of glucose levels are located in the periphery, including in the pancreas (German, 1993), the liver (Hamilton-Wessler *et al*., 1994), and the portal vein (Havener *et al*, 1997) and in specialized intestinal neuroendocrince cells, the L- cells (Reimann & Gribble, 2002).

The putative glucose sensing properties of the CB remains a hot topic. Whereas some *in vivo* and *in vitro* studies, performed in cultured CB type I cells or slices, showed that CBs respond to blood glucose levels, (Koyama *et al.*, 2000; Pardal & Lopez-Barneo, 2002; Zhang *et al*., 2007) others have completely denied a direct involvement of the CB in glucose sensing (Bin-Jaliah *et al*., 2004, 2005; Conde *et al*., 2007; Fitzgerald *et al*., 2009; Gallego-Martin *et al*., 2012) and, due to these controversial results, the sensitivity of the CB to blood glucose is still controversial.

In cultured CB slices, perfusion with low or glucose-free solutions at a PO_2 ≈150 mmHg produced an increase in CAs release from type I cells, with a magnitude comparable to the response evoked by hypoxia, and potentiated hypoxic responses (Pardal & Lopez-Barneo, 2002). This hypoglycemic mediated secretory response has also been observed in post mortem human CBs slices cultured (Ortega-Saenz *et al.,* 2013). Moreover, it was found that low glucose inhibited K^+ currents (Pardarl & Lopez-Barneo, 2002) in an extent similar to the observed by Peers during intense hypoxia (Peers, 1990) and was also able to promote Ca^{2+} entry in type I cells (Pardal & Lopez-Barneo, 2002). Lopez-Barneo's group published that sensitivity to low glucose and to hypoxia depends on distinct signal transduction mechanisms, although they converge on the final steps causing transmembrane Ca^{2+} influx and transmitter release, which stimulates afferent sensory fibers to evoked sympathoadrenal activation (Gao *et a*l., 2014; García-Fernández *et al*., 2007). Almost at the same time, but using an experimental model of co-culture of type I clusters and afferent petrosal neurons, Zhang et al. (2007) described that low glucose increased the spiking activity in the neurons, this increase being sensitive to purinergic and nicotinic blockers, implying that low glucose stimulates type I cells and promotes the release of ATP and ACh.

For the glucose sensing, specialized cells possess specific molecules that are expressed in presence of high glucose sensing like GLUT2 and GK, molecules that are lacking in CB cells (Garcia-Fernandez *et al*., 2007; Schuit *et al.,* 2001; Thorens, 2001;). However, CB expresses others type of glucose transporters with a great importance in glucose homeostasis, like GLUT4, GLUT3 and GLUT1 receptors, but not compatible with glucose sensing properties (Garciz-Fernandez *et al*., 2007). Therefore, it can be postulated that the role of CB in the control of metabolism might be due to the detection of another mediator apart from glucose by the CB.

6. HYPERBARIC OXYGEN THERAPY

Hyperbaric Oxygen Therapy (HBOT) is defined as the therapeutic process by which a patient breathes 100% oxygen at pressures higher than one absolute atmosphere (ATA) (Hampson, 1999). It has been used in the medical field to increase the partial pressure of

oxygen in the blood to allow the tissues to absorb more oxygen and enhance healing ability (Aydin *et al.,* 2013). Knowing that hyperoxia abolish CB activity (Gonzalez *et al.,*. 1994), HBOT might be a way of modulating CB function.

The HBOT procedure consists in placing the patient in a monoplace (single person, breathes oxygen directly) or in a multiplace chamber (can accommodate more than one person and persons breaths through a mask), for periods between 60 and 120 min once or twice daily where the vessels are pressurized to 1.5 to 3.0 ATA (Bennett *et al.,* 2005) (**Figure 1.18**).

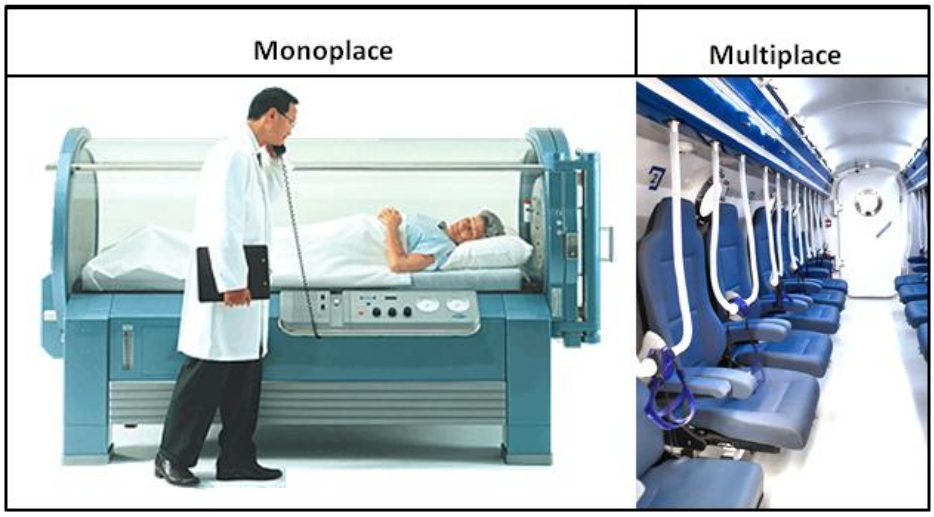

Figure 1.18. The two general styles of hyperbaric chambers used in clinical practice, the monoplace chamber (one person) and multiplace chamber (more than one person).

The treatment application mode of HBOT and effects depends of the number of sessions employed, the oxygen pressure, as well as, the session duration and frequency of sessions applied. There are several protocols described in literature however the oxygen pressure recommend by Undersea and Hyperbaric Medical society (UHMS) and used in most protocols is least 2.5 except for special cases (e.g childrens) where pressure of 2.1 to 2.4 ATA may be employed (D´Agostino *et al*., 2008). However a pressure less than 2 ATA should be never be used and duration of each session is usually 90 min (Brown *et al*., 1994) in order to prevent the occurrence of toxic neurological and respiratory effects of hyperoxia (Al-Wali *et al*., 2006). The first medical application of HBOT was for the treatment of decompression sickness, a hazard of scuba diving (Monn, 2014). Nowadays, it is a technique used in the treatment of several conditions, such as, carbon monoxide intoxication (Weaver *et al*., 2002), infections, arterial gas embolism, radio-

induced lesions and delayed wound healing resulting from diabetes or arteriosclerosis (Abidia *et al*., 2003; Kessler *et al.,* 2003; Sing & Gambert, 2014).

The exact mechanism and efficacy of HBOT are not fully understood, but it is believed that the success of this therapy comes from the increased concentration of oxygen in the blood and also in the tissues, by other words, during HBOT the concentration of oxygen in plasma increases by 10-15 times (Nikitopoulou & Papalimperi, 2015). This increase corresponds to a partial pressure of oxygen as high as 1500 mmHg to 2000 mmHg (at sea level the air pressure is 760 mmHg), creating a four time increase in the diffusion of oxygen from the capillaries (Al-Wali *et al*., 2006; Nikitopoulou & Papalimperi, 2015). Hyperbaric Oxygen Therapy is generally regarded as a safe treatment, however there are potential side effects with a low incidence, as barotrauma in the tissues surrounding trapped air inside the body, such as the lungs (Broome & Smith, 1992) and the middle ear (Fitzpatrick *et al*., 1999; Fiesseler *et al*., 2006), and seizures as a result of too much oxygen (oxygen toxicity) in central nervous system. Reversible myopia is also problem normally seen due to oxygen toxicity (Plafki *et al*., 2000).

6.1. Hyperbaric Oxygen Therapy in Type 2 Diabetes complications

In last decades several studies came out supporting HBOT as a potential treatment to some complications of T2D, such as diabetic foot ulcers (Desola *et al.,* 1998; Oliveira *et al*., 2014; Londahl, 2012). The benefits, of this treatment are associated with stimulation of angiogenesis and neovascularization, optimization of cellular and oxygen levels, promotion of osteoblast and fibroblast proliferation, and formation of collagen (Desola *et al*., 1998; Falanfa, 2005). More specifically, in would healing, HBOT promotes healing probably by a combination of systemic events, as well as regional alterations within the wound margin. It was described that the therapeutic value is related with stimulation of the release of growth factors and stem cells, which promote healing (Stephen & Thom, 2011). Several clinical trials have been conducted to assess the efficacy of HBOT on wound healing in diabetic foot ulcers, however even though the majority of the studies reported positive effects, some methodological issues preclude a clear conclusion. A lot of these studies were retrospective, or when performed prospectively they were non-blinded or unclearly randomized (Game *et al*., 2012).

However, in the prospective, double blinded and randomized studies it was found that HBOT improved wound healing and improved quality of life after one year in diabetic patients with chronic foot ulcers (Londahl *et al*., 2010; 2011).

More recently, animal studies demonstrated that HBOT is effective on the treatment of kidney complications resulting from diabetes, like diabetic nephropathy. In a study performed in diabetic mice, HBOT was able to suppress biomarkers of cell stress and kidney injury (Verma *et al.,* 2015). Also, in rats, HBOT treatment protects renal tissue, thus delaying occurrence and retaining development of diabetic nephropathy (Nie *et al*., 2014).

Apart from the wound healing in T2D and diabetic nephropathy, there is evidence that HBOT improves fasting glycemia by 20% (Desola *et al.,* 1998; Ekanayake & Doolette, 2001;Karadurmus et al., 2010; Wilkinson *et al*., 2012) and lowers C-reactive protein and IR in diabetic patients (Ekanayake & Doolette, 2001; Chateau-Degat *et al*., 2012) without changing insulin levels (Desola *et al*., 1998). Also, it was observed in T2D patients, submitted to 100% oxygen at 2.0 ATA for 2 h, six sessions per week for 5 weeks that insulin sensitivity increased after 3 days of HBOT, being the effect maintained during the 30 sessions (Wilkinson *et al*., 2012). Moreover, an acute session of 90 min of HBOT decreases blood glucose levels, in T2D, stroke and in traumatic brain injury patients (Peleg *et al*., 2013), however no statistically significant differences were observed in blood glucose levels when HBOT was compared to normobaric air in any of the three subgroups (Peleg *et al.,* 2013). This suggests that the decrease in blood glucose should probably not be attributed to the hyperbaric environment *per se* (Peleg *et al*., 2013). One of the reasons that can account for these contradictory effects is the duration of HBOT protocol: while some authors have performed an acute HBOT protocol (Desola *et al.,* 1998; Peleg *et al*., 2013), others submitted patients to 90 min HBOT sessions during 2 weeks (Wilkinson *et al*., 2012) or 2 h during 5 weeks (Chateau-Degat *et al.,* 2012). Also, the majority of these studies included a very small number of T2D patients, and some of them reported that the decrease in blood glucose was not related with HBOT.

In contrast with the beneficial effects described for HBOT in diabetes, severe side effects of HBOT such as oxidative stress and oxygen toxicity have been also described, leading inclusively to cytotoxic effects in the β-cell and hyperglycemia in rats

(Matsunami *et al.,* 2008). Therefore more research on HBOT mechanisms on T2D are needed to clarify its efficacy as well as its mechanism of action on T2D and its comorbidities.

CHAPTER II – HYPOTHESIS & AIMS

2.1. GENERAL AIM:

A growing body of evidence suggests a common pathophysiological process in different metabolic diseases where increased sympathetic nervous system activity, HT, obesity and IR are present. The involvement of sympathetic nervous system activation in the pathogenesis of deregulated metabolism is agreed however the stimulus that promotes its sustained activation remains unknown. Knowing that CB activates the sympathetic nervous system, the general hypothesis of the present thesis is that CB overactivation is involved in the early pathogenesis of IR. Therefore, the overall purpose of the work is to investigate the role of the CB in the development of IR in diet induced insulin-resistant animal models.

2.1.1. Specific aims:

The project was divided into 3 chapters, according to specific aims.

Aim 1: To investigate the role of CB in the genesis of IR. The sub-specific aims were:

1) To investigate if CB activity is increased in animal models of IR and HT induced by hypercaloric diets;
2) To study if blunting of CB activity through chronic CSN bilateral resection prevents the development of IR in rats submitted to hypercaloric diets;
3) To evaluate if insulin is a stimulus for CB activation;

Aim 2: To investigate if Kv1.3 channels mediate insulin action in the rat CB. The sub-specific aims were:

1) To study if voltage activated K^+ currents are sensitive to insulin in the CB type I cells;
2) To determine if Kv1.3 channels are involved in insulin sensitive K^+ currents in CB type I cells;
3) To study if Kv1.3 channels are present in type I CB dissociated cells;
4) To evaluate if insulin modulates Kv1.3 channel activity through phosphorylation in type I CB cells;

5) To test if the release of neurotransmitters evoked by insulin is mediated by Kv1.3 channels;

Aim 3: **To evaluate the impact of HBOT on glucose homeostasis in T2D patients.** The sub-specific aims:

1) To study the impact of HBOT on fasting glycemia;

2) To determine the impact of HBOT on glucose tolerance.

CHAPTER III

This chapter is based on the following manuscript:

Ribeiro MJ, Sacramento JF, Gonzalez C, Guarino MP, Monteio EC, Conde SV. Carotid body denervation prevents the development of insulin resistance and hypertension induced by hypercaloric diets. Diabetes 2013; 62(8):2905-16.

Carotid body denervation prevents the development of insulin resistance and hypertension induced by hypercaloric diets.

Ribeiro MJ[1], Sacramento JF[1], Gonzalez C[2], Guarino MP[1], Monteio EC[1], Conde SV[1].

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ABSTRACT

Increased sympathetic activity is a well-known pathophysiological mechanism in IR and HT. The CBs are peripheral chemoreceptors that classically respond to hypoxia by increasing chemosensory activity in the CSN, causing hyperventilation and activation of the sympathoadrenal system. Besides its role in the control of ventilation, the CB has been proposed as a glucose sensor being implicated in the control of energy homeostasis. However, to date no studies have anticipated its role in the development of IR. Herein we propose that CB overstimulation is involved in the aetiology of IR and HT, core metabolic and hemodynamic disturbances of highly prevalent diseases like the MS, T2D and OSA. We demonstrated CB activity is increased in IR animal models and that CSN resection prevents CB-overactivation and diet-induced IR and HT. Moreover we showed that insulin triggers CB, highlighting a new role for hyperinsulinemia as a stimulus for CB-overactivation. We propose that CB is implicated in the pathogenesis of metabolic and hemodynamic disturbances through sympathoadrenal overactivation and may represent a novel therapeutic target in these diseases.

3.1. INTRODUCTION AND AIM

Insulin Resistance, arterial HT, obesity and dyslipidemia are core features of widespread diseases in western societies such as the MS, T2D and OSA. Visceral obesity has been proposed to play a fundamental role in the simultaneous development of IR and HT that characterize these diseases (Katagiri *et al*., 2007). Recent findings suggest that peripheral IR is also a common feature in lean OSA (West *et al*., 2006) as well as lean polycystic ovarian syndrome (Dunaif *et al*., 1989), despite its strong relationship with visceral obesity. Similarly, the association of HT with OSA is independent from obesity (Peppard *et al*., 2000), as demonstrated by hypertensive lean sleep apnoea patients. Altogether, these findings point out to the existence of an obesity-independent etiological factor that simultaneously causes IR and HT: the activation of the CBs has recently been suggested as a putative candidate (Iturriaga *et al*., 2005).

The CBs are arterial chemoreceptors that sense changes in arterial blood O_2, CO_2 and pH levels. Hypoxia and acidosis/hypercapnia activate the CBs, which respond by increasing the action potential frequency in their sensory nerve, the CSN. CSN activity is integrated in the brain stem to induce a fan of respiratory reflexes aimed, primarily, to normalize the altered blood gases via hyperventilation (Gonzalez *et al*., 1994) and to regulate blood pressure and cardiac performance via sympathetic nervous system activation (Marshall, 1994) .The CB directly activates the adrenals via increased sympathetic drive and also increases sympathetic vasoconstrictor outflow to muscle, splanchnic, and renal beds (Cao *et al*., 2001; Marshall, 1994). Enhanced sympathetic nerve activity is known to contribute to skeletal muscle IR and to impaired glucose tolerance, mainly due to sympathetic mediated lipolysis (Esler *et al*., 2006; Kahn & Flier, 2000) and also to increased arterial pressure (Esler *et al*., 2006). Recently, the CB was proposed to be a glucose sensor (Pardal & López-Barneo, 2002) and implicated in energy homeostasis control (Koyama *et al*., 2000).

The objective of this study was to investigate the role of the CB in the pathogenesis of metabolic and hemodynamic disturbances by testing the hypothesis that CB activity is increased in IR and HT animal models independently of obesity. Also, to clarify the role of obesity as an independent factor in CB activation, we compared CB function in both obese and lean models of IR. The second hypothesis tested was that insulin is a trigger for CB activation. *In vivo* experiments have previously shown that intravenous infusion

of insulin causes a CB-dependent increase in ventilation (Bin-Jaliah *et al*., 2004). The authors concluded that this effect was associated with the hypoglycemia caused by insulin administration, however others have shown that low glucose is not a direct stimulus for rat CB chemoreceptors (Conde *et al*., 2007; Gallego-Martin *et al*., 2012). These discordant results point towards insulin as a good alternative candidate to activate the CBs.

Finally, we performed chronic CSN bilateral resections to test the hypothesis that preventing the CBs from being overactivated averts the development of IR and HT, and also the increase in sympathoadrenal activity, induced by hypercaloric diets in animals. The data presented herein clarify the role of the CB in the pathogenesis of diet-induced IR and HT and unveil a new promising target for intervention in T2D, MS and OSA.

3.2. RESEARCH DESIGN AND METHODS

3.2.1. Animals and experimental procedures

Experiments were performed in Wistar rats (200–420 g) of both sexes, aged 3 months, obtained from the *vivarium* of NOVA Medical School/Faculdade de Ciências Médicas. Two diet-induced IR and HT animal models were used: the rat submitted to a HF diet, a model that combines obesity, IR and HT (Conde *et al*., 2012b; Shearer *et al.,* 2009) and the rat submitted to a high-sucrose (HSu) diet, a lean model of combined IR and HT (Conde *et al*., 2012b; Ribeiro *et al*., 2005). Briefly, the control group fed a sham diet (7.4% fat+75% carbohydrate (4% sugar)+17% protein, SDS diets RM1, Probiológica, Portugal); the HSu model was obtained by administration of 35% sucrose (Panlab, Portugal) in drinking water during 28 days. The HF model fed a lipid rich diet (45% fat+35% carbohydrate+20% protein, Mucedola, Italy) during 21 days (**Figure 3.1**). The HSu and HF animals are validated in the literature as animal models of the MS (Panchal *et al*., 2011). To demonstrate that CB activity was increased in hypercaloric fed animals we compared HF and HSu with control group. To evaluate the contribution of CB to the genesis of IR and HT, bilateral resection of CSN was performed 5 days prior to submitting the animals to *standard* or hypercaloric diets. The carotid artery bifurcations were located bilaterally and CSN were identified and either sectioned bilaterally or left

intact (sham). These procedures were performed in aseptic conditions under ketamine (30 mg/kg)/xylazine (4 mg/kg) anaesthesia and brupenorphine (10 μg/kg) analgesia. Chronic resection of CSN was confirmed by absence of ischemic hypoxia-induced hyperventilation prior to experiments. Rats fed with standard diet were used to investigate if insulin triggers CB activation. All test groups included equal number of males and females. Whenever an odd experimental number is displayed, this refers to the death of experimental units during the experimental procedure. Also, food and liquid intake was monitored during the treatments, in all groups of animals. Body weight and animal behavioural changes were assessed twice per week. All measurements were performed with animals under sodium pentobarbital (60 mg/kg i.p.) anaesthesia, since pentobarbital was shown not to alter the metabolic parameters tested herein (constant of insulin tolerance test (K_{ITT}), fasting glycemia, insulinemia and FFAs) in comparison to conscious animals (Guarino *et al*., 2013) nor insulin responses to glucose (Davidson, 1971). At the end of the experiments the rats were euthanized by an intracardiac overdose of pentobarbital, except when heart puncture was performed to collect blood. Principles of laboratory care were followed in accordance with the European Union Directive for Protection of Vertebrates Used for Experimental and Other Scientific Ends (2010/63/EU). Experimental protocols were approved by the Ethics Committee of the NOVA Medical School/Faculdade de Ciências Médicas.

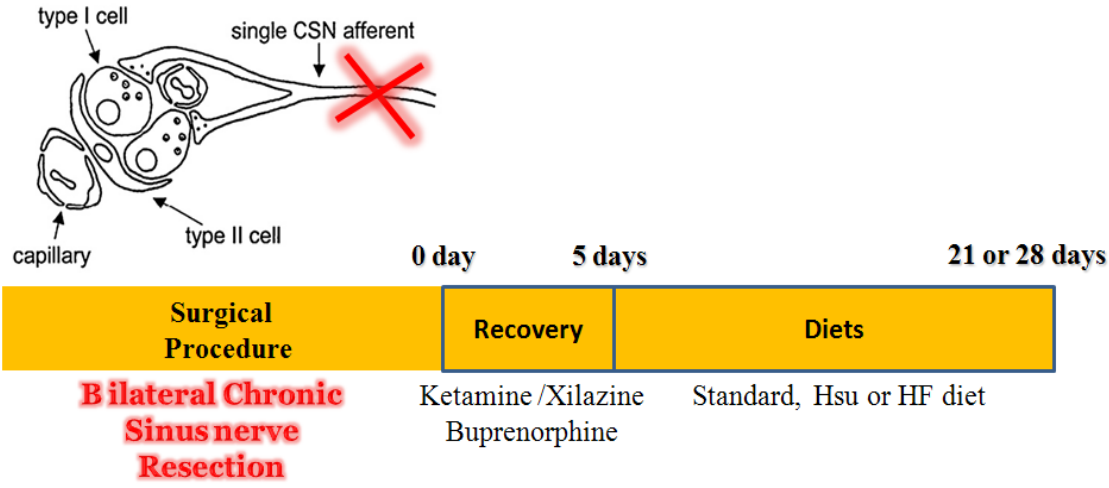

Figure 3.1: Representation of experimental timeline after surgical procedures. The chemical compounds were administered during recovery time and after 5 days of recovery period the animals were submitted to high-fat, high-sucrose or standard diets. CSN: chronic sinus nerve; Hsu: high sucrose; HF: high fat.

3.2.2. Evaluation of basal ventilation and ischemic ventilatory responses in animal models of insulin resistance and hypertension

A detailed description of these methods was previously published (Monteiro & Ribeiro, 1989). Shortly, respiratory rate (RR) and TV were obtained by pneumotachography (Hugo SACHS Elektronik, Harvard Apparatus, Madrid, Spain) in anaesthetized and tracheostomized control rats and in rats submitted to hypercaloric diets. These respiratory parameters and blood pressure were continuously recorded in anesthetized and vagotomized rats breathing spontaneously and submitted to either bilateral occlusions (5–15 sec) of CCA. Bilateral midcervical vagotomy was performed to abolish the role of vagal afferents innervating the lungs and the aortic chemoreceptors with a major influence on respiratory activity (Marek *et al*., 2008). Control experiments were performed in animals submitted to bilateral cut of the CSN in order to distinguish central and peripherally mediated effects.

3.2.3. Effect of insulin on spontaneous ventilation in control animals

Insulin effect on ventilation was assessed in control rats anesthetized, tracheostomized and vagotomized. Briefly, an insulin bolus (1, 5, 10, 50,100 and 200 mU/kg) was administered in external carotid artery and reaches the CB by being pushed by the blood flow of CCA (**Figure 3.2**).

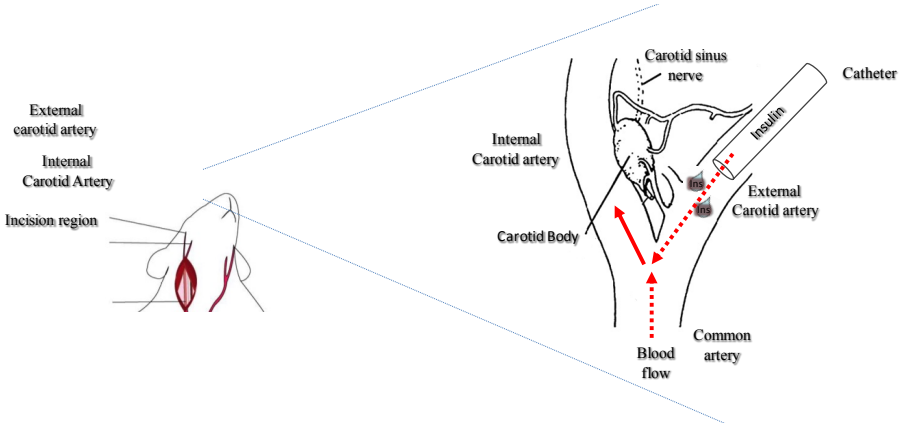

Figure 3.2: Surgical procedure made in control anesthetized rats before evaluation of ventilation. Insulin *bolus* was administered in external carotid artery and reaches the carotid body (CB) by being pushed by the blood flow of common carotid artery.

Ventilatory parameters as described above were monitored. Euglycemic clamp was maintained through glucose (10 mg/kg/min) perfusion into the femoral vein. Confirmation of CB-insulin mediated effect was done by measurement of ventilation after CSN cut.

3.2.4. Measurement of insulin sensitivity and mean arterial pressure

Insulin tolerance test (ITT) was used to measure insulin sensitivity (Conde *et al.,* 2012b; Monzillo & Hamdy, 2003). Mean arterial pressure (MAP) was measured in the femoral artery (Conde *et al.,* 2012b). After insulin sensitivity and MAP evaluation, blood was collected by heart puncture and treated for quantification of soluble biomarkers (Conde *et al.,* 2012b). Visceral fat and adrenal medulla were collected after an abdominal laparotomy and weighted. Adrenal medullas were frozen in liquid nitrogen and stored placed at -80ºC.

3.2.5. Measurement of plasma insulin, circulating free fatty acids, corticosterone and catecholamines levels and adrenal medulla catecholamines content

Plasma and serum were collected after heart puncture to ethylenediamine tetraacetic acid (EDTA) precoated tubes and to eppendorfs, respectively. Insulin concentrations and FFAs were determined in plasma and corticosterone was determined in serum (Conde *et al*., 2012b). Corticosterone determination was obtained with a DetectX corticosterone Immunoassay kit (Arbor Assays, Madrid, Spain). For CAs quantification in plasma, 400 µl of plasma samples were purified and CAs were extracted and quantified as previously described (Conde *et al*., 2012). For quantification of CAs content in adrenal medulla, adrenal medullas previously frozen were homogenized in perchloric acid (PCA) 0.6N and their endogenous CAs content was quantified as described (Gallego-Martin *et al.*, 2012).

3.2.6. Carotid body dopamine and ATP release in response to hypoxia and to insulin

Carotid bodies were cleaned free of nearby connective tissues under dissection microscope and incubated in Tyrode solution (Gallego-Martin *et al*., 2012). To evaluate CB activity in IR and HT animal models, CB DA (plus 3, 4-Dihydroxyphenylacetic acid (DOPAC), its major metabolite) release was determiend. Carotid body ATP and DA release in response to insulin were monitored in control animals. In brief, CBs were incubated in 500 µl (250 µl for ATP and 250 µl for DA for insulin effects) of Tyrode bicarbonate solution and cofactors for tyrosine hydroxylase (TH) and DA-β-hydroxylase (20 µM tyrosine, 100 mM ascorbic acid and 500 nM 6-methyl-tetrahydroptine) or Tyrode bicarbonate plus insulin (0.01-100 nM). Solutions were kept at 37ºC and continuously bubbled with normoxia (20%O_2/5%CO_2/75%N_2), except when hypoxic stimuli were applied. Protocols for DA release in overfeeding rats include two 10 min normoxic incubations, followed by 10 min incubation in hypoxia (5%O_2/5%CO_2/75%N_2) and 2 post-hypoxic incubations in normoxia. Protocols for insulin effect on DA and ATP release include two 10 min incubation in normoxia, followed by 3 incubations with different insulin concentrations and 2 post-insulin incubations in normoxia. The solutions were renewed at each fixed time and all fractions were collected and quantified as previously described (Gallego-Martin *et al*., 2012).

3.2.7. Western Blot analysis of insulin receptor, insulin receptor phosphorylation (phosphor-Tyr 1322) and tyrosine hydroxylase expression

For evaluation of InsR phosphorylation, CBs were isolated, cleaned and incubated at 37ºC during 30 min in Tyrode-solution containing 1 and 100 nM of insulin and bubbled with 20%O_2/5%CO_2/75%N_2. After, CBs were immediately frozen in liquid nitrogen and placed -80ºC. For CB InsR and TH expression, CBs after cleaned were frozen in liquid nitrogen. CBs were homogenized in Zurich medium containing a cocktail of protease inhibitors (Caceres *et al*., 2007). Proteins were separated in a 10 or 12% dodecyl sulfate (SDS) PAGE gel electrophoresis and electroblotted on nitrocellulose membrane (0.2 µM BioRad, Madrid, Spain). To enhance detection sensitivity we used a three step Western blot protocol (Johnson *et al.*, 2009). After blocking, membranes were

incubated with primary antibodies against InsR (1:100, Sta Cruz Biotech, Madrid, Spain), InsR phosphorylated (phosphor-Tyr1322, 1:50, Assay Designs, Portugal) and TH (1:10000, Sigma, Madrid, Spain). The membranes were incubated in tris-buffered saline tween (TBST) (0.1%) containing biotin-conjugated goat anti-mouse IgG (1:10000, Millipore, Madrid, Spain) for 1 h, washed in TBST (0.02%), and incubated for 30 min in TBST (0.1%) containing horseradish peroxidase (HRP)-conjugated streptavidin (1:10000, Pierce, Madrid, Spain). Membranes were then washed in TBST (0.02%) and developed with enhanced chemiluminescence reagents (Immobilon Western, Millipore, Spain). Intensity of the signals was detected in a Chemidoc Molecular Imager (Chemidoc BioRad, Spain) and quantified using the Quantity-One software (BioRad, USA). The membranes were re-probed and tested for β-actin immunoreactivity (bands in the 42 kDa region) to compare and normalise the expression of proteins with the amount of protein loaded.

3.2.8. Type I cell culture and intracellular Ca^{2+} measurements

Cleaned CBs were enzymatically dispersed, and dissociated cells were plated on poly-L-lysine-coated coverslips maintained in culture for up to 24 h as previously described (Pérez –García *et al*., 1992). Coverslips were incubated with fura-2 AM, mounted in a perfusion chamber, and fura-2 fluorescence was measured as the ratio of the fluorescent emission at 340/380 nm of type I cells (Gomez-Niño *et al*., 2009). General protocol for Ca^{2+} measurements consisted in a sequential incubation hypoxia (N_2; 1 min), 5 min normoxic incubation (20%O_2), 3 min incubation with 1 nM insulin, combination of both hypoxia (N_2) and insulin (1 nM), 5 min normoxia (20%O_2), 1 min hypoxia (N_2) and finally 30 sec of high external KCl.

3.3. RESULTS

Administration of hypercaloric diets to Wistar rats produced changes in body weight, sympathetic nervous system, blood pressure and insulin sensitivity similar to the ones observed in humans (Conde *et al*., 2012b; Landsberg *et al*., 1978; West *et al*., 2006). Liquid intake was similar in all animals tested (control group: 101.21±3.09 ml/kg/day;

HF animals: 89.50±3.93 ml/kg/day; and HSu animals: 93.22±2.59 ml/kg/day). No significant differences were observed in food intake (Control: 57.78±2.05 mg/kg/day; HF: 62.56±1.99 mg/kg/day; HSu: 51.22±4.51 mg/kg/day). The daily caloric intake was 164.7±5.8 kcal/kg/day in control animals, 299.0±9.4 kcal/kg/day in HF animals ($p<0.001$ vs control) and 332.8±12.8 kcal/kg/day for HSu animals ($p<0.001$ vs control). After CSN cut, the daily caloric intake was: 179.6±10.1 in the control group, 289.2±6.5 in the HF group and 327.6±10.5 kcal/kg/day in the HSu group. The daily caloric intake was not changed by CSN cut and there were no significant differences among the HF and HSu rats. Insulin resistance and HT were confirmed by measurement of insulin sensitivity and blood pressure in HF and HSu animals. The HF diet caused a decrease in K_{ITT} from 4.69±0.33% glucose/min in Control animals to 2.98±0.34 % glucose/min ($P<0.01$). The HSu diet decreased K_{ITT} to 2.68±0.32 %glucose/min ($P<0.01$). HF and HSu diets caused a significant increase in MAP compared with controls (MAP control = 95.99±3.21mmHg; MAP HF = 142.31±2.47 mmHg; MAP HSu = 136.71±4.51 mmHg). Fasting glycemia was not significantly different in control and HF groups, although the HSu diet significantly increased fasting glycaemia in comparison with the control group ($P<0·001$) (data not shown).

3.3.1. Carotid body is overactivated in insulin resistant and hypertensive rats

Figure 3.3 demonstrates that CB activity is increased in animal models of IR and HT. Spontaneous ventilatory parameters (RR, TV and the product of these two parameters, VE) were increased in both HF and HSu animals, with a more pronounced effect in HF animals (**Figure 3.3 A, D**). Surgical CSN cut completely abolished the increase in spontaneous ventilation induced by the diets (**Figure 3.3 C, D**), showing that this effect is mediated by the CB. In addition, ventilatory responses to ischemic hypoxia, assessed as the increase in ventilation produced by CCA occlusions for periods of 5, 10 and 15 sec were augmented in HF animals (**Figure 3.3 B, E**). This increase in ventilation, which was proportional to the duration of the stimulus and was mediated through the CBs as it was abolished by CSN cut (**Figure 3.3 C**).

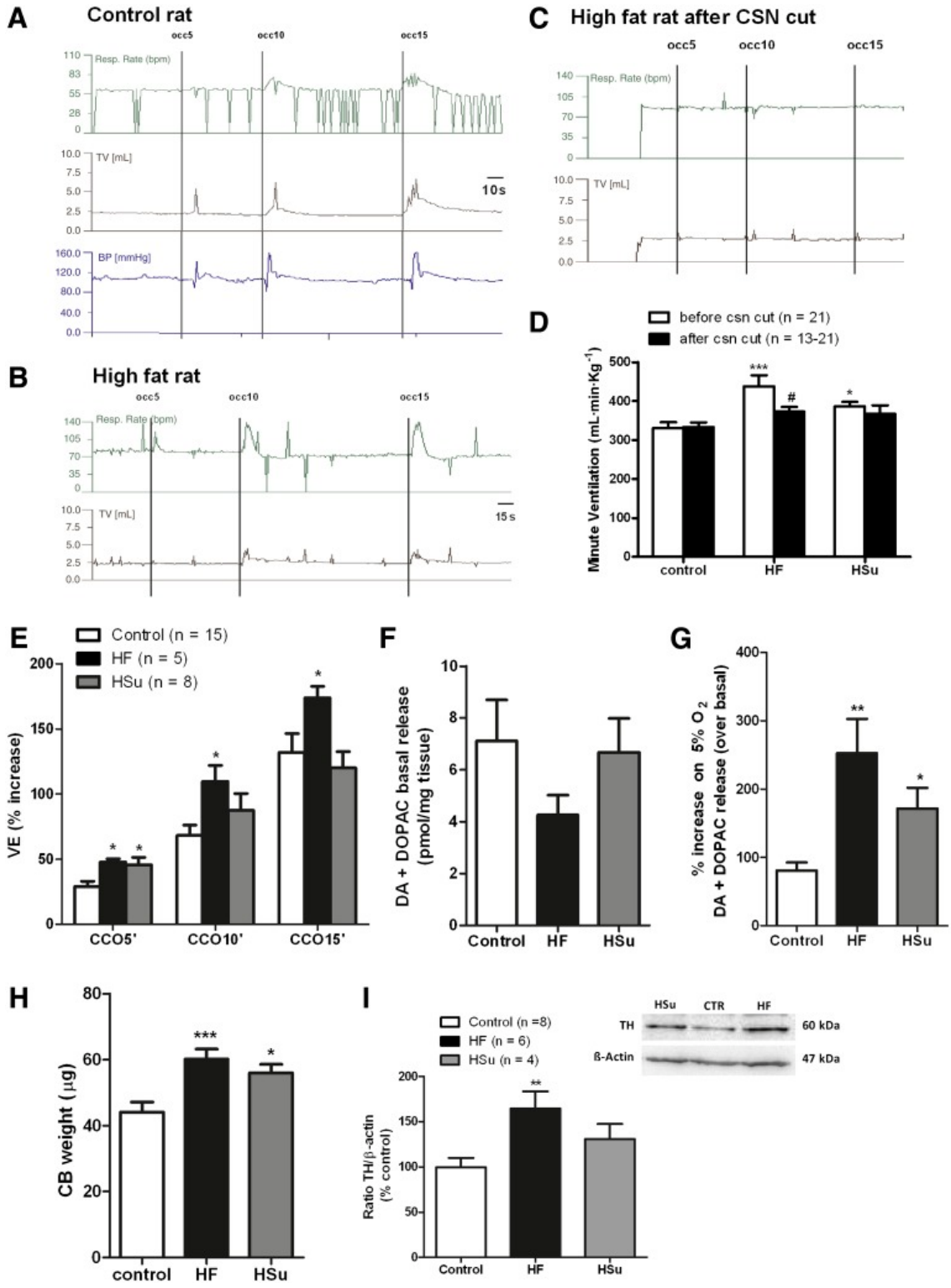

Figure 3.3: Carotid body (CB) activity is increased in rat models of insulin resistance (IR) and hypertension (HT). A), B) Typical recordings of respiratory rate (RR) (bpm), tidal volume (TV) (ml) and blood pressure (BP) in basal conditions and in response to ischemic hypoxia, induced by occlusions of common carotid (OCC) artery, in a control rat and in a rat submitted to a high fat (HF) diet. C) Typical recording of ventilatory parameters after carotid sinus nerve (CSN) cut in an HF rat. D) Mean minute ventilation (VE, product of RR and TV) in control, HF and Hsu rats. E) Effect of OCC during 5, 10 and 15 sec on VE in control, HF and HSu rats. F) Effect of hypercaloric diets on CB catecholamines (CAs) (dopamine (DA) +DOPAC) basal release ($20\%O_2 + 5\%O_2$ balanced N_2) (n=5). G) Effect of hypercaloric

diets on the release of CAs from CB evoked by hypoxia (5%O_2 + 5%CO_2 balanced N_2) (n=5). H) Effect of HF and HSu diets in CBs weight; control n = 19, HF n=27, HSu n=24. I) Effect of HF and HSu diets on the inmmunoreactivity for tyrosine hydroxylase (TH, 60 kDa) expressed as the ratio TH/β-actin (43 kDa) expression. Left panel shows representative immunoreactivity for TH and β-actin in the CB in of control, HF and, HSu, animals. Bars (D, E, F, G, H and I) represent mean ± SEM. One and Two-Way ANOVA with Dunnett's and Bonferroni multicomparison tests, respectively; *p<0.05, **p<0.01, ***p<0.001 vs control; #p<0.05 vs values within the same group.

In HSu animals only the response to an ischemic hypoxia of 5 sec was significantly increased, and as observed in the HF model this was also abolished by CSN cut. We concluded that both the HF and the HSu rat models of IR and HT present an overstimulated CB, however, the more pronounced increases in spontaneous ventilation and in ischemic hypoxia induced-hyperventilation observed in HF animals suggest that these animals hold a higher degree of CB activation. Catecolamines, namely DA, are the best well characterized neurotransmitters in the CB (Gonzalez *et al*., 1994), and its release in all mammalian species depends on extracellular Ca^{2+}, is proportional to stimulus intensity and to the increase in CSN activity and therefore to CB function (Obeso *et al*., 1985; Vicario *et al.,* 2000) .Thus, to confirm CB overactivation in HF and HSu animals, we measured both basal and hypoxia evoked-release of DA (plus DOPAC), the main metabolite of DA in the CB). We observed that basal release of DA was not significantly modified by hypercaloric diets (**Figure 3.3 F**), however the release induced by hypoxia (5%O_2) was increased 3.15 fold in HF and 2.12 fold in HSu rat models (**Figure 3.3 G)**. Also, CBs weight was significantly increased by 36.71% and 27.13% in HF and HSu models, respectively (**Figure 3.3 H**), which suggests that overactivation of CB is due to hyperplasia of the organ. In fact, western blot analysis confirmed that the TH expression, the rating enzyme for CAs biosynthesis increased by 64.4% in HF (p<0.01) and 30.8% in HSu animals (p=0.12) (**Figure 3.3 I**), confirming CB overactivity in these pathological animal models.

3.3.2 Chronic carotid sinus nerve resection prevents insulin resistance and hypertension

To test the involvement of the CB in the development of IR and HT, we performed a chronic CSN bilateral resection prior to hypercaloric diet administration, therefore blocking CB activity during the induction of IR.

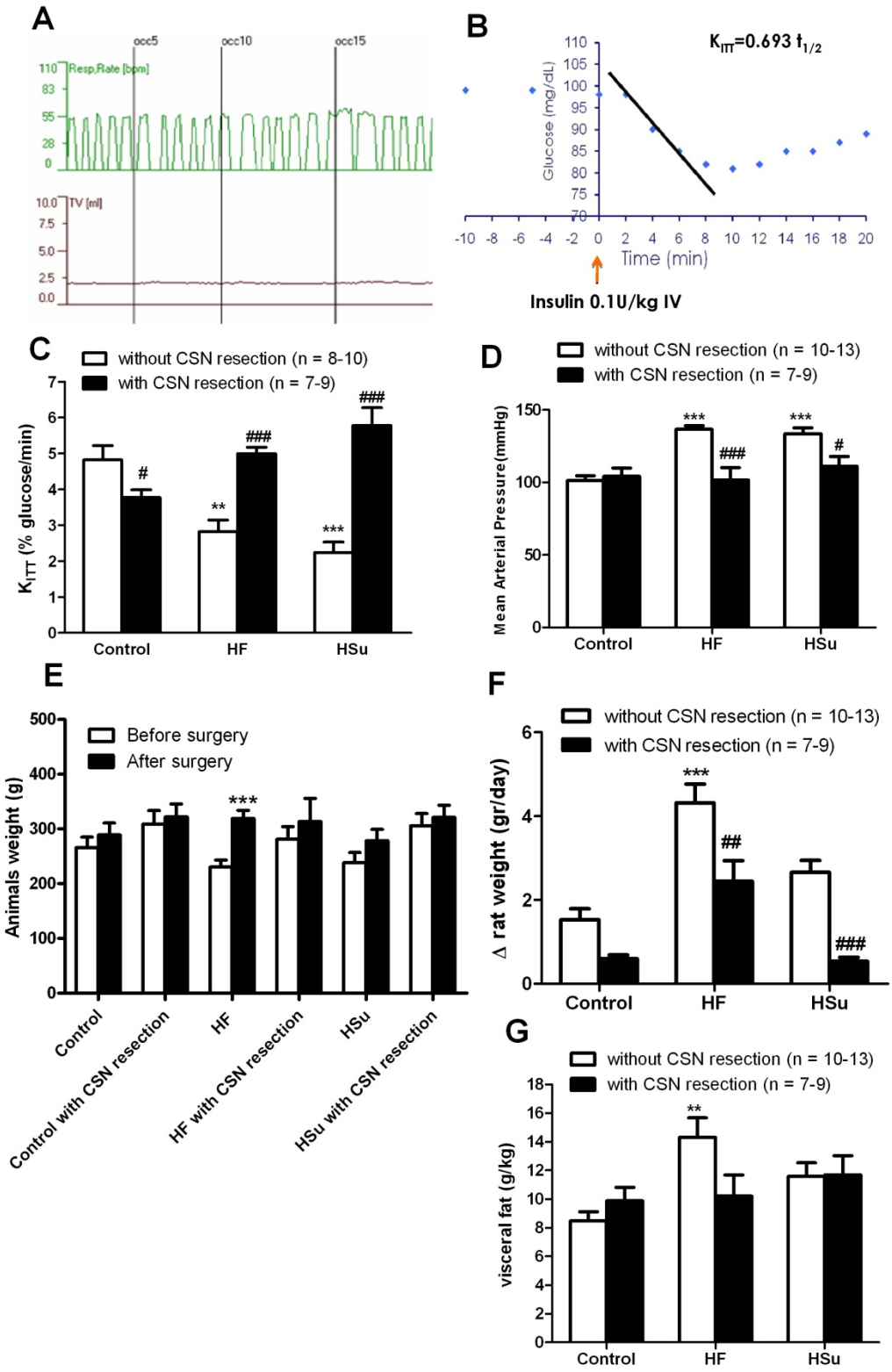

Figure 3.4. Carotid sinus nerve (CSN) bilateral resection prevents insulin resistance (IR) and hypertension (HT) in high fat (HF) and high sucrose animal (Hsu) models. A) Typical recording of respiratory rate (RR) (bpm) and tidal volume (TV) (ml) in response to ischemic hypoxia, induced by occlusion of common carotid (OCC) artery, in a rat submitted to CSN bilateral resection. The absence of increment in the ventilatory responses confirms CSN. B) Representative glucose excursion curve for insulin tolerance test (ITT) in a control rat. Details on constant of insulin tolerance test (K_{ITT}) calculation are described methods section. A, C) Effect of CSN resection on insulin sensitivity determined by the

ITT, expressed as constant rate for glucose disappearance K_{ITT} in control, HF and HSu diet rats. D) Effect of CSN resection on mean arterial pressure (MAP) in control, HF and HSu rats. E), absolute weight before and after hypercaloric diets administration and surgery (CSN resection). F) Increment in body-weight, calculated as total weight variation during the experimental period, in control, HF and HSu rats with and without CSN resection. G) Visceral fat, weighed post-mortem and corrected to body weight in control, HF and HSu rats with and without CSN resection. Bars represent mean ± SEM One and Two-Way ANOVA with Dunnett's and Bonferroni multicomparison tests, respectively; *p<0.05, **p<0.01, *** p<0.001 vs control; #p<0.05; ##p<0.01, ###p<0.001 comparing values with and without CSN resection.

Rats submitted to CSN bilateral resection were compared with animals submitted to the same surgical procedure but in which CSN was left intact (sham). Chornic sinus nerve bilateral resection was confirmed by the lack of increase in the ventilatory responses to ischemic hypoxia, assessed as CCA occlusion (**Figure 3.4 A**). Sham procedure did not modify any of parameters evaluated (insulin sensitivity, MAP, glycemia, insulinemia, FFAs, corticosterone, visceral fat, plasma CAs) when compared with animals control, HF and HSu animals not submitted to any surgical procedure (first paragraph results section, Conde *et al*., 2012b). Also, CSN bilateral resection did not alter liquid and food intake in any of the groups tested (data not shown). **Figure 3.4 B** depicts a representative curve of a typical ITT in a control rat. Insulin sensitivity was significantly decreased by 42.08% and 53.61% in HF and HSu rats respectively (**Figure 3.4 C).** Insulin resistance produced by hypercaloric diets, was completely prevented by CSN resection **(Figure 3.4.C),** linking CB dysfunction with the development of IR. In addition, we observed that CSN resection in control animals decreased insulin sensitivity, suggesting that CB also contributes to maintain metabolic control in physiological conditions. Mean arterial pressure, as previously described (Conde *et al*., 2012b), was increased by 38.79% and 35.70% in HF and HSu rats, respectively, and this effect was totally prevented by CSN chronic resection (**Figure 3.4 D).** Glucose homeostasis and insulin secretion became normalized since fasting hyperglycemia and hyperinsulinemia returned to control values after CSN chronic denervation (**Table 3.1**). The increase in serum FFAs observed in HSu rats was abolished by CSN resection **(Table 3.1)**. Neither HF and HSu diets nor CSN resection modified corticosterone levels **(Table 3.1).** Due to the strong association between obesity and visceral fat with IR and HT (Katagiri *et al.,* 2007; Koyama *et al*., 2000; Pardal & López-Barneo, 2002), we tested if CSN resection could alter weight gain and visceral fat.

Table 3.1: Effect of carotid sinus nerve chronic (CSN) resection on fasting plasma glucose, plasma insulin, serum free fatty acids (FFAs) and corticosterone levels in control, high fat (HF) and high sucrose (HSu) diet rats. Data with and without CSN resection are means of 7-9 and 10-13 values, respectively.

Treatments		Glycemia (mg/dL)	Insulinemia (μg/L)	FFAs (μM)	Corticosterone (ng/mL)
Control	Without CSN resection	100.4 ± 4.2	1.9 ± 0.5	389.1 ± 40.5	4.34 ± 0.3
	With CSN resection	95.4 ± 3.5	2.2 ± 0.0	468.6 ± 42.3	4.89 ± 0.1
High fat diet	Without CSN resection	106.3 ± 2.5	4.6 ± 0.6 ***	436.5 ± 36.2	4.51 ± 0.1
	With CSN resection	112.7 ± 3.9	2.0 ± 0.1 ###	377.8 ± 37.5	5.1 ± 0.1
High sucrose diet	Without CSN resection	145.8 ± 9.6 ***	5.27 ± 0.3 ***	891.1 ± 93.3***	3.9 ± 0.3
	With CSN resection	95.6 ± 5.8 ###	1.9 ± 0.2 ###	431.8 ± 76.5###	4.6 ± 0.1

In **Figure 3.4 E** absolute weights before and after administration of hypercaloric diets and also before and after CSN resection are depicted. HF, but not control or HF, animals significantly gained weight during the experimental period **(Figure 3.4 E, F)**. We found that CSN resection significantly decreases weight gain in HF animals **(Figure 3.4 E, F)** and avoids visceral fat deposition **(Figure 3.4 G)**. Since IR, HT and obesity are associated with sympathetic nervous system overactivity (Katagiri *et al.,* 2007; Pardal & López-Barneo, 2002; Koyama *et al*., 2000), and CB controls sympathetic outflow and sympathetic nerve activity (Cao *et al.,* 2001;Marshall, 1994;), we also analysed sympathoadrenal activity, measured both as circulating and adrenal medulla CAs in our animal models. Plasma norepinephrine significantly increased in both HF and HSu rats in relation to control animals (HF = 48.40±7.72 pmol/ml; HSu = 71.32±9.04 pmol/ml; Control = 22.23±2.98 pmol/ml) **(Figure 3.5 A).** Also, as depicted in **figure 3.5 B**, plasma epinephrine increased 151.52% and 178.31% in HF and HSu, respectively (Control= 30.80±4.25 pmol/ml). These results suggest an increased sympathoadrenal activity **(Figure 3.5 A, B)** that was confirmed by the augmented CAs content in adrenal medulla of these animals **(Figure 3.5 C, D).** High fat and HSu rats exhibited significant increases of 29.72% and 44.52% in adrenal medulla norepinephrine respectively, and of 34.27% and 69.50% adrenal medulla epinephrine content, compared with the controls (norepinephrine= 11.75±0.58 nmol/mg tissue; epinephrine control = 24.28±2.62 nmol/mg tissue, **Figure 3.5 C, D**).

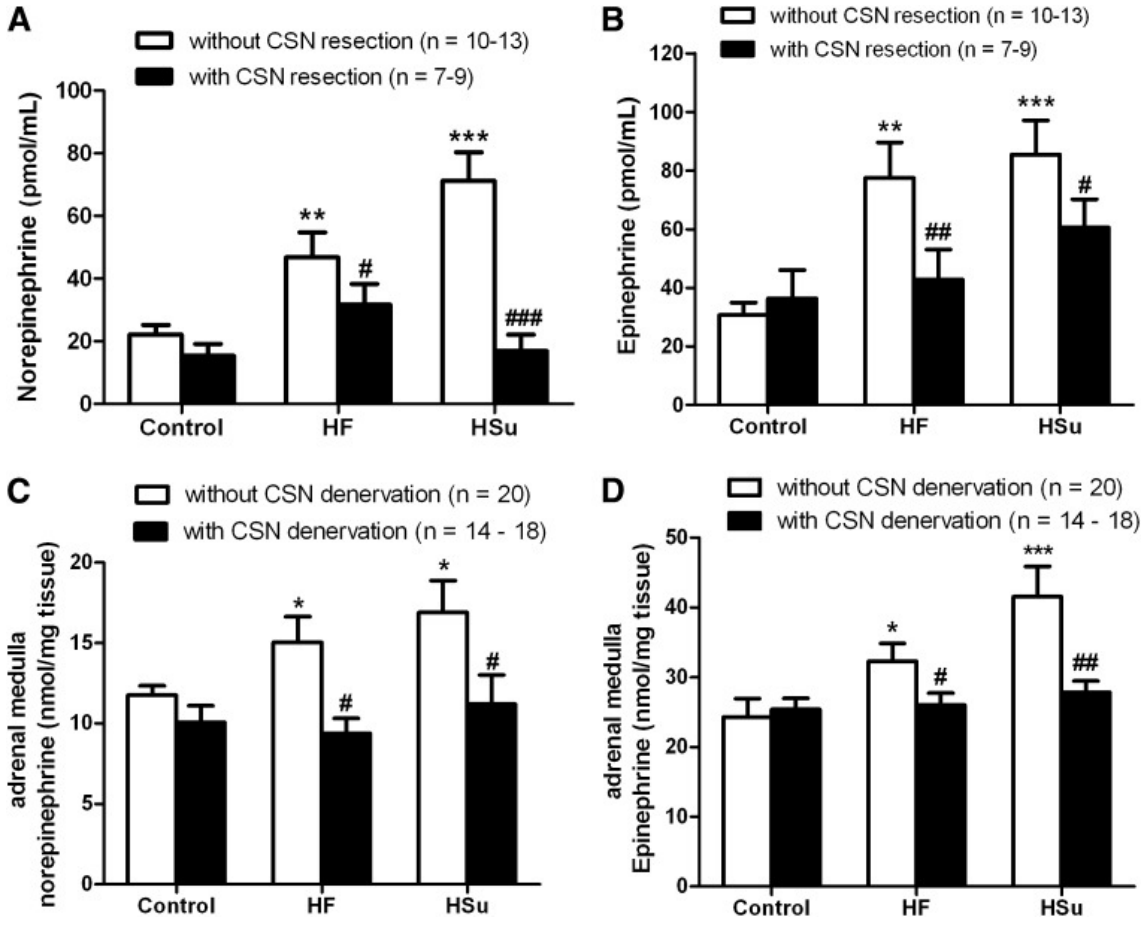

Figure 3.5: Carotid sinus nerve (CSN) bilateral resection prevents sympathoadrenal overactivation in high fat (HF) and high sucrose (Hsu) animal models. A), B) Effect of CSN resection on circulating catecholamines (CAs), norepinephrine and epinephrine), respectively. C), D) Effect of CSN resection on adrenal medulla norepinephrine and epinephrine content, respectively. Bars represent mean ± SEM. Two-Way ANOVA with Bonferroni multicomparison tests, respectively; *p<0.05, **p<0.01, *** p<0.001 vs control; #p<0.05; ##p<0.01, ###p<0.001 comparing values with and without CSN resection.

Chronic CSN cut did not affect sympathoadrenal activity in control animals; however, sympathoadrenal overactivation induced by hypercaloric diets was abolished in rats with CSN bilateral resection **(Figure 3.5. A - D).** These results demonstrate that CB plays a role in the genesis of IR and HT in animal models of T2D and MS.

3.3.3. Insulin triggers carotid body activation

In the present work we propose that the stimulus for CB overactivation responsible for IR and HT is increased plasma insulin and therefore we hypothesize that insulin is capable of triggering CB activation. We used a 3 step western blot approach (Johnson *et al.,* 2009) to examine the presence of the InsRs in the CB and its phosphorylation in

response to insulin. Western blot analysis demonstrated that InsRs are present in the CB **(Figure 3.6 A)** and that their phosphorylation increases in the presence of 1 and 100 nM insulin (**Figure 3.6 A, B)**. Incubation of the CBs with 1 and 100 nM insulin significantly increased InsR phosphorylation by 98.6% and by 47%, respectively (**Figure 3.6 B)**.

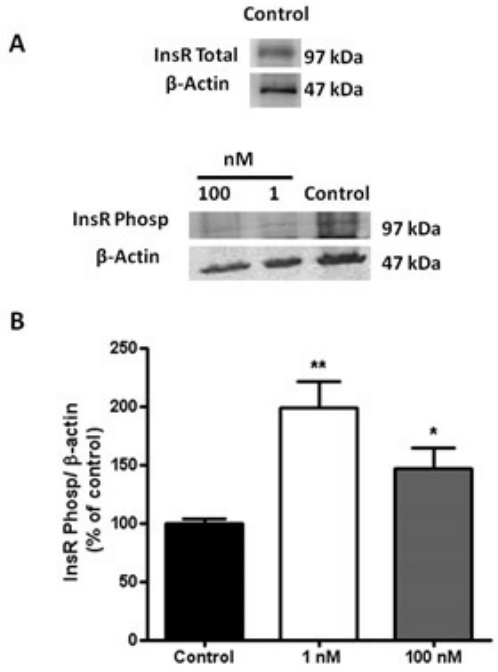

Figure 3.6. Insulin receptors (InsRs) are present in the carotid bodies (CBs) and its phosphorylation increases in response to insulin. A) Representative Western blot showing InsRs immunoreactivity in the CB and InsRs phosphorylation immunoreactivity in control CBs and in response to 1 and 100 nM insulin (30 min incubation), respectively, corresponding to the 97 kDa band. A) Reprobing of the membranes with an anti-β-actin antibody, corresponding to the 42 kDa band is shown below the gels. B) Average InsRs in control and in CBs incubated with 1 and 100 nM insulin in relation to β-actin immunoreactivity (n = 3-4). **p<0.01, * p<0.05; One –Way ANOVA with Dunnett's multicomparison test comparing the groups with the control. Data represent mean ± SEM.

We also tested if InsRs activation in the CB elicits a neurosecretory response by measuring $[Ca^{2+}]_i$ and the release of CAs and ATP, two of the neurotransmitters released from CBs in response to hypoxia (Gonzalez *et al*., 1994; Conde *et al*., 2007; Conde *et al*., 2012; Obeso *et al*., 1985; Vicario *et al*., 2000). **Figure 3.7 A** depicts, a bright-field image of a 20-h-old cell culture of dissociated CB and typical recording of $[Ca^{2+}]_i$, measured as the ratio of the fluorescent emission at 340/380 nm of type I cells in basal conditions, in response to hypoxia (N_2), to 1 nM of insulin and to 35mM of K^+ in left and right panels, respectively. Hypoxia significantly increased $[Ca^{2+}]_i$ by 15.97%.

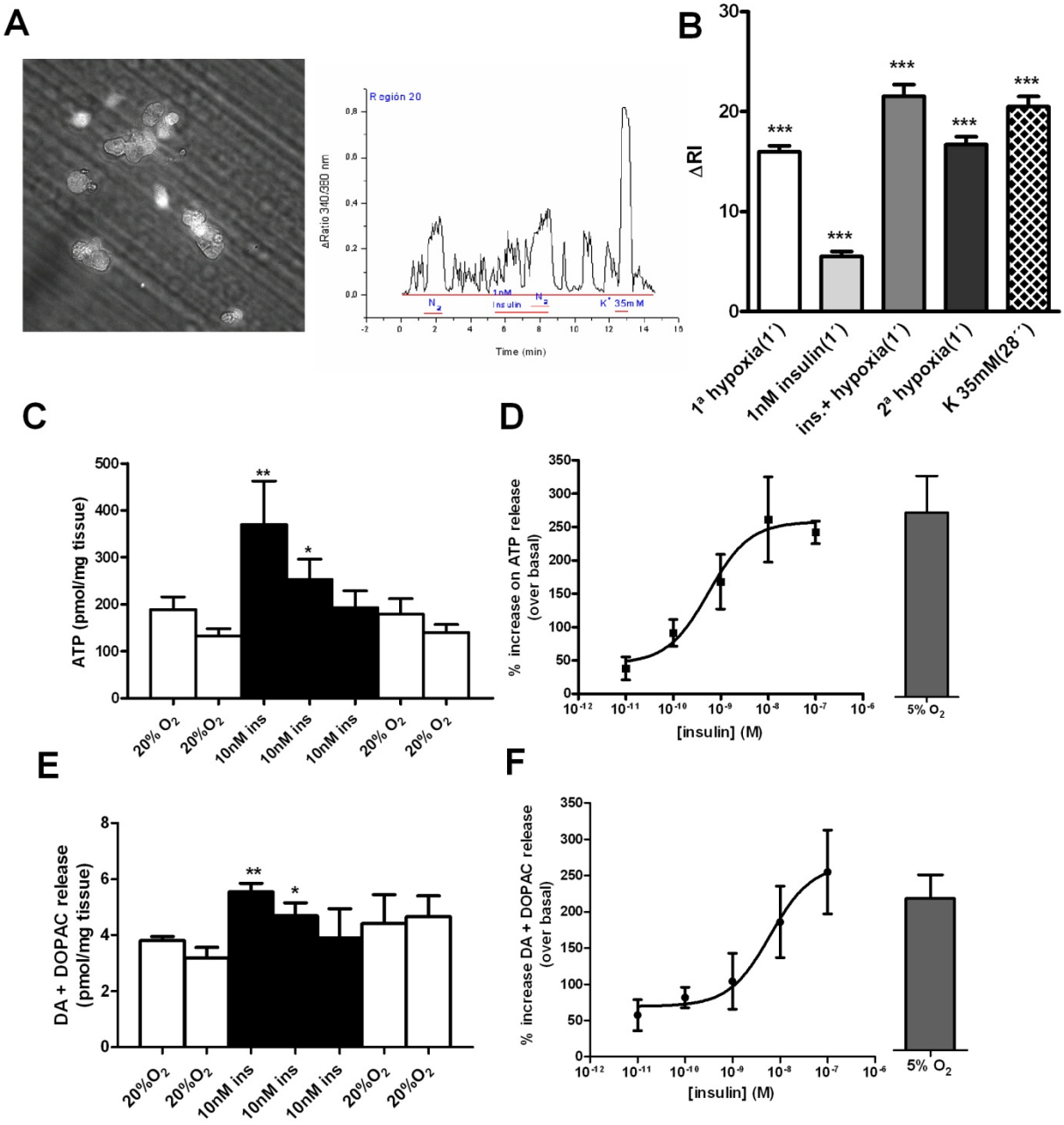

Figure 3.7: Insulin increases the neurosecretory responses in the carotid bodies (CBs). A) Microscope field of dissociated rat CB cell culture and the typical recording of $[Ca^{2+}]_i$, measured as the ratio of the fluorescent emission at 340/380 nm of type I cells in basal conditions, in response to hypoxia (N_2), to 1 nM of insulin and to 35mM of K^+. B) Effect of insulin on $[Ca^{2+}]_i$, measured as means of the ΔRI in 179 type I cells. In every cell the fluorescence signal was integrated as a function of time (running integral; RI). C and D) time course for the release of ATP from CB in response to insulin (10 nM) and dose-response curve for insulin action on ATP release and its comparison with the effect of hypoxia (5%O_2 + 5%CO_2 balanced N_2). Release protocol consisted in a 2 incubations of CBs in normoxic solutions (20% O2 + 5%CO_2 balanced N_2, 10 min), followed by insulin application during 30 min in normoxia and two final normoxic incubations. E), F) Identical group of experiments than C) and D) but measuring catecholamines (CAs) (dopamine (DA) + DOPAC) release from CB instead of ATP. ATP and CAs quantification in the CB are means of 4-6 data. Bars represent mean ± SEM. One and Two-Way ANOVA with Dunnett's and Bonferroni multicomparison tests, respectively; *p<0.05, **p<0.01, *** p<0.001 vs control. Controls in the release experiments correspond to the period prior to insulin application.

Also, 1nM insulin significantly increased $[Ca^{2+}]_i$ by 6.53%. When applied simultaneously, insulin and hypoxia increased $[Ca^{2+}]_i$ concentration by 21.53% suggesting that the transduction mechanisms by which the two stimuli operate are different. To evoke a neurosecretory response, the increase in $[Ca^{2+}]_i$ produced by insulin must be transduced into the release of neurotransmitters from the CB. **Figure 3.7 C, E** show that insulin (10 nM) produced an increase in the basal release (black bars) of ATP and DA (plus DOPAC) from the whole CB in incubating solutions, and the effect was reversed after drug washout. The dose–response curves for the effect of insulin in neurotransmitter release in the whole CB are depicted in **Figure 3.7 D, F**. The curves fitted a sigmoid with EC_{50} of 0.552 nM and 6.17 nM and maximal effects of 257.9% and 265.1% for CB ATP and DA release, respectively. Note that concentrations above 400-500 pM are already compatible with an hyperinsulinemic state (Kronmal *et al.,* 2004; Stegenga *et al.,* 2006) and that when insulin was applied above 10 nM concentrations it evoked the release of ATP and DA (plus DOPAC) from CB in a similar magnitude as produced by hypoxia (5%O_2) (**Figure 3.7 D, F)**. Knowing that stimuli-induced CB activation results in hyperventilation (Gonzalez *et al*., 1994), we assessed the effects of insulin on ventilation. *In vivo* experiments have previously showed that intravenous infusion of insulin-caused a CB-dependent increase in ventilation (Bin-Jaliah *et al.*, 2004), an effect that was not due to hypoglycaemia *per se*, since low glucose is not a direct stimulus for rat CB chemoreceptors (Conde *et al*., 2007; Gallego-Martin *et al*., 2012). Therefore, we tested the effect of an intracarotid *bolus* of insulin on ventilation during an euglycemic clamp, to avoid the confounding effects of systemic hypoglycaemia. **Figure 3.8 A** depicts a typical recording of pulmonary flow and VT before and after an intracarotid administration of an insulin (50mU/kg) *bolus*. Insulin increased RR, VT (**Figure 3.8 A**, left panel **Figure 3.8 D**) and the product of both parameters, VE (**Figure 3.8 C)** in a dose-dependent manner. The increase in ventilation induced by insulin is not immediate, showing a significant latency period (time to the onset of the response) comprised within the 106.0±4.04 and 188.5±3.51 sec range (**Figure 3.8 D).** This observation is in accordance with the time-scale necessary for the activation of tyrosine kinase receptors, namely InsRs (Czech, 1985). Full dose-response curve for the effect of insulin in VE is depicted in **Figure 3.8 C** fitting a sigmoid with an EC_{50} of 35 mU/kg and a maximal effect of 60.41%. **Figure 3.8 E** depicts a typical euglycemic clamp following an intracarotid administration of an insulin *bolus* of 50 mU/kg. As expected the amount of glucose infused to maintain

euglycemia increased in an insulin-dose dependent manner (**Figure 3.8 F**). The effect of insulin on ventilation was totally mediated by the CB, since CSN cut completely abolished the increase in ventilation induced by insulin (right panel **Figure 3.8 D**).

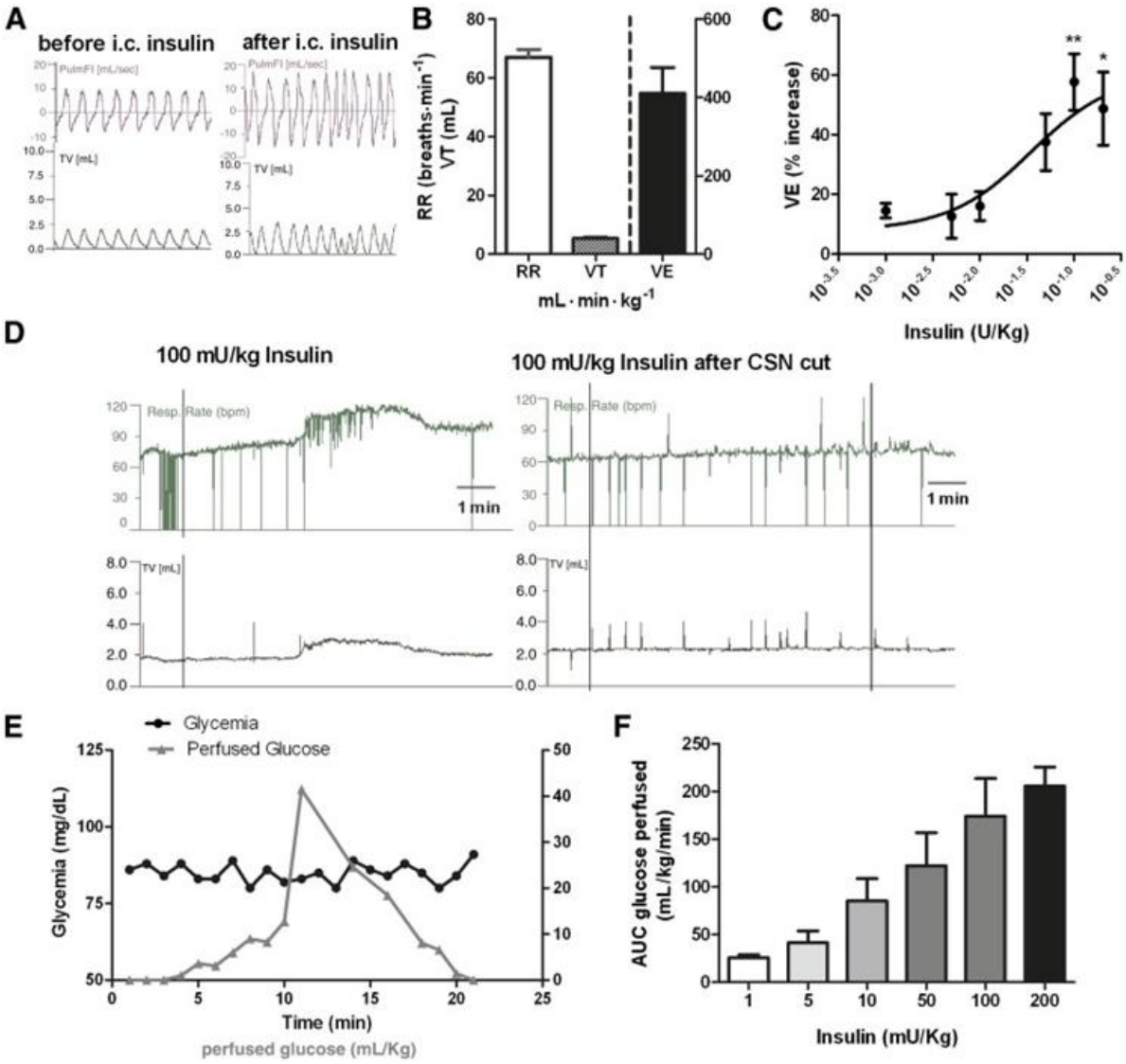

Figure 3.8 Insulin increases ventilation through a carotid body (CB) mediated effect. A) Respiratory rate (RR) and tidal volume (TV) recordings before and after administration of an intracarotid insulin (100mg/kg) *bolus***.** B) Mean basal ventilatory parameters, RR, VT and minute ventilation (VE) before insulin administration. C) Dose-response curve for the effect of intracarotid insulin (1, 5, 10, 50, 100, 200 mU/kg) on VE. To avoid the effect of hypoglycemia the study the study of insulin effect on ventilation was made performed in euglycaemic conditions. Insulin effects on ventilation are means of 5-7 data. D) Typical RR and VT recordings due to the administration of an intracarotid insulin (100 mg/kg) *bolus* before and after carotid sinus nerve (CSN) cut. E) Graph depicting a typical glucose perfusion curve to maintain euglycemia after insulin *bolus* and the levels of glycemia throughout the experiment. F) Show total glucose concentrations perfused to maintain euglycemic clamp in response to the insulin concentrations (1, 5, 10, 50, 100, 200 mU/kg) tested. Values represent means ± SEM. One-Way ANOVA with Dunnett's multicomparison test; *p<0.05, **p<0.01vs basal values. AUC: Area under the curve.

3.4. DISCUSSION

This study represents a new conceptual framework regarding the pathogenesis of IR. Using a combination of neurochemical, physiological and cellular biology techniques we showed that CB activity is increased in models of MS and T2D and that CB dysfunction is involved in the development of IR and HT. In addition, we demonstrated for the first time that insulin triggers the peripheral chemoreceptors located in the CBs, suggesting that hyperinsulinemia may trigger CB-induced sympathoadrenal overactivity associated with metabolic disturbances.

Hyperinsulinemia is a known early pathological feature caused by increased secretory stress on the β cell caused by hypercaloric diets. Increased insulin levels trigger the CBs to activate the sympathetic nervous system, initiating a vicious cycle that worsens peripheral insulin action, impairs β-cell function and causes systemic HT. In line with these results, the CB rises as a new therapeutic target for intervention in metabolic disturbances. We show herein, and also for the first time that CB activity is increased in diet-induced animal models of IR and HT. Carotid body mediated basal ventilation and ventilation in response to ischemic hypoxia were increased in the pathological models tested, as well as the CB chemoreceptor cell function - assessed both as hypoxia induced-release of DA and as TH expression. The increase in CB cell function, together with increased CB weight observed in our experimental setting, are in agreement with the previous observations of Clarke et al. (1999) showing that CB volume is increased in spontaneous insulin-dependent diabetic rats (strain BB/s), an effect that could not be attributed to an increase in the vascular component of the organ. We have also observed that HF animals exhibited more pronounced increases in both spontaneous ventilation and ischemic-hypoxia-induced-hyperventilation than HSu animals, suggesting that the HF animal-model is characterized by a higher degree of CB activation. Our results strongly suggest that there is an obesity-related factor that contributes to CB stimulation. Although some authors have suggested that obesity does not enhance peripheral chemoreflex sensitivity (Narkiewicz *et al*., 1999) this topic remains controversial. It was shown that chronic intermittent hypoxia increases expression of TNF-α and interleukin 1 beta (IL-1β) within the CB (Del Rio *et al*., 2012) and that these pro-inflammatory cytokines may contribute directly to CB-mediated cardio respiratory changes evoked by intermittent hypoxia. Obesity is also characterized by a

sub-clinical pro-inflammatory condition with increased secretion of adipokines, including leptin, TNFα, IL-1β and IL-6, (Trayhurn *et al.,* 2005) the same cytokines proposed as having a role in chemoreceptor changes observed in sleep apnoea. On the other hand, obesity has been associated with increased sympathetic nervous system activity through a leptin-mediated mechanism that is still unclear (Landsberg *et al.,* 1978). Recently it was described that type I cells in the CB express leptin receptors and are activated by intermittent hypoxia and systemic leptin injections (Messenger *et al.,* 2012), which suggests that leptin may be also represent an independent factor in CB activation.

Besides demonstrating that CB overactivity is present in animal models of IR and HT we have also shown that CSN bilateral resection totally prevented diet-induced IR and HT, as well as increased fasting plasma glucose, fasting plasma insulin, FFAs and systemic sympathoadrenal overactivity. In accordance with our results, it was previously observed by other authors that CB stimulation by corconium, a nicotinomimetic agent, causes a rise in circulating insulin that is reversed by CSN resection (Anichkov *et al*., 1962). We have also found that CSN resection decreased insulin sensitivity in control animals, which suggests a role for CB in metabolic control, not only in pathological but also in physiological conditions. This kind of mechanism is not novel in CB physiology, since it was recently proposed that the CB is involved in the counterregulatory response to hypoglycemia and in baroreflex control of blood pressure in humans (Wehrwein *et al.,* 2010).

Regarding the contribution of the CB to the development and maintenance of HT, our work agrees with previous results obtained by other groups in which it was observed that CSN denervation prevented arterial pressure increase and decreased sympathetic activity in spontaneous hypertensive young rats (Abdala *et al*., 2012). It is known that, apart from chemoreceptor activity, CSN carries information related with baroreceptor activity. However, we would like to emphasize that the results obtained herein, both in the common carotid occlusion experiments and the CSN denervation experiments reflect a CB chemoreceptor mediated effect. If there was a significant baroreceptor-mediated effect the animals would have become hypotensive in response to acute ischemic hypoxia and hypertensive after CSN denervation (Scheffers *et al.,* 2010 for review), which was not observed.

Our results show, for the first time that insulin triggers CB activation and that high insulin doses are an effective stimulus for CB overactivation. It is generally accepted that insulin stimulates the sympathetic nervous system, being fasting hyperinsulinemia one of the components of the sympathetic overactivation present in diabetes and the MS (Reaven *et al*., 1988; Landsberg, 2005). However, insulin-induced sympathetic activity has been attributed to a central nervous system effect, since the infusion of insulin into the third cerebral ventricle increased sympathetic outflow, without significantly increasing adrenal and renal sympathetic activity (Munztel *et al.,* 1994; 1995). Without contradicting with these results, we show that insulin can also act on the CBs to increase sympathoadrenal outflow. We demonstrated that InsRs are present in the CB and that its phosphorylation increases in response to insulin in euglycemic conditions. As depicted in **Figure 3.6 B**, 1 nM produced a higher degree of InsR phosphorylation than 100 nM. We expected to find a concentration-dependent relationship in CB InsR phosphorylation, which we did not observe at high insulin concentrations. At high insulin levels InsRs are possibly saturated inducing a functional desensitization either by decreasing tyrosine kinase activity or by promoting InsR endocytosis and degradation as it happens in human HepG2 cell line (Blake *et al*., 1987) and in rat Fao cells (Cettaz *et al*., 1984). Also, we showed that insulin was capable of initiating a neurosecretory response measured as the increase in $[Ca^{2+}]i$ and the release of the neurotransmitters, ATP and DA, that is transduced into an increase in ventilation. The increase of ventilation induced by insulin is not novel (Conde *et al.,* 2007), however in Bin-Jaliah´s work insulin was administered intravenously aiming to study the effects of insulin induced-hypoglycaemia in ventilation. Herein we administered insulin intracarotidally, to guarantee that the first site of insulin action is the CB; also we performed the experiments in euglycemic conditions, to avoid the confounding effects of systemic hypoglycaemia. These results together with the finding that the effect of insulin on ventilation disappears after CSN cut suggests that insulin action on ventilation is mediated by the CB.

In conclusion, we propose that insulin-triggered CB activation is responsible for increased sympathoadrenal activity and outflow creating a vicious cycle that culminates in severe IR and arterial HT, the core features of the MS and T2D.

CHAPTER IV

This chapter is based on the following manuscript:

Ribeiro MJ, Lima PA, Obeso A, Patricio C, Conde SV (2016) Kv1.3 channels mediate insulin action in the rat carotid body (Submitted to Journal of General Physiology).

Kv1.3 channels mediate insulin action in rat carotid body

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ABSTRACT

The CBs are the main peripheral chemoreceptor classically seen as the sensor of arterial O_2, CO_2 and pH levels. Recently, we have described that CB regulates peripheral insulin sensitivity. Also, insulin stimulates the CB through its action on InsRs enabling a neurosecretory response, with the associated $[Ca^{2+}]i$ rise and consequent release of ATP and dopamine, from the whole CB. Herein, we report the presence of Kv1.3 mediated currents and their role as effectors on insulin signaling in rat CB. For that, the effects of insulin (30 nM) on voltage-activated K^+-currents and of Kv1.3 channels blockers, MgTx (1-10nM) and ShK-Dap^{22} (100pM), were investigated by whole-cell voltage-clamp recordings from CB type I cells. Also, the expression of Kv1.3 channels and its phosphorylation in response to insulin was shown. Concordantly, we found that MgTx (10nM) (as well as insulin) induced DA release from the CB. Also, insulin decreases the voltage activated K^+ currents in CB type I cells. In addition, insulin was able to modulate the Kv1.3 channels activity through phosphorylation at residue tyrosine 135 and MgTx was capable of blocking the DA evoked-release by insulin in the whole-CB. We demonstrate for the first time that Kv1.3 channels are functional in CB and mediate insulin action in the CB. Modulation of their activity may serve as a novel therapeutic target for IR.

4.1. INTRODUCTION AND AIM

The CBs are peripheral chemoreceptors that sense arterial O_2, CO_2 and pH levels (Gonzalez *et al*., 1994) and are involved in insulin sensitivity (**Chapter III**). Carotid bodies are constituted by two types of cells, the type I or glomus (chemoreceptor) cells with neurosecretory properties (containing secretory vesicles with neurotransmitters, like ATP, DA, ACh, among others) and the type II or sustentacular cells with glia-like properties (Gonzalez *et al*. 1994; Nurse *et al*. 2005). Type I cells express a broad variety of voltage and ligand gated ion channels, as well as transient receptor potential and background K^+ channels (Gonzalez *et al*., 2009). Under hypoxia, the O_2-sensitive K^+ channels close in the plasma membrane, triggering membrane depolarization originating the influx of the Ca^{2+} to the cytosol, and neurotransmitter release. These neurotransmitters act on the CSN to increase its activity, which is integrated in the brainstem to induce a fan of cardiorespiratory reflex responses to normalize the altered blood gases. Besides its role in the cardiorespiratory control, the CB has been proposed as a metabolic sensor implicated in the control of energy homeostasis (Conde *et al*., 2014) for review and, more recently, we described that the CB regulates peripheral insulin sensitivity (**Chapter III**). We demonstrated that CB overactivation is involved in the development of diet-induced IR and that insulin stimulates the CB through its action on CB InsRs. Also, insulin elicited a neurosecretory response, as it increased the $[Ca^{2+}]i$ and ATP and dopamine release from the CB (**Chapter III**), suggesting that hyperinsulinemia is responsible for CB overactivation in insulin-resistant animal models.

Kv1.3 is a delayed-rectifier of the *Shaker*-like family of Kv channels that present a highly expression in olfactory bulb (OB) in the brain being widely distributed through the body (Bielanska *et al.,* 2010; Stuhmer *et al.* 1989; Swanson *et al*., 1990). In response to voltage stimulation, Kv1.3 channels show a fast activation followed by, and intermediately placed, c-type inactivation and exhibiting prominent cumulative inactivation with repetitive stimulation (Marom & Levitan, 1994; Spencer *et al.* 1997). In addition to the well described function in regulating electrical excitability (Hille, 2001), in the last decade, several studies demonstrated that Kv1.3 channels participate in a wide variety of nontraditional functions outside the cellular electrical excitability, like cellular proliferation, axonal targeting, insulin sensitivity, glucose metabolism,

body weight, apoptosis, protein expression and scaffolding (Kaczmarek, 2006 for review) . This channel has 17 tyrosine residues, six of which lie within good recognition motifs for tyrosine phosphorylation (Hunter, 1995; Songyang *et al*., 1995). Several studies performed in the OB, demonstrated that Kv1.3 channels are modulated by tyrosine kinases and other enzymes linked to signaling pathways, as receptor tyrosine kinases, tropomyosin-related kinase B, the InsR, as well as the cellular tyrosine kinase c-Src (Colley *et al.,* 2004; Cook & Fadool 2002; Fadool *et al*., 2004; Tucker & Fadool 2002;). Each enzyme phosphorylates kinase-specific channel tyrosine residues, which evokes a decrease in Kv1.3 peak current magnitude (Bowlby *et al*., 1997; Fadool *et al*. 1997; Fadool & Levitan, 1998). In the OB, insulin stimulation causes multiple phosphorylation of Kv1.3 at discreet tyrosine residues to induce current suppression of the ion channel (Fadool *et al*., 2000). Additionally, it has been shown that Kv1.3 channels regulate food intake, body control and energy homeostasis in which the hypothalamus is the key (Xu *et al*., 2003). In the skeletal muscle and in adipose tissue, Kv1.3 channels have also been mechanistically related with systemic insulin sensitivity and in glucose uptake (Xu *et al*., 2004). Therefore, knowing that Kv1.3 channels are involved in insulin signaling in central nervous system and in the periphery (Fadool *et al*. 2000; Xu *et al*., 2003; 2004), our hypothesis is that Kv1.3 channels are effectors of insulin signaling in the CB. Herein, we demonstrated, using voltage clamp, that insulin promotes a decrease in K^+-current in CB type I cells and that MgTx and ShK-Dap^{22} mimic insulin action. Also, the reduction of the effect of insulin in K^+-currents with increasing concentrations of MgTx demonstrates the involvement of Kv1.3 channels in this mechanism. We also described for the first time the presence of Kv1.3 channels in CB type I cells. Additionally, we showed that insulin was able to modulate the Kv1.3 channels activity through phosphorylation at residue tyrosine 135 and MgTx was capable of blocking the DA evoked-release by insulin in the whole-CB.

4.2. MATERIALS AND METHODS

4.2.1. Animals and surgical procedure

Experiments were performed in Wistar rats (200–420 g) of both sexes, aged 3 months, obtained from the animal house of NOVA Medical School and from the Faculty of Medicine of the University of Valladolid. The animals were kept under temperature and humidity control (21 ± 1 °C; 55 ± 10% humidity) with a 12 h light–12 h dark cycle. On the day before the experimental procedures, rats were fasted overnight and allowed free access to water. Rats were anaesthetized with sodium pentobarbital (60 mg/kg^{-1} i.p), tracheostomized and the carotid arteries were dissected past the carotid bifurcation. At the end of the experiments, rats were sacrificed by an intracardiac overdose of pentobarbital. Principles of laboratory care were followed in accordance with the European Union Directive for Protection of Vertebrates Used for Experimental and Other Scientific Ends (2010/63/EU). Experimental protocols were approved by the ethics committee of the NOVA Medical school and of the Faculty of Medicine of the University of Valladolid.

4.2.2. Electrophysiology Methods

Type I cells have been selected from the CB-dissociated cell culture based on the response to hypoxia (2% O_2+5%CO_2+93% N_2). Whole-cell voltage-clamp recordings were made in dissociated type I cells at room temperature (20-24ºC) using an Axopatch 200B (Axon Instruments, Inc). Microelectrodes (1.8-4.0 MΩ), pulled from borosilicate glass (Science Products GMBH) were filled with the pipette-solution which contained (in mM): 140 $KMeSO_4$, 1 $MgCl_2$, 10 HEPES, 10 EGTA, 1 $CaCl_2$, 2 Na_2ATP, 0.4 Na-GTP, pH 7.2-7.3 titrated with KOH; (calculated free [Ca^{2+}] = 60 nM by Webmaxclite v1.15, MaxChelator). The external bathing solution was constantly superfused (~ 2-3 ml/min) and contained (in mM): 140 NaCl, 5 KCl, 2 CaCl2, 1.1 MgCl2, 10 HEPES, 5.5 glucose, pH 7.4 titrated with NaOH. The estimated junction potential (jPCalc) for the present pair of solutions was -9.2 mV; data were not corrected for the junction potential. Currents were measured with capacitance compensation and series resistance compensation (80%) filtered at 2 KHz sampled at 5 kHz, using a Digidata 1200 AC converter (Axon Instruments) and pClamp software (v6). Only cells with negligible current rundown after stabilization of whole-cell recording conditions were used for experiments. Time was allowed for the stabilization of the recording before experiments were conducted. To monitor drug effects, currents were recorded every 30 sec or every

minute with a set of two command pulses to 0 and +30 mV lasting 900 milliseconds, from a holding potential of -60mV; a pulse to -20mV applied after the command pulse to study deactivation. To detect eventual current-components highly dependent to voltage, such as, for example, type-A K^+ currents, the same command pulse to +30 mV was preceded either by a prepulse to -30 mV or -120 mV.

To obtain activation-voltage profiles, a set of 9 command pulses from -50mV to +30mV (10 mV increment lasting 900 ms) was used (holding potential of -60 mV); command pulses were preceded by a set of nine 100 ms duration prepulses (from –75 mV, increments of 2 mV) to calculate and subtract leak current during data processing. Sensitivity of the K^+-currents of CB type I cells to insulin (30 nM), was accessed by prediluting it in the superfusing solution. To investigate Kv1.3 mediated current, recordings were monitored under treatment with MgTx, (1-10nM) but also with ShK-Dap[22] (100 pM). Since, in comparison with other ions channels, the Kv1.3 channel is sensitive to many pharmacological agents including small organic compounds and peptide toxins such as MgTx, agitoxin-2 (AgTx2) and ShK-Dap[22] among others (Anangi *et al.,* 2012; Rashid *et al*., 2013), we have tested the sensitivity to MgTx (1-100 nM) and ShK-Dapp[22] (100 pM) applied alone or together with insulin.

To access if the insulin sensitive current was coincidental with the MgTx sensitive component, insulin was applied during the treatment with different concentration of MgTx (1-10 nM). In reverse, MgTx was applied after the insulin effect.

Whole cell data were analysed using Clampfit (v9) (Axon Instruments, Inc.) and Origin (v5) (Microcal Origin). Peak current was taken for current amplitude voltage dependence of activation data were converted to conductance using the relationship $G=I/(V-E_{K+})$, where I is current amplitude, V is the step command potential and E_{K+} the estimated equilibrium potential for K^+. Results were plotted against the step command potential and fit with the following equation:

$$G/Gmax= A1-A2/\{1+\exp [V-V_{1/2})/Vs]\}+ A2 \quad (Eq.1)$$

4.2.3. Immunocytochemistry

For the immunocytochemistry of Kv1.3 channels and their co-localization with TH in the Type I cells of CB, specific antibodies to these two proteins were used: a mouse

monoclonal anti-TH (1:1000, Sigma, Madrid, Spain) and a rabbit polyclonal anti-Kv1.3 primary antibody (1:50, Alomone Labs, Jerusalem, Israel). Coverslips were washed with phosphate-buffered saline (PBS; 2 x1 min) and fixed with 4% paraformaldehyde in PBS at room temperature (15 min), and finally the fixative was washed away with PBS at room temperature (3 x 5 min). Cells were then exposed to a blocking permeabilizing solution containing 2% of goat serum and 0.1% of Triton X-100 in PBS for 20 min at room temperature, followed by an overnight incubation at 4ºC in a humidified chamber with the following cocktail of primary antibody: mouse anti-TH (1:1000) and rabbit anti-Kv1.3 (1:50). Incubation without primary antibodies yielded only background levels of signal (**Figure 4.4 F**). After this step, cells were washed and re-incubated in dark with secondary antibodies with fluoresce in isothiocyanate (FITC)-conjugated goat anti-mouse (1:1000, Sigma Immuno-Chemicals, Madrid, Spain), goat anti-rabbit conjugated to Alexa 594 (1:1000, Molecular Probes, Eugene, OR, USA) in PBS containing 2% normal goat serum for 2 h at room temperature. The incubation medium also contained 4-6 diamina-2-phenylndole (DAPI, 1:1000). After washed in PBS and distilled water, coverslips were mounted in a photobleaching protective medium Vectashield H-1000 (Vector Laboratories, Burlingame, CA, USA). Cells were photographed by fluorescence microscopy with appropriate filters, using Zeiss Axioscop 2 (mot plus) microscope equipped with a digital camera (Cool Snap cf) and analyzed with Metamorph 6.3 software.

4.2.4. Western Blot Analysis of Kv1.3 channels and Kv1.3 phosphorylation at tyrosine 135 residue

Kv1.3 expression was evaluated in skeletal muscle and CBs, respectively collected from rats submitted to an overnight fasting and frozen liquid nitrogen. Both tissues were homogenized in Zurich (10 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, 1% sodium cholate, 1% SDS) with a cocktail of protease inhibitors. For Kv1.3 expression evaluation, samples were separated by SDS-PAGE under reducing conditions and polyvinylidenedifluoride membranes (0.45µM, Millipore, Madrid, Spain). After blocking, the membranes were incubated overnight at 4 °C with the primary antibodies against Kv1.3 protein (1:500, Alomone Labs, Jerusalem, Israel). The membranes were washed with TBST (0.1%) and incubated with goat anti rabbit-HRP

(1:2000, Santa Cruz Biotechnology, Heidelberg, Germany) in TBS for 2 h at room temperature and developed with Clarity Western ECL (BioRad, Madrid, Spain). Intensity of the signals was detected in a Chemidoc Molecular Imager (Chemidoc, BioRad, Madrid Spain) and quantified using the Quantity-one software (BioRad, Madrid, Spain). The membranes were re-probed and tested for β-Actin (bands in the 42 kDa).

For evaluation of Kv1.3 phosphorylation, the CBs were isolated from fasted animals, cleaned, and incubated at 37°C during 30 min in Tyrode solution (nM) (NaCl 116; KCl 5; $CaCl_2$ 2; $MgCl_2$ 1,1; HEPES 10; Glucose 5.5) containing 10 nmol/L of insulin (Humulin Regular, Lilly, Portugal) and bubbled with 20% O_2, 5% CO_2, and 75% N_2. Carotid bodies were homogenized in Zurich medium as described in the previous paragraph. Proteins were separated by SDS–PAGE (10% with a 5% concentrating gel) under reducing conditions and electro-transferred to nitrocellulose membrane (0.2 mM; BioRad, Madrid, Spain). For Kv1.3 phosphorylation evaluation a three-step Western blot protocol adapted (Johnson *et al.* 2009) was used, to enhance detection sensitivity in order to maximize the signal that is low due the small size of the CBs. After blocking membranes were incubated with a rabbit polyclonal anti-Kv1.3/KCNA3 phospho-Tyr135 antibody (1:100, Assaybiotech, Sunnyvale, US). The membranes were washed with TBST (0.02%) and incubated in TBST (0.1%) containing biotin conjugated goat anti-mouse IgG or goat anti-rabbit IgG (1:10,000; Millipore, Madrid, Spain). After, membranes were incubated in TBST (0.1%) containing HPR conjugated streptavidin (1:10,000, Pierce, ThermoFisher Scientific, and Rockford, USA). Membranes were then washed in TBST (0.02%) and developed through enhanced with clarity western ECL substrate (BioRad, Madrid, Spain). Intensity of the signals was detected in -DiGit® Blot Scanner (LI-COR) and using the image studio software 4.0. The membranes were re-probed and tested for α-tubulin (1:5000, Sigma, Madrid, Spain) immunoreactivity to compare and normalize the expression of proteins with the amount of protein loaded.

4.2.5. Effect of insulin and margatoxin on dopamine release in the carotid body

For the release of DA and its major metabolite, DOPAC, the carotid bifurcation was placed in a Lucite chamber in ice-cold/95% O_2-equilibrated Tyrode (in mM: 140 NaCl,

5 KCl, 2 CaCl2, 1.1 MgCl2, 10 HEPES, 5.5 glucose, pH 7.40) and the CBs were cleaned of CSN and nearby connective tissue. Afterwards, the CBs were incubated in bicarbonate-CO_2 buffered solution of composition identical to above except for the absence of HEPES, substitution of 24 mM NaCl by equimolar amounts of $NaHCO_3$ and for the fact that all equilibrating gas mixtures contained 5% CO_2. This solution also contained cofactors for TH and DA-β-hydroxylase (20 μmol/L tyrosine, 100 mmol/L ascorbic acid, and 500 nmol/L 6-methyl-tetrahydroptine). Solutions were kept at 37°C and continuously bubbled with normoxia (20% O_2, 5% CO_2, and 75% N_2), except when hypoxic (5% O_2, 5% CO_2, and 75% N_2) stimuli were applied. Due to their small size (CB wet weight ≈50 μg) four CBs were used in each experiment. To test insulin or MgTx alone on the release of DA/DOPAC, the protocols consist in two 10-min incubations in normoxia coditions, followed by three incubations with insulin (10 nM) or MgTx (10 nM) alone, followed by two normoxia periods one incubation in hypoxic conditions and the last one, in normoxic. To test the effect of MgTx on the insulin-induced dopamine release the protocol consists in two 10-min incubations in normoxia, followed by 1 incubation with MgTx (10 nM), followed by two incubations with insulin (10 nM) plus MgTx (10 nM), two incubation in normoxia, one incubation in hypoxia and the last one, one incubation in normoxia. The solutions were renewed at each fixed time, and all fractions were collected and quantified as previously described (Conde *et al*. 2006; 2012b).

4.2.6. Statistical Analysis:

Data were evaluated using Graph Pad Prism Software, version5 (GraphPad Software Inc., San Diego, CA, USA) and adobe illustrator CS6 and presented as mean values with their standard errors. The significance of the differences between the mean values was calculated by Student t- test and one- way ANOVA with Dunnett's multiple comparison tests, respectively. Differences were considered significant at $^{**}p<0.01$ and $^{***}p<0.001$.

4.3. RESULTS

4.3.1. Presence of insulin sensitive voltage-activated K^+ currents in the rat carotid body

Whole-cell voltage clamp recordings allowed the characterisation of the voltage-activated K^+ currents in isolated rat CB cells. Depolarization evoked outward currents with threshold for activation of around-30 mV and little inactivation. **Figure 4.1 Ai** shows typical current traces elicited by the voltage protocol of incremental pulses. The consequent voltage profile, pulled together with similar recordings, is plotted in the voltage/conductance relationship (**Figure 4.1 Aii**). The parameters of the fitting function (e.q1, see page 95) showed typical values of $V_{1/2}$=-2.4±1.8 mV and slope=11.5 ±1.6 mV n=12). The nature of the currents found and indeed their voltage sensitivity were similar to what was published before for mouse type I CB cells (e.g. Pérez-Garcia *et al*., 2004).

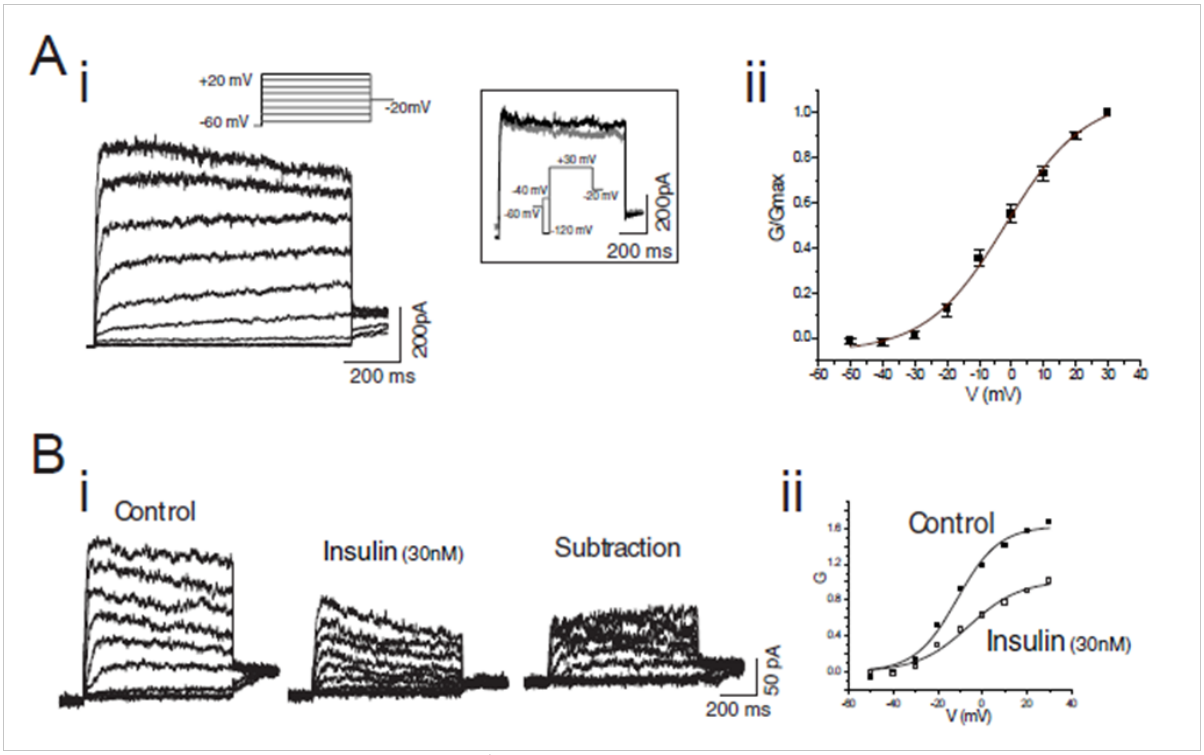

Figure 4.1: Voltage-activated outward K^+ currents in carotid body (CB) type I cells. A) and sensitive to insulin B). Ai) Currents evoked by a voltage protocol aiming at the characterization of voltage dependence of activation (depolarizing steps in increments of 10mV holding potential -60 mV; see insert). Aii) Amplitudes were converted to conductance and normalized to maximal conductance. Figure refers to consequent activation curve fit with a Boltzman equation (Eq.1) from averaged recordings (n=12) such as the one presented in 'a' (V1/2=-3.9 mV; Vs=11.4 mV; n=12). Box: currents evoked by depolarizing step to +30 mV (holding potential -60mV), which were preceded either by a prepulse to -40 mV (trace in grey) or -120 mV (trace in black). Current-traces were similar. B) Effect of insulin (30 nM)

on voltage activated currents elicited by same protocol as 'Ai and Aii'. Typical example of current traces (i) and subsequent activation curves (ii) obtained before (black symbols) and under 30 nM insulin (open symbols). Amplitudes were converted to conductance; data points were fit with Eq.1. (Control, V1/2=-9.2mV, Vs=9.4mV/e; insulin V1/2=-9.3 mV, Vs=9.2 mV/e).

Additionally, we investigated for the existence of current components with inactivation dependent to voltage, using a prepulse to -120 or -40 mV (inset-box of **Figure 4.1**). As shown in the example presented, no differences were detected, result that points out for the inexistence of A-type currents/channels. **Figure 4.1 B** shows the effect of insulin (30 nM) on such currents. From **Figure 4.1 Bi**, one can observe the insulin-sensitive currents, obtained by subtracting the currents recoded under the effect of insulin with those obtained prior to insulin. The voltage dependence of activation before and during insulin can be depicted from the voltage profiles presented in **Figure 4.1 Bii**. Interestingly, insulin did not evoke significant changes in the I-V curves ($\Delta V_{1/2}$= -0.32±0.35mV; n=4).

4.3.2. Voltage-activated K^+ currents are sensitive to specific Kv1.3 antagonists.

To investigate the presence of Kv1.3 mediated currents, two antagonists of the Kv1.3 channels were applied in similar CB cells under voltage clamp recordings (MgTx and ShK-Dap^{22}; **Figure 4.2**). The effects of both blockers were tested on the outward currents elicited in a single step to +20 mV (**Figure 4.2 Ai**, **Bi**) and possible effects on voltage dependence activation (**Figure 4.2**. **Aii, Bii**). Margatoxin (1nM) evoked a clear reduction of the current (**Figure 4.2 Ai**), an inhibitory effect very similar to the one induced by ShK-Dap^{22} (100pM, **Figure 4.2 Bi**). The current sensitive to such pharmacological tools are presented in the subtracted currents, showing obvious similarities. In both cases, there were little changes in the current voltage dependence evoked by the inhibitory effects (before MgTx $V_{1/2}$=2.98mV and after MgTx V1/2=-1.3mV; before ShK-Dap^{22} $V_{1/2}$= -1.2mV and after ShK-Dap^{22} $V_{1/2}$=3.9mV (**Figure 4.2 Aii**, **Bii**). Such subtle shifts do not appear to be relevant considering that voltage clamp recordings tend to show small hyperpolarizing shifts during considerable time periods when using whole-cell configuration (Vicente *et al*. 2010). As whole, the figure shows that Kv1.3 channels contribute to the whole-cell voltage activated K^+ current.

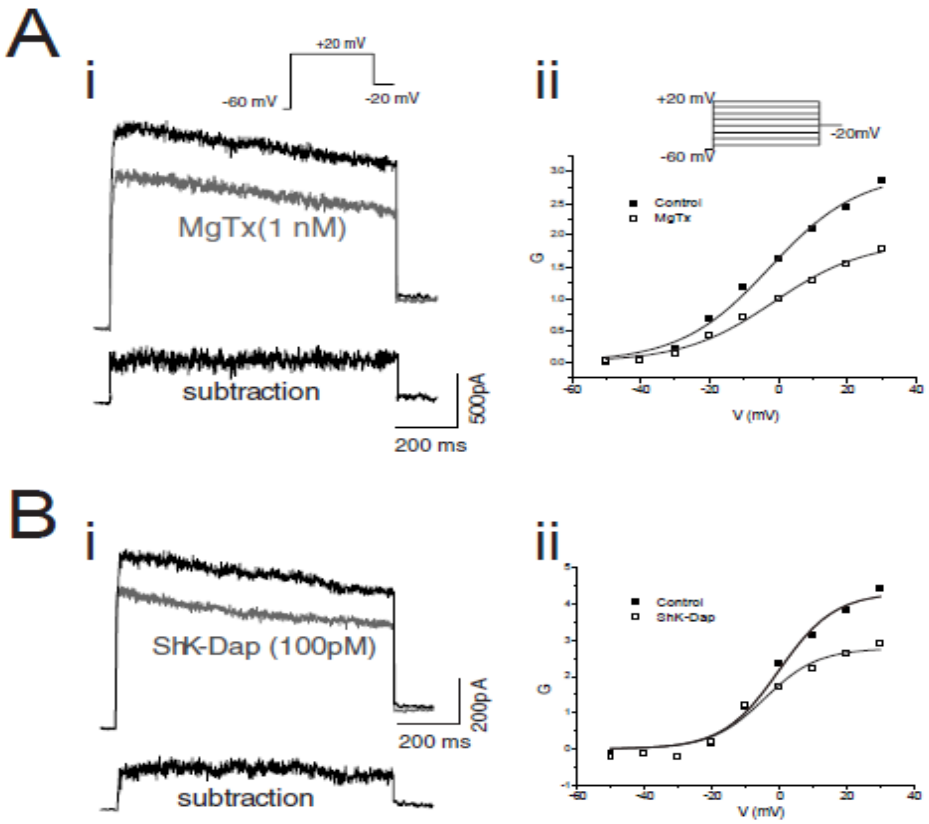

Figure 4.2: Effect of the Kv1.3 blockers on voltage activated outward K^+ currents carotid body (CB) type I cells**.** Current-traces evoked by depolarizing pulse to +20mV before and under application of Ai) 1nM Margatoxin (MgTx) and Bi) 100 pM ShK-Dap[22]; lower traces corresponding to the current subtraction. Aii) and Bii) correspond to the effects of the same antagonists on the voltage dependence of activation; activation curves derived from currents obtained using same protocol as in Figure 4.1 Ai) and Aii). Typical examples of activation curves before (black symbols) and under application of antagonists (open symbols). Current amplitudes were converted to conductance and were fit with Eq. (1). The fitting parameters were as follows: for MgTx Aii), control, V1/2=-2.4mV, Vs=13.0mV/e; MgTx V1/2=-1.3 mV, Vs=13.1 mV/e); for ShK-Dap[22] Bii), control, V1/2=-1.2mV, Vs=8.3mV/e; MgTx V1/2=-4.2 mV, Vs=8.0 mV/e);

4.3.3. Kv1.3 channels underlie the insulin-sensitive K^+ currents

Although the current-component sensitive to insulin appear to be very similar to the ones affected by both Kv1.3 blockers, we set-up to investigate if the same current component was indeed in play during the insulin mediated inhibition and the one inhibited with the Kv1.3 blockers. In other words, it is pivotal, for the context of the present publication, to investigate if insulin activated signaling cascade leads to the inhibition of Kv1.3. Hence we conduct the experiments presented in **Figure 4.3,** in which successive application of insulin and MgTx (and vice versa) were made. In **Figure 4.3 A**, we investigate the effects of different concentrations of MgTx (1-10 nM)

on the record K^+ outward currents (evoked by single steps to + 20mV) and consequently, we co-apply insulin (at a fixed concentration of - 30nM) to compare the late effect with the different concentrations of MgTx. Hence, in **Figure 4.3 Ai**, one can be observe the time course of the peak current amplitude during the experiment.

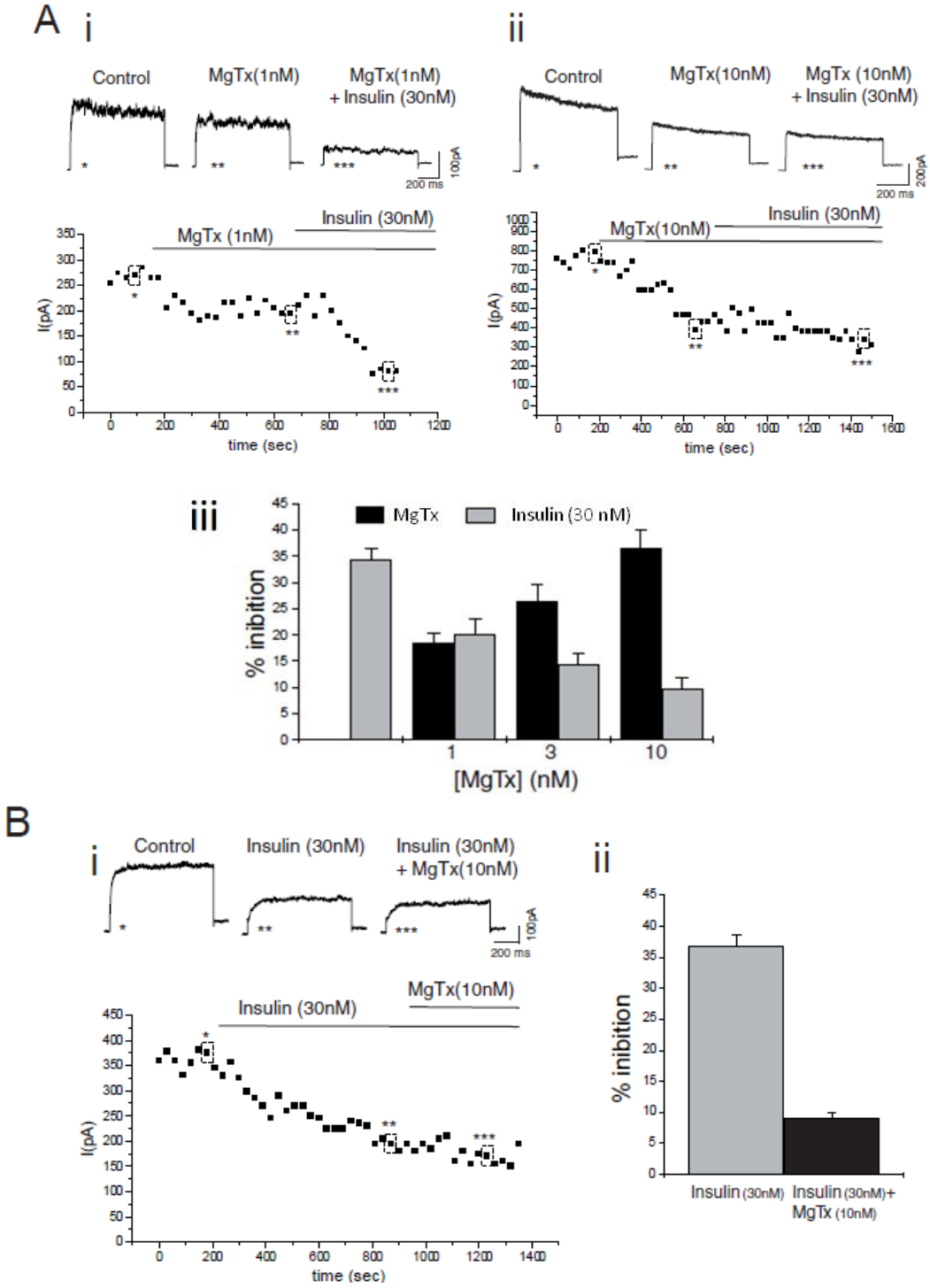

Figure 4.3. Margatoxin (MgTx) sensitive currents (Kv1.3) underlie the insulin sensitive K^+ currents. A) Effects on voltage activated K^+ currents of different concentrations of MgTx and consequent co-application of insulin (30 nM) and. Typical experiments with pharmacological treatments of 1nM MgTx Ai) and 10 nM Aii), both followed by application of 30 nM insulin (still in the presence of MgTx); Top panels show typical current traces of control, during MgTx application and Insulin+MgTx; bottom panels

are the time-courses of the peak-current amplitude over the course of the experiment, also showing the time points of the chosen illustrative current-traces (above). Aiii) Graph showing relationship between effects of applications, in % of inhibition, of different concentrations of MgTx (1, 3 and 10 nM, black columns) and consequent co-application of Insulin (30nM; grey columns) (n=3 to 6). B) Effects on voltage activated K^+ currents of insulin (30nM) and consequent co-application of MgTx (10nM). Bi-time course of the effects on current amplitude, as well as, respective illustrative current traces. Bii) Graph showing % of inhibitions obtained from 4 similar experiments. Bars represent means ±SEM.

The application of the lowest concentration of MgTx (1nM) resulted in a moderate reduction of the current (see respective current-traces above). Still, in the presence of 1nM MgTx, the co-application of insulin resulted in a clear, severe even, reduction of the whole-cell current. In contrast, in **Figure 4.3 Aii**, a larger concentration of MgTx (10 nM) evoked a much larger reduction of K^+ current (in comparison with the one obtained with 1 nM of MgTx). Most interestingly, the consequent co-application of insulin (30 nM) resulted in a modest effect on the current. Such trend gains clarity in the pulled data presented in **Figure 4.3 Aiii**. The graph shows that there is a clear dose-dependency of MgTx in what current inhibition is concerned (columns in black). Such tendency is the inverse of the insulin dependent inhibition when applied after the respective concentration of MgTx. In other words, the insulin effect on the current was as little as large the prior MgTx mediated inhibition was. This strongly indicates that both insulin and MgTx were acting on the same channel and those were mediating the same effected current-components.

In order to confirm this further, we conduct the inverted experiment (**Figure 4.3 B**), in which, we applied MgTx (at the larger concentration) but after the pre-treatment with insulin. From **Figure 4.3 Bi**, we can note that MgTx, after the application of insulin (30 nM), even at 10 nM, failed to evoke a substantial reduction on K^+ currents. In **Figure 4.3 Bii** this phenomena became even clearer with the pulled data. Altogether, this shows that the activation of the InsRs leads to an inhibition of Kv1.3 channels.

4.3.4. Kv1.3 channels are present in type I cells at the rat carotid body.

The presence of insulin sensitive voltage-dependent K^+ currents in the rat chemoreceptor cells strongly suggested that the insulin sensitive-Kv1.3 channel could be located in type I cells. To test that further, we have performed immunocytochemical studies (**Figure 4.4).** In **Figure 4.4 A,** a bright field image of a 24-h-old culture is shown. In **Figure 4.4 B** and **4.4 C** depicts the immunostaining for TH positive type I

cells (green colour) and for DAPI (in blue), respectively, in the same microscopy field. Note that TH is the gold standard marker for type I cells (see for ex. Conde *et al.* 2006; Gauda *et al*. 2002).

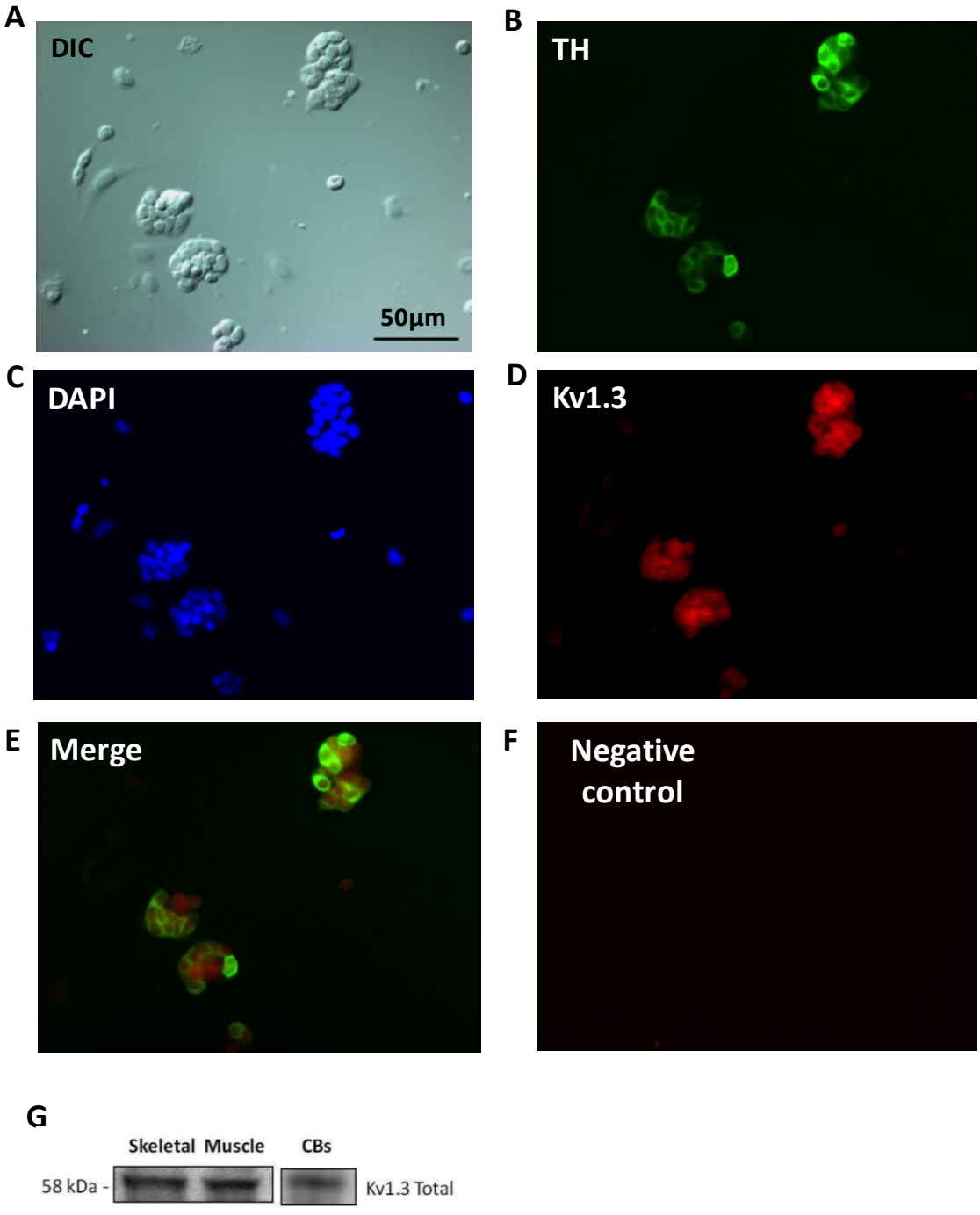

Figure 4.4; Immunocytochemical demonstration of Kv1.3 channels presence in carotid body (CBs) type I cells. A), E) Correspond to images from microscopic field of a 24-h-old culture of CB dissociated cells. A) Show a bright field of the CB culture; B) Depicts the tyrosine hydroxylase (TH) staining in green, and therefore CB type I cells; C) Show cells immunopositive staining for DAPI, and therefore represent all the nucleus of cells in culture; D) Shows the immunostaining for Kv1.3 channels, in red colour, in the CB cell culture;E) Depicts the image resulting from the superimposition of images in B) and D); F) Shows the negative control for Kv1.3 channels, where CB- dissociated cell culture was incubated only with the

secondary antibody. In G) is depicted a representative Western blot for Kv1.3 channels expression in the CB and in the skeletal muscle. Scale bar 50 μm.

Also note, from the immunostaining with DAPI, that apart from type I cells, there is evidence of another types of cells (**Figure 4.4 C**). **Figure 4.4 D** shows, in the same microscopy field, the immunostaining for Kv1.3 channels (red colour) in the CB cell culture. **Figure 4.4 E** shows the image resulting from the superimposition of images in panels B) and D). Overall, it is evident that all TH^+ type I cells are also positive to Kv1.3 channels. In addition we can see TH^-cells that are immunoreactive to Kv1.3 channels, as well as some cells that are unreactive to both antibodies (**Figure 4.4 E**). The immunocytochemical results described herein are in agreement with the Western blot analysis for the expression of Kv1.3 channels in the CB showed in **Figure 4.4 G**. Positive controls for Kv1.3 channels in the skeletal muscle were used to validate the Western Blot technique used (**Figure 4.4 G**). Altogether, these results demonstrated for the first time that Kv1.3 channels are present in the rat CB type I cells.

4.3.5. Kv1.3 channels are phosphorylated by insulin.

Using a Western blot approach we have examined if the Kv1.3 channels are phosphorylated in response to insulin, more specifically in tyrosine residue 135 of the channel. Western blot analysis demonstrated that incubation during 30 min in presence of 10 nM of insulin increases the tyrosine phosphorylation in Tyr 135 residue and consequently the activity of Kv1.3 channels. The incubation of CB with 10 nM of insulin significantly increased by 42.24% the Kv1.3 phosphorylation (**Figure 4.5**). Note that we have used 10 nM of insulin, since we have previously described that concentrations of 1 and 100 nM increase InsR phosphorylation and 10 nM produced a maximal release of ATP from rat CB (**Chapter III, Figure 3.6**).

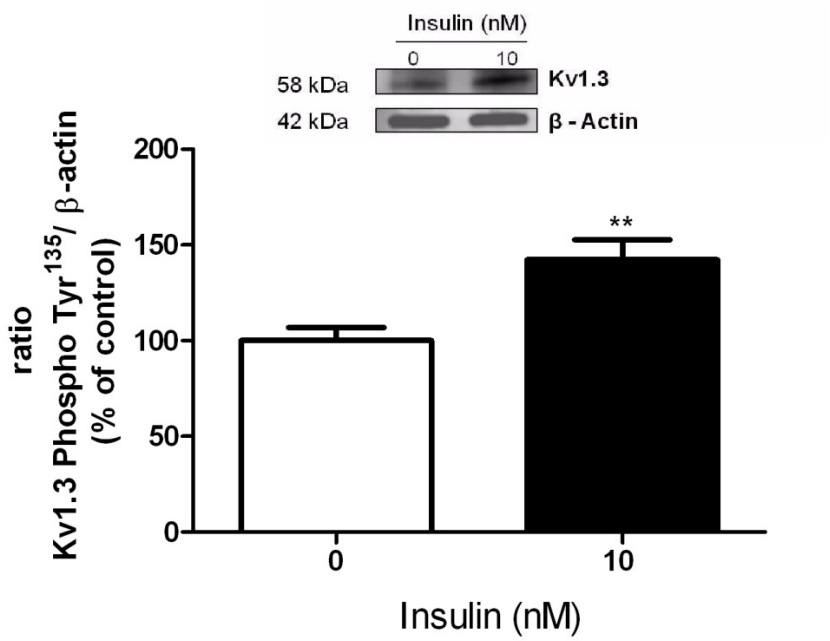

Figure 4.5: Kv1.3 activity, measured as its phosphorylation in tyrosine motifs, increases in response in insulin in the carotid body (CB). Top: representative western blot showing the immunoreactivity for Kv1.3 in CBs in absence of insulin and when incubated during 30 min with 10 nM of insulin. Kv1.3 channels correspond to the 58 kDa band. A reprobing of the membranes with an anti–β-actin antibody, corresponding to the 42 kDa band, is shown below the gels. The graph shows average phosphorylation of Kv1.3 channels in residue tyrosine (Tyr) 135 in CBs incubated in the absence and in the presence of 10 nM of insulin. Averaged phosphorylation was expressed in relation to β-actin immunoreactivity (n = 6–8). **p<0.001 Unpaired Student´s t test. Bars represente means ±SEM.

4.3.6. Kv1.3 channels mediate insulin-evoked dopamine release from carotid body type I cells

To investigate if Kv1.3 channels mediate insulin-evoked DA release from CB chemoreceptor cells, the effects of MgTx alone and combined with insulin were tested on the basal (20% O_2, normoxia) and hypoxic-evoked (5%O_2) release of DA and its metabolite DOPAC from CB (**Figure 4.6**). With the application of 10 nM of insulin the release of dopamine and its metabolite DOPAC increase by 29.46% in relation to control condition (absence of insulin in 20% O_2) (**Figure 4.6 A**). When CBs were incubated in the presence of 10 nM of MgTx alone, the release of DA+DOPAC increases, mimicking the effect of insulin administration (**Figure 4.6 B**). In fact, the increase in the release of DA, in the presence of MgTx, occurs in a similar magnitude to the one released by insulin, suggesting that both substances share the same effectors (**Figure 4.6 A**, **B**). The co-application of MgTx plus insulin at concentration 10 nM (see **Figure 3.7 D**, page 81) decreased CB DA+DOPAC released, implying that MgTx

blocks the effect of insulin on dopamine release. Being MgTx a Kv1.3 channels blocker, these results mean that insulin affects the release of DA from CB type I cells through a mechanism that involves Kv1.3 channels.

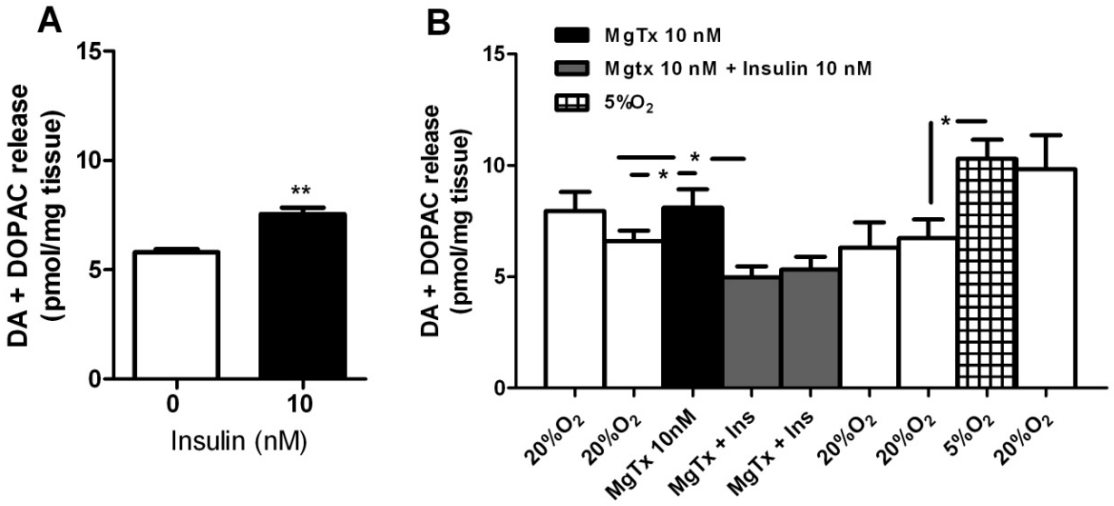

Figure 4.6: Kv1.3 channels mediate insulin-induced dopamine (DA) release from the carotid body (CB). A) Represent the effect of insulin (10 nM) on the release of DA plus its main metabolite, DOPAC, from the rat CB. In panel B) is depicted the effect of MgTx (10 nM), applied alone and together with insulin (10 nM) on the release of DA+DOPAC from the CB. In the same graph is represented the response of the same CBs to a hypoxic stimulus (5%O_2). The release protocol consisted of incubations during 10 min of CBs in normoxic solutions (20% O_2 plus 5%CO_2 balanced N_2, 10 min) in presence of insulin (10 nM) or MgTx (10 nM) alone or MgTx plus insulin. Additionally, a hypoxic stimulus (5% O_2 plus 5%CO_2 balanced N_2, 10 min) was delivered at the end of the release protocol to compare the magnitude of the responses. Dopamine+DOPAC quantification in the CBs are means of 5-6 data. Bars represent means ± SEM. *p<0.05; **<0.01; One Way ANOVA with Bonferroni's multi comparison test

4.4. DISCUSSION

Using a combination of electrophysiological, neurochemical, pharmacological and immunocytochemical techniques we demonstrated for the first time that Kv1.3 channels are expressed in CB type I cells and mediate insulin action at the rat CB. Herein, we showed that the CB has insulin sensitive K^+ voltage dependent channels and also MgTx and ShK-Dap^{22}mimic the effect of insulin in K^+ currents. Importantly, we have shown that insulin failed to reduce K^+ currents when in the presence of MgTx, a Kv1.3 channels blocker, and vice versa, meaning that insulin and MgTx are mediating the same affected current-components. Additionally, we showed that Kv1.3 channels are present in the CB, more specifically in the CB type I cells, and that they are phosphorylated in the presence of insulin. Moreover, MgTx mimics the effect of insulin

on the release of DA from the rat CB. Overall, these findings point out for cellular mechanism underlying the previously reported role of CB in IR/sensitivity (**Chapter III**), mechanism of which involving cellular excitability controlled by insulin via the modulation of Kv1.3 channels.

Carotid body type I cells express a number of different K^+ channels, such as TASK, BK and/or Kv channels with an O_2 sensitive background (Buckler *et al*., 2000; Pérez-Garcia *et al*., 2004; Pichard *et al*., 2015, Sanchez *et al*., 2002). Using whole-cell voltage clamp recordings we have characterised the voltage-activated K^+ currents in isolated rat CB cells. The nature of the K^+ currents found and their voltage sensitivity were similar to what was published before for mouse CB cells (Pérez-Garcia *et al*. 2004) but not that similar to those of rabbit CBs (Sanchez *et al,* 2002). In the latest, more rapidly inactivating K^+ currents, with a strong voltage dependence of inactivation, are reported: A-type currents mediated by Kv channels like Kv1.4, Kv3.4 and Kv4.3. Such currents were clearly not present in our recordings from rat CB (box in **Figure 4.1**). The Kv1.3 mediated slow insulin sensitive current reported in the present manuscript is likely to be different that is the recruited for O_2 sensing, at least in the rabbit CB, that shows clear A-type faster K^+ currents (Sanchez *et al*. 2002).

We have recently shown that insulin activates the CB promoting the increase in $[Ca^{2+}]i$ and the release of neurotransmitters (**Chapter III, Figure 3.7**), therefore in an attempt to find the mechanism of insulin action on the CB we have hypothesized that insulin acts on the type I cells to suppress voltage-activate K^+ currents. Such inhibition, evoke a depolarization, which would trigger the activation of voltage-activated currents. Herein we have shown for the first time, that insulin suppresses voltage-activated K^+-currents in the CB type I cells, similarly to what happens in neurons of the OB (Fadool *et al*., 2000), differentiated N1E-115 neuroblastoma cells and in hippocampus neurons (Lima *et al.* 2008). We demonstrated that insulin promoted a reduction in K^+ conductance without changing the sensitivity to voltage. These biophysical properties, as well as the profile of the currents and its kinetics are compatible with Kv1.3 channels, as it was described in several studies that these currents are characterized by a fast activation outward current that takes few milliseconds and a slow deactivation (Coetzee *et al*., 1999; Gutman *et al*., 2005).

In order to confirm the Kv1.3 channels identity and contribution in CB whole-cell K^+ currents, we used two specific blockers of Kv1.3 channels, MgTx and ShK-Dap^{22}, with MgTx being the most common agent used to selectively inhibit the Kv1.3 channels (Toldi *et al*., 2013; Zhao *et al*. 2013; 2015). However, nowadays it is known that MgTx is also a blocker of closely related Kv1.1 and Kv1.2 channels (Grissmer *et al.*, 1994; Koch *et al.,* 1997) and therefore, we have also tested. ShK-Dap^{22}, that presents a lower affinity for Kv1.1 (Kalman *et al.*, 1998, Zhao *et al.,* 2015). Interestingly, both MgTx and the ShK-Dap^{22} inhibit the current acting on the conductance of the channel, not in the voltage sensitivity (hence in the voltage sensor). Both compounds are derived from scorpion poisons, which, are supposed to act as 'pore blockers', in contrast to spider toxins, that block Kv1.3 channels by shifting the channel sensitivity to voltage (Zhao *et al*., 2015)

The data from patch clamp demonstrated the presence of MgTx and ShK-Dap^{22} sensitive currents in CB with a similar profile of the sensitive current obtained with insulin administration (a slow inactivation kinetics). The data from administration of MgTx and ShK-Dap^{22} did not differ suggesting that independently of the specificity probably only Kv1.3 channels are being blocked. The presence of sensitive MgTx and ShK-Dap^{22} are consonant with the contribution of the rat CB currents of insulin belonging to Kv1.3 channels. In order to make sure that we were looking for the same current, to investigate if the target of MgTx and insulin is the same, we performed time course experiences. The application of MgTx and then co-application of insulin demonstrated that in fact we are acting on the same target.

By the time courses we observed that in both tested concentrations, MgTx was able to reduce the voltage activated K^+ currents with a distinct final effect after insulin co-application. In presence of a higher dose of MgTx insulin effect on voltage activated K^+ is not so marked, which contrasts with the effect if insulin in presence of a lower dose of MgTx. Altogether these results demonstrated that MgTx and insulin have an inverted correlation telling that Kv1.3 channel mediate the insulin sensitive currents in CB cells.

Data from immunocytochemistry and Western blot confirmed the presence of the Kv1.3 channel in the CB type I cells. As far as we are aware, the presence of Kv1.3 channels in type I cells had never been studied. In previous studies performed in the CB of rabbits and mice, several members of the Kv subfamily have been described. Sanchez et

al. (2002) have demonstrated the presence of Kv1.4, Kv3.4, and Kv4.1 and Kv4.3 mRNAs transcripts in the rabbit CB however, immunocytochemistry studies demonstrated that only Kv3.4 and Kv4.3 were present in type I cells, while Kv1.4 was found in nerve fibres (Sanchez *et al*., 2002). Additionally, in mice CB, mRNA transcripts for Kv2.2, Kv3.1, Kv3.2 and Kv3.3 have been described, as well as the presence of Kv3.1, Kv3.2 and Kv3.3 channels in type I cells (Perez-Garcia *et al*., 2004). Therefore, the present manuscript adds to the knowledge of subtypes of Kv channels that are present and contribute to K^+ currents in the rat type I cells.

Knowing that insulin causes a reduction in Kv1.3 currents in the CB type I cells (**Figure 4.2** and **4 .3**) and that InsRs are present in the CB and that its activity increases in the presence of insulin (**Chapter III, Figure 3.6**), we decided to investigate if insulin modulates Kv1.3 channel activity through the phosphorylation of tyrosine motifs. In fact, the modulation of Kv1.3 channels activity at multiple discrete sites, similar to the insulin receptor substrate, has been described in the OB neurons and in cell lines, where the activation of tyrosine kinase receptors, as the InsR, the epidermal growth factor receptor and neurotrophin receptor B, cause a suppression of Kv1.3 channels (Bowlby *et al*., 1997; Colley *et al.,* 2004). Herein, we found that Kv1.3 channels are phosphorylated on Tyrosine 135 residue in presence of 10 nM of insulin (**Figure 4.5**), meaning that the activation of InsR suppress Kv1.3-mediated currents in the type I cells by phosphorylation of tyrosine residues at these channels. This kind of modulation of Kv1.3 channels activity is not new, as similar effects were observed in human embryonic kidney (HEK) 293 cells, where increased tyrosine phosphorylation of Kv1.3 was accompanied by a time dependent decreased in Kv1.3 current (Holmes *et al*., 1996), as well as in the neuronal primary cultures from OB of Sprague Dawley rats (Fadool *et al*., 2000). The results described in the present manuscript do not allow to deep into the mechanism by which the phosphorylation of tyrosine residues results in the suppression of Kv1.3 currents, however two mechanisms can be postulated as suggested by several authors: 1) Kv1.3 channels may undergo conformational changes that decrease channel activity and/or 2) tyrosine phosphorylation of Kv1.3 channels may lead to a decrease in the number of functional channels at the cell surface, or both (Bowlby *et al*. 1997; Colley *et al.,* 2007; Holmes *et al*., 1996). For example, growth factors receptors internalize rapidly after activation (Schessinger & Ullrich 1992), which might result in nonspecific internalization of other proteins, such as ion channels

as suggested by Holmes et al. (1996). So, it is not surprising that functional suppression of K^+ currents could be associated with phosphorylation of tyrosine residues within the channel protein, leading to conformational changes that are able to decrease the channel activity, and/or to the decrease the number of functional channels at the cell surface.

In the CB it is very well described that K^+ channels inhibition, for example by hypoxia, leads to an increase in membrane potential that opens Ca^{2+} channels originating the influx of Ca^{2+} into the type I cells that induces the release of neurotransmitters (for a review see Gonzalez *et al*., 1994). Recently, we have demonstrated that insulin induces an increase in $[Ca^{2+}]i$ in type I cells that culminates in the release of DA and ATP from the CB (**Chapter III**). In the present we report that MgTx, a Kv1.3 channel blocker, mimics the effect of insulin on the release of DA+DOPAC from the CB. Additionally, we have found that in the presence of both drugs (insulin + MgTx) the release of DA+DOPAC is attenuated suggesting that they share the same effector, the Kv1.3 channel. Our results are in agreement with the finding that Kv1.3 channels modulate the influx of Ca^{2+} into the cells, namely in T lymphocytes (Kollár *et al.,* 2015) and in smooth muscle cells (Cheong *et al*., 2011) and with the fact that these channels are implicated on neurotransmitter release, such as glycine from rat spinal neurons (Shoudai *et al*., 2007) and GABA from dentate granule cells and interneurons (He *et al.,* 2012). Therefore, we can postulate that Kv1.3 channels activation, will allow the influx of Ca^{2+} in to type I cells that lead to the release of neurotransmitters from the CB.

Considering that IR is associated with an overactivation of the CB and that CB denervation prevents the development of IR (**Chapter III**), one can assume that modulation of the excitability of the CB would affect for insulin sensitivity. Hence the findings insulin sensitive K^+ current (mediated by Kv1.3), in the CB gains high relevance as it gives clues to how the CB can be 'switched off'. In this context, Kv1.3 channel may emerge as an attractive target for IR syndromes. In fact, Kv1.3 has been already referred as pivotal in peripheral insulin sensitivity (Xu *et al.,* 2004).

In conclusion, we have demonstrated for the first time that the CB have insulin-sensitive voltage-activate K^+ currents, and more importantly, that insulin failed to reduce K^+ currents when in the presence of MgTx, a Kv1.3 channels blocker, and vice versa. Additionally, we described the presence of Kv1.3 channels in CB type I cells and its phosphorylation in the presence of insulin. Moreover, MgTx mimic the effect of insulin

on the release of DA from the rat CB, meaning that the effect of insulin on neurotransmitters release is mediated via Kv1.3 channels. Kv1.3 channels mediate insulin action at the rat CB and we can suggest that modulation of Kv1.3 activity in this organ may serve as a novel therapeutic target for IR

CHAPTER V

Vera-Cruz P, Guerreiro F, **Ribeiro MJ**, Guarino MP and Conde SV (2015) Hyperbaric oxygen therapy improves glucose homeostasis in type 2 diabetes patients: a likely involvement of the carotid bodies. Advance in Experimental Medicine and Biology, 860:221-225.

Hyperbaric oxygen therapy improves glucose homeostasis in type 2 diabetes patients: a likely involvement of the carotid bodies

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*

ABSTRACT

The CBs are peripheral chemoreceptors that respond to hypoxia increasing VE and activating the sympathetic nervous system. Besides its role in ventilation we recently described that CB regulate peripheral insulin sensitivity. Knowing that the CB is functionally blocked by hyperoxia and that HBOT improves fasting blood glucose in diabetic patients, we have investigated the effect of HBOT on glucose tolerance in T2D patients. Volunteers with indication for HBOT were recruited at the Subaquatic and Hyperbaric Medicine Center of Portuguese Navy and divided into two groups: T2D patients and controls. Groups were submitted to 20 sessions of HBOT. Oral glucose tolerance test were done before the first and after the last HBOT session. Sixteen diabetic patients and 16 control individuals were included. Fasting glycemia was143.5±12.62 mg/dl in diabetic patients and 92.06±2.99 mg/dl in controls. In diabetic patients glycemia post-OGTT was 280.25±22.29 mg/dl before the first HBOT session. After 20 sessions, fasting and 2 h post-OGTT glycemia decreased significantly. In control group HBOT did not modify fasting glycemia and post-OGTT glycemia. Our results showed that HBOT ameliorates glucose tolerance in diabetic patients and suggest that HBOT could be used as a therapeutic intervention for T2D.

Keywords: glucose tolerance, hyperbaric oxygen therapy, type 2 diabetes

5.1. INTRODUCTION AND AIM

Hyperbaric oxygen therapy is a well-established treatment for decompression sickness and other conditions like carbon monoxide intoxication, infections, arterial gas embolism, radio-induced lesions and delayed wound healing as a result of diabetes or arteriosclerosis. In a hyperbaric chamber, patients breathe pure oxygen (100%) and the air pressure is raised up to two and a half times higher than at the sea level air pressure allowing lungs to gather more oxygen than would be possible breathing pure oxygen at sea level air pressure (Al-Wali *et al.,* 2006).

The maximum duration and working pressure in routine sessions of HBOT are 90 min at 2.5 ATA, in order to prevent the occurrence of toxic neurological and respiratory effects of hyperoxia (Al-Wali *et al.*, 2006). The benefits of HBOT are well documented in diabetic patients with foot ulcers (Desola *et al*., 1998). Additionally, there is some evidence that HBOT improves fasting glycemia by 20% in T2D (Desola *et al.,* 1998; Ekanayake & Doolette, 2001; Karadurmus *et al.,* 2010; Wilkinson *et al*., 2012) and that lowers C-reactive protein and IR in diabetic patients (Chateau-Degat *et al*., 2012; Ekanayake & Doolette, 2001;) without changing insulin levels (Desola *et al.,* 1998). Moreover, it has been seen that HBOT significantly decreases systolic blood pressure, both T2D and in hypertensive patients (Peleg *et al*., 2013). In contrast, severe side effects of HBOT, such as oxidative stress and oxygen toxicity have also been described, leading inclusively to cytotoxic effects in the β-cell and hyperglycemia (Matsunami *et al.,* 2008).

One of the reasons that can account for these contradictory effects is the duration of HBOT protocol: while some authors have performed an acute HBOT protocol (Desola *et al*., 1998; Peleg *et al*., 2013), others submit patients to 90 min HBOT sessions during 2 weeks (Wilkinson *et al*., 2012) or 2 h during 5 weeks (Chateau-Degat *et al.*, 2012). Also, the majority of these studies included a very small number of T2D patients and some of them reported that the decrease in blood glucose is not related with HBOT. In third chapter of this work we demonstrated that CB is involved in the development of IR and HT associated with the consumption of hypercaloric diets. Knowing that CB activity is functionally blocked by hyperoxia the aim of this work is to investigate the effect of HBOT on glucose tolerance in T2D patients.

5.2. MATERIAL AND METHODS

5.2.1. Volunteers

Volunteers with indication for HBOT were recruited at the Subaquatic and Hyperbaric Medicine Center of the Portuguese Navy. Written informed consent was obtained from all individuals and the study was approved by the Ethical Committee of the Portuguese Navy Hospital. Volunteers were divided into two groups: T2D patients and control. Inclusion criteria for the group with diabetes *mellitus* were those defined by the American Diabetes Association in 2010: HbA1C ≥6.5% or FPG ≥126 mg/dl or 2-h plasma ≥200 mg/dl during an OGTT or in a patient with classic symptoms of hyperglycemia or hyperglycemic crisis, a random plasma glucose ≥200 mg/dl. Exclusion criteria were respiratory disease, renal disease and psychiatric illness previously identified. Indications for HBOT included sudden deafness, radio-induced cystitis and diabetic foot ulcers for the T2D group. No medications were changed during the study.

Anthropometric data, like weight, height and abdominal perimeter were collected from all volunteers. All patients performed a daily HBOT sessions protocol, five times a week for a total of 20 sessions. Each session lasted for 100 min, comprising two periods of 35 min breathing 100% oxygen at 2.5 ATA separate by a 5 min air break interval or 70 min, plus 25 min for compression and decompression. Patients were submitted to an OGTT in two different time points: before initiating the HBOT protocol and after completing 20 HBOT sessions. The OGTT consisted in the administration of a beverage with orange flavour containing 75 g glucose (Top Star 75, Toplabs, Portugal) and the measurement of blood glucose immediately before and 2 h after ingestion.

Data were presented as mean values with their standard errors, unless stated otherwise. Normally distributed variables were analysed using unpaired Student´s t-test while non-normally distributed variables were compared using the Mann-Whitney U-test. Differences were considered significant at $P<0.05$. The significance of the differences between the mean values was calculated by two-way ANOVA with Bonferroni multiple comparison test. GraphPad Prism version 4 (GraphPad Software Inc., San Diego, CA, USA) was used to perform statistical analysis.

5.3. RESULTS

The study was conducted in 32 patients (**Table 5.1**): 16 controls (non-T2D patients – control group) and 16 T2D (study group). Demographic and baseline patient characteristics are presented in **Table 5.1**. Body mass index were not different between the control and study group, however individuals with T2D had a higher abdominal perimeter ($p<0.01$).

Table 5.1: Comparison between demographic and baseline variables in controls and type 2 diabetes (T2D) patients

Parameters	Non-type-2-diabetes	Type 2 diabetes	p-value
n	16	16	
Male	11	15	
Age (years)	58 (52,64)	64 (58,69)	NS
Body Mass Index (kg m^{-2})	25 (23,28)	26 (24,28)	NS
Abdominal perimeter (cm)	91 (83,98)	110 (96,124)	<0.01
Fasting glucose levels (mg/dl)	92.15 ± 3.68	143.5 ± 12.62	<0.001

Data are expressed has mean (95%CI) or as mean ± SEM. NS, nonsignificant (P>0.05); n, number of patients

As depicted in **Table 5.1**, fasting plasma glucose was significantly higher in T2D patients than in controls (controls = 92.15 ± 3.68 mg/dl, diabetes = 143.5 ± 12.62 mg/dl). Hyperbaric oxygen therapy did not change fasting glucose levels in control subjects, but it decreased these levels to 119.1 ± 4.80 mg/dl in T2D patients, however without reaching statistical significance ($p = 0.089$) (**Figure 5.1**). Additionally, in control subjects, plasma glucose levels measured 2 h after an OGTT were not different at the 1st and 20th session of HBOT (**Figure 5.1 A**).

In contrast, glycemia measured 2 h after the ingestion of 75 g of glucose significantly decreased from 280.25 ± 22.29 mg/dl to 185.78 ± 11.70 mg/dl after 20 sessions of HBOT in T2D patients (**Figure 5.1 B**).

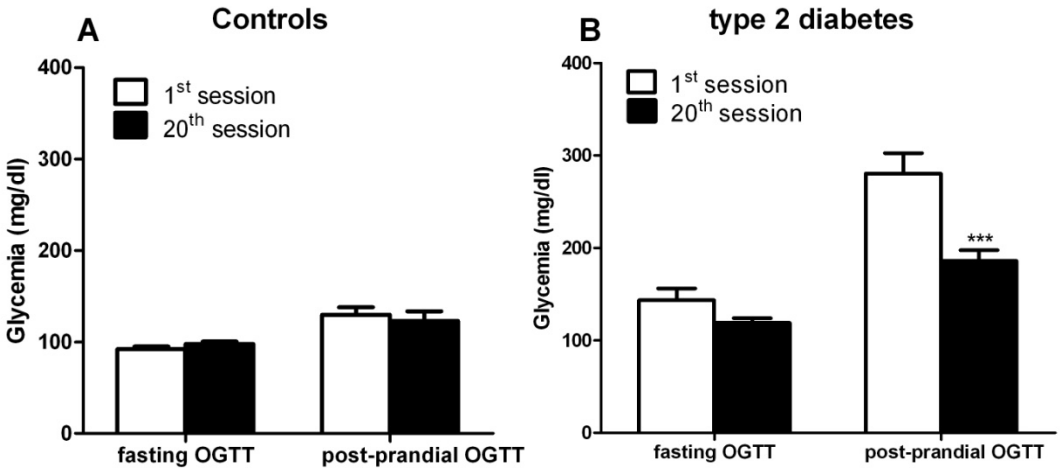

Figure 5.1. Effect of 20 sessions of hyperbaric oxygen therapy (HBOT) in fasting glycemia and glucose tolerance in controls and type 2 diabetes (T2D) patients. Glucose tolerance was assessed through an oral glucose tolerance test (OGTT), which consisted in the oral administration of a glucose drink containing 75 g glucose and measurement of blood glucose before and 2 h after ingestion. All patients performed a hyperbaric oxygen protocol of 100% oxygen at 2.5 absolute atmospheres (ATA) for 60 min five times a week for a total of 20 sessions. Data are means ± SEM. ***p<0.001 compared with values in the 1st hyperbaric oxygen therapy session (two-way ANOVA with Bonferroni multicomparison test

5.4. DISCUSSION

These results indicate that HBOT ameliorates glucose tolerance on T2D patients. The effect of HBOT in glucose tolerance is powerful as it accompanies the previously described hypoglycaemic effect of this intervention in fasting plasma glucose. Additionally we show that HBOT did not alter glucose homeostasis in control subjects.

In the last decade several studies have demonstrated that HBOT improves fasting plasma glucose (Desola *et al*., 1998; Ekanayake & Doolette, 2001; Karadurmus *et al*., 2010; Wilkinson *et al.,* 2012) and insulin sensitivity in T2D patients (Chateau-Degat *et al*., 2012; Ekanayake & Doolette, 2001), however without modifying HbA1c (Chateau-Degat et al., 2012), Herein we showed that 20 sessions of HBOT decreased fasting glucose levels by 21%, being this value in agreement with the literature (Desola *et al*., 1998; Ekanayake & Doolette, 2001; Karadurmus *et al*., 2010; Wilkinson *et al.,* 2012). Additionally we also show, for the first time, that HBOT ameliorates glucose tolerance in T2D patients by 34%, meaning that HBOT was capable of improving the ability of the body to stimulate glucose metabolism, either by increasing glucose uptake or glucose oxidation in the post-prandial state. Although glucose tolerance and insulin

sensitivity are not the same, they are positively correlated and this improvement in glucose tolerance is probably due to an increase in insulin sensitivity, since it was already observed that HBOT does not change insulin secretion (Desola *et al*., 1998). Herein we did not observe any effect of HBOT either in fasting glucose levels or in glucose tolerance in control subjects. However, our results disagree with the previous findings of Peleg et al. (2013) and Wilkinson et al. (2012) in which a decrease in fasting glucose levels and in HbA1c in control subjects was observed.

In the present work the mechanisms underlying the effect of HBOT in glucose tolerance in T2D patients were not tested, although it was previously shown in diabetic rats that exposure to HBOT improves glucose and lipid oxidative metabolism in skeletal muscle (Fujita *et al*., 2012). Another plausible mechanism of action for HBOT on glucose tolerance is the inhibition of the CB chemoreceptors. It is known that hyperoxia blunts the peripheral chemoreceptor activity (Fidone *et al*., 1986) and we have recently described that the CB is a powerful glucose and insulin sensor and that surgical ablation of its sensitive nerve prevents the development of diet-induced metabolic diseases (**Chapter III**). Therefore we postulate that the functional inhibition of CB chemoreceptor activity obtained by exposure to HBOT accounts for the improvement in glucose tolerance observed in T2D patients. Hyperoxia is commonly used to acutely block the CB chemoreceptors and in fact it was recently seen that deactivation of CB chemoreceptors by hyperoxia decreases blood pressure in hypertensive patients (Sinski *et al.,* 2014).

We conclude that HBOT improves both fasting glycemia and glucose tolerance in T2D patients suggesting a novel application for this technology as a therapeutic intervention for controlling post-prandial glucose excursions in T2D.

CHAPTER VI – GENERAL DISCUSSION

GENERAL DISCUSSION

In the present thesis we have demonstrated for the first time that CB is involved in the genesis of IR. We have shown that animal models of diet-induced prediabetes develop an overactivation of the CB. This overactivation of the CB results in an increase in sympathetic nervous activity and in a reduction in insulin sensitivity, as well as in HT, dyslipidemia and weight. All these characteristic features of metabolic diseases were prevented by chronic CSN resection, meaning that the CB is primordial in controlling peripheral insulin sensitivity and that CB dysfunction is involved in the genesis of these disturbances. Moreover, we showed that insulin triggers the CB, being its action mediated by the activation of Kv1.3 channels, suggesting that is hyperinsulinemia that causes CB overactivation. In agreement with our pre-clinic data, we have demonstrated that functional blockade of the CB by HBTO ameliorates fasting glycemia and glucose tolerance in T2D patients.

What causes carotid body overactivation in metabolic diseases?

Glucose sensing by the carotid body

The first hypothesis that was raised to explain the involvement of CB in glucose homeostasis was the ability of the organ to sense glucose, more precisely, the ability of the CB to respond to hypoglycemia. Several *in vivo* and *in vitro* evidences pointed out a role for the CB as a glucose sensor (Koyama et al. 2000; Pardal and Lopez-Barneo, 2002; Zhang et al., 2007) (**Chapter I, Section 5.5**), however reports from several labs come out questioning this CB glucose sensing properties. In contrast with the findings that CB sense low glucose levels, CSN activity in freshly isolated cat and rat CB–CSN preparation was not modified by perfusion with glucose-free or low-glucose solutions (Almaraz *et al*., 1984; Bin-Jaliah *et al.,* 2004, 2005). Also, Conde et al. (2007) demonstrated that low glucose concentrations neither activate the release of neurotransmitters, namely CAs and ATP, from the CB, nor altered basal and hypoxia (5% O_2)-induced CSN action potential frequency in freshly isolated whole CB preparations (Conde *et al.,* 2007). In the same line, Fitzgerald et al. (2009) showed that the release of ATP from the cat CB was not modified in the presence of hypoglycemia but, surprisingly, they observed an increase in the release of ACh in the same conditions

(Fitzgerald *et al*., 2009). Additionally, it was shown that withdrawal of glucose from the perfusion media did not activate K_{ATP} channels, suggesting that this channel was insensitive to hypoglycemia (Kim *et al*., 2011). Altogether these results suggest that low glucose is not a direct stimulus for the CB chemoreceptors and do not support a significant physiological role of the CB as a glucose sensor.

Several differences can account for these discrepant results regarding glucose sensing in the CB, namely species differences, different dissociation protocols or culture conditions that lead to an altered cells phenotype, as suggested by Kumar (2007), or even the differences in the PO_2 levels used by some authors, as postulated by Zhang et al. (2007). However, Conde et al. (2007) have shown in the whole CB that low or absent glucose does not activate either type I cells or the CB–CSN complex at different PO_2 tested in a very wide range (~133, 66, 46, and 33 mmHg) and thus, differences in the PO_2 used in the experiments in intact preparations *vs.* slices or co-cultures is not the factor determining divergent findings, as suggested by Zhang et al. (2007). More recently, Gallego-Martin et al. (2012) demonstrated that in intact CBs cultured during 1 day, but not in freshly isolated organs, 0 mM glucose media potentiates the release of CAs elicited by hypoxia and that type I cells in culture become transiently more dependent on glycolysis suggesting that the scarcity of glucose leads the cells to acquire the ability to increase their neurosecretory response to hypoxia. Another relevant issue in the discussion is the duration of glucose deprivation. While glucose reduction or deprivation did not have an effect when applied for short periods of time (<15 min), either in basal conditions or in response to hypoxia, when applied for longer periods of time (up to 120 min) it caused a spontaneous increase in basal release of CAs observable after 40 min of glucose deprivation. Concomitantly, bursts of CSN activity were observed with a comparable time course to the release of CAs that culminated in a complete loss of the capacity of the CSN to respond to hypoxia (Conde *et al*., 2007). Consistent with these findings Holmes et al. (2014) have recently demonstrated that basal CSN activity was sustained during glucose deprivation approximately for 30 min before irreversible failure following a brief period of increased activity. Also, they showed that pharmacological inhibition of glycogenolysis and depletion of glycogen reduced the time to glycolytic run down, suggesting that glycogen metabolism in chemoreceptor cells allows glycogenolysis and the maintenance of CSN basal activity during hypoglycemia (Holmes *et al*., 2014). Therefore, glycogen metabolism may

account for the differences reported in the capacity of the CB to sense glycemia and could contribute to CB responses in pathological conditions associated with an overstimulation of the organ.

Insulin action at the carotid body

The fact that peripheral insulin administration elicited a higher increase in sympathetic activity than systemic administration (Pereda *et al*. 1962, See **Chapter I, Section 4.2**) together with the evidence that CB overactivation characterizes essential HT, OSA, chronic heart failure as well other sympathetic mediated diseases lead us to the hypothesis that the CB is a peripheral insulin sensor. According to this new paradigm, CB overstimulation by inadequate insulin levels contributes to the genesis of peripheral IR and HT present in metabolic diseases via sympathetic nervous system activation

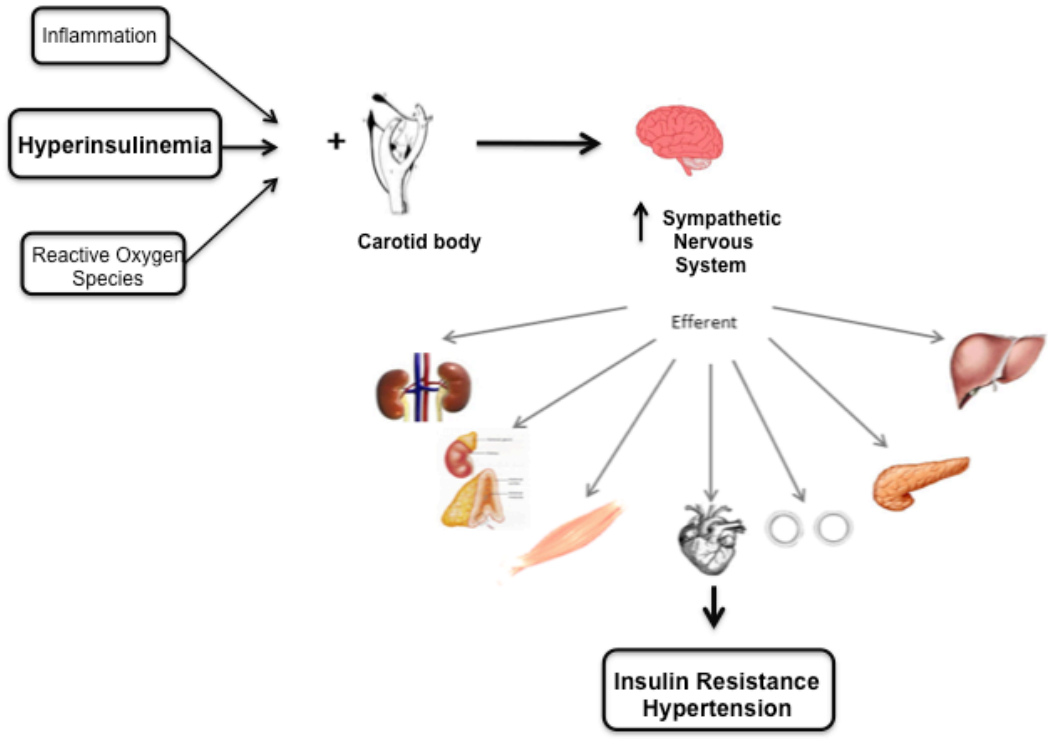

Figure 6.1: Schematic representation of the stimuli that activates the carotid body (CB) to induce an increase in sympathetic activity that promotes insulin resistance (IR) and glucose deregulation and hypertension (HT). Hyperinsulinemia, inflammation and reactive oxygen species (ROS) induces CB overactivation leading to an increase in sympathetic nervous system activity that promotes IR and HT. Adapted from (Conde *et al*., 2016).

We have demonstrated the presence of InsRs in the rat CB by western-blot and its

phosphorylation in response to insulin (**Chapter III**, **Figure 3.6**). The presence of InsRs was also confirmed on finding that isolated whole CBs incubated with insulin accumulate more 2-deoxiglucose than the diaphragm muscle (Gallego Martin *et al*., 2014). Insulin is also capable to induce a rise in $[Ca^{2+}]i$ in type I cells and to elicit the release of ATP and DA from the whole CB in a concentration-dependent manner (**Chapter III, Figure 3.7**). We have also shown that this neurosecretory response is transduced into an increase in ventilation in the whole animal, as insulin increased the spontaneous ventilation in a dose-dependent manner during an euglycemic clamp **(Chapter III, Figure 3.8 C).** The increase in ventilation induced by insulin is mediated by the CB, since it is absent in animals that had their CSN resected (**Chapter III, Figure 3.8 D).** Contrarily to our results, Bin-Jaliah et al. (2004) proposed that the ventilatory and metabolic effects observed *in vivo* were not due to insulin *per se*, since the increase in ventilation produced by insulin was absent during an euglycemic clamp. However, some differences in the methodology used can be in the basis of these discrepancies. In our study we have administrated a *bolus* of insulin intracarotidally to guarantee that the first site of insulin action is the CB, and not systemically as Bin-Jaliah et al. (2004; 2005). Also we performed a dose-response curve in which several concentrations of insulin were tested, making the results more robust in terms of concluding on a role of insulin in CB modulation. In fact, the neurosecretory response and the increase in ventilation elicited by insulin in our experimental setting support the idea that insulin is a very powerful stimulus for CB activation. Nevertheless, these findings do not exclude that the central nervous system is also involved in the sympathetic activation observed in response to circulating insulin and more studies are required to clarify the exact contribution of both the peripheral and the central nervous system in this process. It is undoubtedly however, that the overactivation of the sympathetic nervous system, measured as the increase in plasmatic CAs (norepinephrine + epinephrine) and in CAs (norepinephrine + epinephrine) content of the adrenal medulla (**Chapter III, Figure 3.5)** and the IR (**Chapter III, Figure 3.4 C)** seen in hypercaloric animal models are prevented by surgical resection of the CSN. These findings point toward a new role for the CB in the regulation of peripheral insulin sensitivity and in the pathogenesis of IR.

In the present thesis we have also demonstrated that insulin action at the CB is mediated through Kv1.3 channels (**Chapter IV**), since insulin suppress voltage activated K^+ in

CB type I cells and MgTx and ShK-Dap[22] (blockers of Kv1.3 channels) inhibit the effect of insulin on voltage K^+ activated currents (**Chapter IV, Figure 4.1** and **4.2**). We also have shown that Kv1.3 channels are present in CB type I cells and that the channels are phosphorylated in the presence of insulin, demonstrating that phosphorylation is one of the mechanisms by which insulin can modulate Kv1.3 activity (**Chapter IV, Figure 4.5**) Additionally, we showed that Kv1.3 channels are involved in the neurosecretory response elicited by insulin, since MgTx modulates the release of dopamine from the CB (**Chapter IV, Figure 4.6**). The results obtained in the present thesis are in agreement with several evidences that demonstrated the involvement of Kv1.3 channels in glucose metabolism, regulating energy homeostasis as well as obesity (Xu *et al.,* 2003). In fact, several evidences have shown that Kv1.3 channels are important effectors of insulin signaling in central nervous system (in OB) and in the periphery, for example in the skeletal muscle (Fadool *et al*., 2000; Xu *et al* 2003; 2004). These findings herein described suggest a functional link Kv1.3 channels and InsRs, since insulin phosphorylates Kv1.3 channels, although other mechanisms for the involvement of Kv1.3 channels in insulin signaling have been described. For example, it has been shown that whole body Kv1.3 inhibition enhanced peripheral insulin sensitivity by increasing the amount of GLUT4 at plasma membrane and the uptake of glucose in skeletal muscle and adipose tissue via intracellular Ca^{2+} signaling (Li *et al.,* 2006; Xu *et al*., 2003). Additionally to Kv1.3 channels, AMPK can also play a role in CB insulin signaling as this kinase is involved both in CB O_2 signaling (Evans *et al*. 2005) and insulin signaling in several metabolic diseases. However, this hypothesis would need further experiments.

As a whole it can be said that insulin stimulates the CB via Kv1.3 channels and that hyperinsulinemia might be one of the factors responsible for the CB overactivation seen in metabolic disturbances.

Other mediators that can contribute to carotid body overactivation

Apart from insulin, other humoral and local factors have been described to activate the CB, as leptin, inflammatory cytokines and ROS (**Figure 6.1**). It has been suggested that leptin may contribute to peripheral ventilatory control, as the administration of the hormone can reverse hypoxia and hypercapnia in animal models with no functional

leptin gene (O'Donnel *et al.,* 1999; Tankersley *et al*., 1998). The results suggest that the ventilatory effects of leptin are mediated by the CB chemoreceptors and, in fact, the CBs express leptin-B receptor (Porzionato *et al.,* 2011). Yet, we have recently shown that leptin do not modify CAs secretion in the rat CB suggesting that the acute stimulatory effect of leptin on ventilation is not CB-controlled (Olea *et al*., 2015).

Other local mediators that are known to activate the CB are ROS (Del Rio *et al;* 2010; Peng *et al.* 2009). It has been described that ROS production and regional oxidative stress play a role in the CB chemosensory potentiation and in the progression of HT in rats exposed to chronic intermittent hypoxia (Del Rio *et al.* 2010; Peng *et al*. 2009), however, we are not aware of any effect of oxidative stress mediators in CB-dependent glucose metabolism. Additionally, it is well established that the CB senses inflammatory mediators. The expression of receptors for IL1, IL6 and interleukin -10 (IL-10), as well as for TNFα, has been shown in the human CB (Mkrtchian *et al.,* 2012). In the cat, Fernandez et al. (2008) demonstrated the co-localization of TNFα receptors and TH in CB type I cells and its functionality. When the authors administered TNFα, this pro-inflammatory cytokine was incapable of modifying basal CSN chemosensory discharge *ex vivo*, but reduced the hypoxia-induced enhanced frequency of chemosensory discharge in a dose-dependent manner (Fernandez *et al*., 2008). This inhibitory effect of TNFα observed in the cat is in contrast with the findings of Lam et al. (2008, 2012), in the rat, where the authors showed in dissociated CB type I cells that TNFα enhances the $[Ca^{2+}]i$ response to acute hypoxia, being this increase significantly larger in cells from the CB of rats exposed to chronic hypoxia or to chronic intermittent hypoxia. Yet, TNFα is not the only cytokine that acts on the CB. Rat CB type I cells showed a strong expression of interleukin-1 (IL-1) receptor type I (Wang *et al.,* 2002) and IL-6 receptor α (Wang *et al.,* 2006). In rat CB type I cells IL-1β significantly decreased the outward K^+ current and triggered a transient rise in $[Ca^{2+}]i$ (Shu *et al*., 2007). Moreover, IL-1β stimulated CSN discharges. In the same way application of exogenous IL-6 induced an increase in $[Ca^{2+}]i$ and the release of CAs from rat CB type I cells (Fan *et al*., 2009). Knowing that both subclinical inflammation and oxidative stress are correlated with IR (de Rooij *et al.,* 2009) and both mediators stimulate the CB, it is possible that these molecules also play a role in the modulation of CB-mediated IR.

The carotid bodies control whole body glucose homeostasis

The idea of a physiological role of the CB on the control of glucose metabolism was first suggested by Petropavlovskaya in the 50's. In this pioneer study it was shown that the stimulation of the CB induces a reflex hyperglycemia, an effect that is mediated by the adrenal medulla, since it was not observed in adrenalectomized animals (Petropavlovskaya, 1953). Twenty five years later, Alvarez-Buylla and de Alvarez-Buylla (1988) confirmed those results by demonstrating that the pharmacological stimulation of the CB with cyanide (NaCN) produced an increase in hepatic glucose output in cats, this reflex response being eliminated by bilateral adrenalectomy or by surgical removal of the neurohypophysis (Alvarez-Buylla *et al*., 1997). Also, it was shown that changes in blood glucose concentration in the CB-CSN, superfused *in vivo*, modify brain glucose retention, suggesting that chemosensory activity in the CSN controls brain glucose metabolism (Alvarez-Buylla & Alvarez-Buylla, 1994). In parallel with the increase in hepatic glucose output, one would expect an increase in plasma insulin levels to ensure an adequate glucose utilization by the peripheral tissues and, in fact, stimulation of CBs by corconium, a nicotinomimetic agent, caused a rise in circulating insulin that was reversed by CSN resection (Anichkov & Tomilina, 1962). Later on, Koyama et al. (2000) demonstrated that CB plays an important role in glucose homeostasis *in vivo*, since dogs that have their CB resected presented lower arterial glucagon in basal conditions and reduced glucagon and cortisol levels during insulin-induced hypoglycemia, together with a marked decrease in endogenous hepatic glucose production in response to hypoglycemia, and with an increase in insulin sensitivity, independent of blood glucose level. These last results suggested for the first time that CB resection affects the response to moderate hyperinsulinemia and therefore, that the CB may play a role in glucose homeostasis that is not related with the hypoglycemic counterregulatory response. The results obtained by Koyama et al. (2000) were supported by clinical studies where it was demonstrated that, the rate of glucose infusion necessary to maintain glucose levels in a hyperinsulinemic-hypoglycemic clamp was significantly higher during hyperoxia than in normoxia (Wehrwein *et al*., 2010). In the same study, the authors also observed that hyperoxia, which blunts CB activity, decreased the release of counter-regulatory hormones such as adrenaline, cortisol, glucagon and growth hormone, which seems to indicate that the CB play an

important role in neuroendocrine responses during hypoglycemia (Wehrwein *et al.,* 2010). Also, blood pressure responses to hyperinsulinemia-induced hypoglycemia are reduced in hyperoxic conditions in healthy humans, suggesting that the sympathetic control of blood pressure is attenuated (Wehrwein *et al*. 2012). Recently, the same authors provided corroborative results that show that the effect of hyperoxia on the hypoglycemia counterregulatory response is mediated by the CBs (Wehrwein *et al*., 2015). However, in patients who had had bilateral CB resection due to type I cell tumours, the counterregulatory response to insulin-induced hypoglycemia was not modified, suggesting that physiological adaptations may occur over time and/or that the response to hypoglycemic conditions in humans do not rely specifically on CB glucose sensing (Wehrwein *et al*., 2015). In all these studies, the absence of adequate controls in hyperinsulinemic-euglycemic conditions does not allow assigning the effects to the hyperinsulinemia per se or to hypoglycemia. In another clinical study designed to determine whether hypo- and hyperglycaemia modulate the ventilatory responses to hypoxia, it was shown that hypoglycemia, as well as hyperglycemia, produced an increase in ventilation and in the hypoxic ventilatory response, being the latter accompanied by an increase in circulating counter-regulatory hormones (Ward *et al.,* 2007). Interestingly, both hypo- and hyperglycemia were obtained under hyperinsulinemic conditions, and therefore it is possible that the effect in ventilation observed was due to hyperinsulinemia rather than to altered glucose concentrations. In the present thesis we have demonstrated that animals submitted to hypercaloric diets exhibit CB overactivation: they present an increase in spontaneous ventilation, an increase in the respiratory responses to ischemic hypoxia, an increase in hypoxia-evoked release of dopamine from the CB and an increase in the CB expression of TH (**Chapter III, Figure 3.3 I**). This chronic overactivation of the CBs is tied to enhanced sympatho-excitation, acknowledged by increased circulating and adrenal medulla CAs that culminates in the development of IR (**Chapter III, Figure 3.4C** and **3.5**). Moreover, we have shown that bilateral CSN resection prevents the development of these features (**Chapter III, Figure 3.4C** and **3.5**) and these results strengthen the link between CB dysfunction and the development of IR (**Chapter III**). The hypothesis of involvement of the CB in the genesis of metabolic disturbances was also supported by the findings of Shin *et al.* (2014). They observed that mice exposed to 4/6 weeks to chronic intermittent hypoxia exhibited increased fasting blood glucose, increased hepatic glucose output and IR. The authors have shown that CSN denervation prevented

the chronic intermittent hypoxia-induced hyperglycemia and the increase in baseline glucose hepatic output, an effect that was associated with the abolishment of sympathetic overactivation induced by the CB (Shin *et al*., 2014). The latter results in chronic intermittent hypoxia animals (Shin *et al*., 2014), as well as our data in hypercaloric animal models (**Chapter III**), are in accordance with the findings by Limberg et al. (2014) where hyperoxic silencing of carotid chemoreceptors reduced MSNA in hyperinsulinemic conditions, suggesting that the CB mediates insulin-dependent sympatho-excitation in humans. Confirming this role, as well as the involvement of the CB in metabolic diseases pathogenesis, we have shown that the suppression of CB activity with HBOT (100% O_2 at 2.5 ATA, 70 min, 20 sessions) ameliorates fasting glycemia and post-prandial glucose tolerance T2D patients (**Chapter V, Figure 5.1**).

In conclusion, we propose that insulin-triggered CB activation is a key step in the development the excessive sympatho-excitation that characterizes metabolic diseases, creating a vicious cycle that originates IR and HT. Therefore, the modulation of CB activity emerges as a possible therapeutic strategy for the treatment of metabolic diseases. Altough, since adaptation can occur with the total abolishement of CB activity, a therapeutic alternative with an intermittent modulation might be more appropriate.

CHAPTER VII – CONCLUSIONS & FINAL CONSIDERATIONS

CONCLUSIONS:

In conclusion, the results presented herein add to knowledge of the pathophysiological mechanisms involved in metabolic dysfunctions, by showing that the CB is involved in the development of IR and that the functional ablation of CB activity improves glucose intolerance.

We concluded that:

1. Carotid body is involved in the genesis of IR and HT in prediabetic animal models, via sympathetic nervous system activation since:

1.1. Carotid body activity, is increased in the pathological animals' models used, the HF and HSu rats, as the CB-mediated basal ventilation and ventilation in response to ischemic-hypoxia were increased, as well as the CB chemoreceptor cell function-assessed both as hypoxia-evoked release of DA and TH expression;

1.2. Chronic sinus nerve bilateral resection totally prevented diet-induced IR and HT, as well as increased fasting glycemia, fasting insulinemia and FFAs in the hypercaloric animals used.

1.3. Chornuc sinus nerve bilateral resection in our prediabetes animal models prevents the heightened sympathetic activity that is characteristic of metabolic diseases

2. Insulin triggers CB activation suggesting that is hyperinsulinemia that promotes CB overactivation present in prediabetes animal models, because:

2.1. Insulin receptors are present at the CB and are phosphorylated in response to insulin.

2.2. Insulin was capable to initiate a neurosecretory response, since it promoted an increase in $[Ca^{2+}]i$ and the release of ATP and DA, two CB neurotransmitters, from the whole CB.

2.3. Insulin, administrated intra-carotidally, increased in a dose-dependent manner the basal ventilation, an effect that is totally mediated by the CB, since CSN cut completely abolished it;

3. Insulin action at the rat CB is mediated through the activation of Kv1.3 channels, since:

3.1. Insulin suppress voltage activated K^+ in CB type I cells.

3.2. The effect of insulin on voltage K^+ activated currents was mimicked by the administration of specific blockers of Kv1.3 channels, the MgTx and ShK-Dap^{22}.

3.3. Margatoxin and ShK-Dap^{22} inhibit the effect of insulin on voltage K^+ activated currents meaning that the effect of insulin at the CB is mediated through Kv1.3 channels.

3.4. Kv1.3 channels are present in CB type I cells and are phosphorylated in the presence of insulin, demonstrating that phosphorylation is one of the mechanisms by which insulin can modulate Kv1.3 activity.

3.5. Margatoxin was able to mimic the effect of insulin on the release of DA from CB, demonstrating that Kv1.3 channels are involved in the cellular mechanism by which insulin promotes the release of DA from CB type I cells.

4. The functional inhibition of CB activity by HBOT improves glucose homeostasis in T2D patients, suggesting a novel application for this technology as a therapeutic intervention for controlling post-prandial glucose excursions in T2D because:

4.1. Twenty sessions of HBOT improved fasting glycemia by 21% in T2D patients without affecting non-diabetic volunteers.

4.2. Twenty sessions of HBOT ameliorated significantly glucose tolerance by 34% in T2D patients without affecting non-diabetic volunteers.

FINAL CONSIDERATIONS:

The present thesis demonstrated that chronic CB overstimulation is implicated in the etiology of diet-induced IR. We showed that surgical resection of the CSN prevents the development of dysmetabolic changes induced by hypercaloric treatments in rats, an observation that contributed to strengthen that CB blockade/modulation represents a novel and unexploited therapeutic approach. Consistent with this, HBOT functional block of CB activity ameliorated fasting glycemia and glucose tolerance in T2D patients.

Besides the surgical resection of the CB and HBOT treatment, its overactivation can also be prevented pharmacologically with an old, well-studied and very safe drug: caffeine. Sustained caffeine administration prevents HT and normalizes blood pressure levels and restores glucose tolerance and insulin sensitivity in prediabetes animal models (Coelho *et al.,* 2016; Conde *et al.*, 2012b; Panchal *et al*., 2012).The effect of chronic caffeine administration was accompanied by prevention of weight gain and decreased visceral fat in obese animals and in fact it was described that the effect of caffeine was due to an impairment of adipose tissue function (Coelho *et al.,* 2016. However, caffeine also exerted its positive metabolic effects in lean models of insulin resistance and hypertension independently of weight loss (Conde *et al*., 2012b). A putative mechanism related with blockade of adenosine receptors in the CBs and, therefore, with the inhibition of CB-mediated sympathetic overactivation by chronic caffeine administration has been proposed as a paradigm shift to explain the reduction of IR, blood pressure and T2D risk induced by sustained consumption of this xanthine (Conde *et al*., 2012b,c; **Chapter III**). The translation of these promising results into human medicine, namely through clinical trials is still lacking—but the epidemiological data available strongly indicate that caffeine should integrate a normal healthy diet, and actually contribute to decrease the incidence of T2D and obesity in high-risk populations (Bhupathiraju *et al*., 2014; Van Dam & Hu, 2005;).

Another way of modulating CB activity would be to directly target its effector, the sympathetic nervous system. The sympathetic nervous system may also represent a putative target to treat metabolic diseases related with IR, particularly if modulated regionally in classical insulin-target tissues like the skeletal muscle. This pinpoint modulation may be achieved through the use on Bioelectronic Medicines, electronic

devices connected to individual peripheral nerve fibers, aiming to correct pathological electrical patterns and restore health (Famm *et al.,* 2013). This new area of therapeutics is emerging right now, with the promise and ambitious goal of modulating specific peripheral nerves. Due to the important role of the CBs seem to play in both the metabolic and hemodynamic control they represent a natural candidate for Bioelectronic Medicines.

ANNEXES

Annex I: Glycolysis

Glycolysis is the pathway of breakdown glucose into pyruvate/lactate (last one during hypoxic conditions) following glucose uptake by cells and glucose phosporylation. This oxygen independent metabolism of glucose provides the substrates for energy production via formation of ATP and substrates for the storage pathways of glycogenesis and lipogenesis (Cairns *et al*., 2011; Guo *et al.*, 2012). The whole pathway of glycolysis contain 10 steps of chemical reactions each catalyzed by specific enzymes (**Figure A1**). Depending on types of cells, glycolysis is regulated at several/different rate limiting steps such as, glucose uptake, glucose phosporylation and/or conversion of fructose-6-phosphate into fructose-1-6-biphosphate. Also, Glut4, glucokinase (GK), and 6 phosphofructo-1-kinase are essential importance in the regulation of rates of glycolysis (Guo *et al.*, 2012; Li *et al*., 2015) (**Figure A1**).

The overall reaction of glycolysis is:

$C_6H_{12}O_6 + 2\ NAD^+ + 2\ ADP + 2\ P$ ——> 2 pyruvic acid, $(CH_3(C{=}O)COOH + 2\ ATP + 2\ NADH + 2\ H^+$

Annex II: Gluconeogenesis

Gluconeogenesis occurs to supply glucose to tissues, such as brain and red blood cells that depend on glucose as their main or sole energy source especially in long periods of fasting. This process is found mainly in the liver, although occurs also in the kidney and involves the synthesis of glucose from non-carbohydrate carbon substrate such as lactate, glycerol and glucogenic amino acids (i.e alanine) formed by metabolically in peripheral tissues. For example, lactate produced in skeletal muscle is released to the blood where it is taken up by the liver, converted to pyruvate and, through the gluconeogenic pathway, converted to glucose. After, glucose returns to blood in order to be use by muscle as an energy source and to replenish glycogen stores (cori cycle).The process of gluconeogenesis uses some of the reactions of glycolysis (in reverse direction) and some reactions unique to this pathway to re-synthesize glucose (Gerich, 2000) (**Figure A1**).

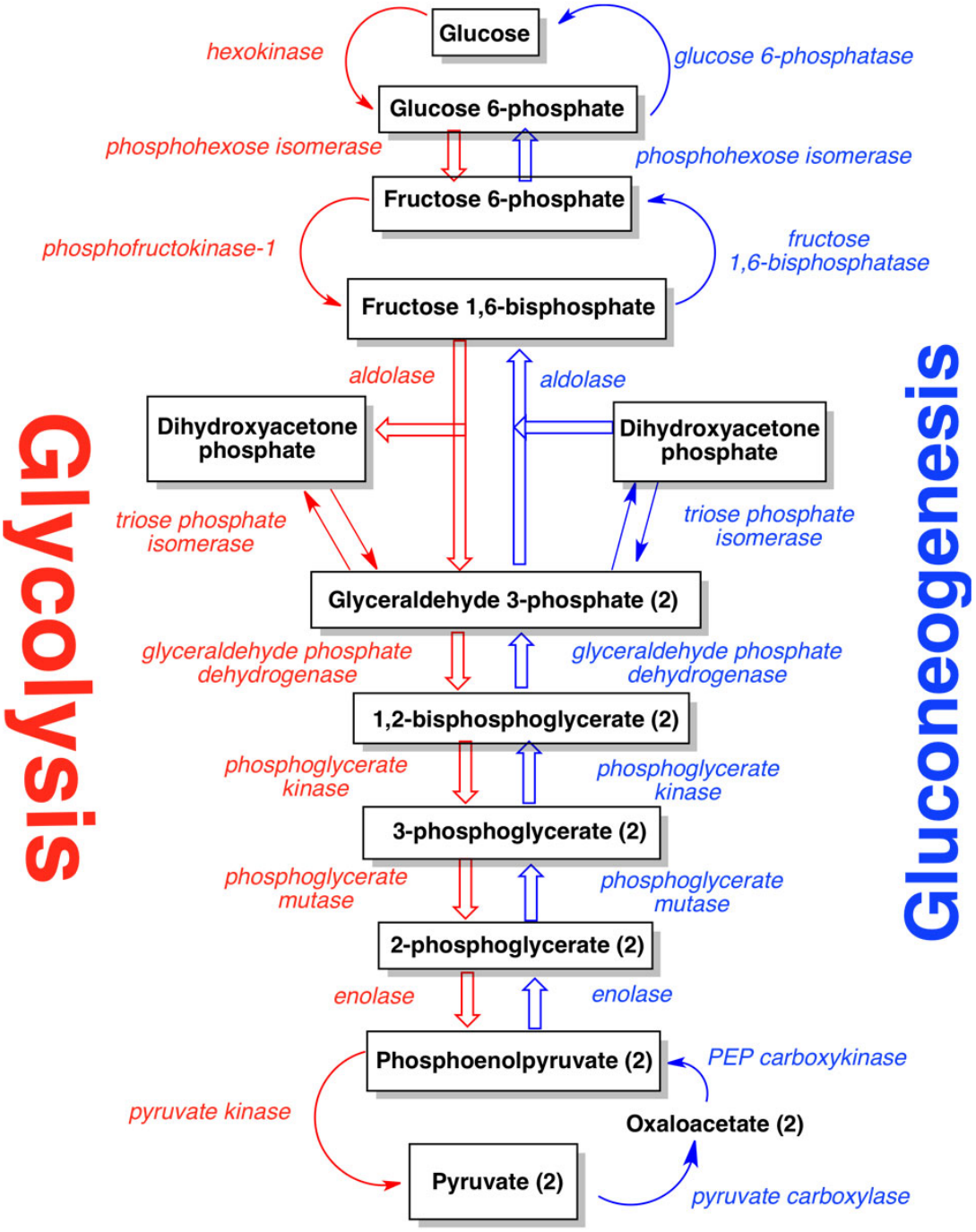

Figure A1. The glycolysis and gluconeogenesis pathways and enzymes involved. Adapted from (http://www.biochemden.com/gluconeogenesis/)

Annex III: Krebs cycle

Krebs cycle also known as the "Citric Acid Cycle" or the "Tricarboxylic Acid Cycle" is a series of chemical reactions that occur in mitochondria used to generated energy through the oxidation of acetil-CoA (**Figure A2**). The citric acid cycle is the final common pathway for the oxidation of carbohydrate, protein and lipids playing an important role in gluconeogenesis, transamination, deamination and lipogenesis. Acetil-CoA is obtained from amino acids like leucyne, tyrosine, isoleucine, lysine, phenylalanine and tryptophan, triacylglycerol, carbohydrates and ketone bodies. In aerobic organisms the TCA is amphibolic pathway, meaning that participates both in the catabolic and anabolic processes. While the Krebs cycle does produce CO_2, this cycle does not produce significant chemical energy directly in the form of ATP (Akram, 2014).

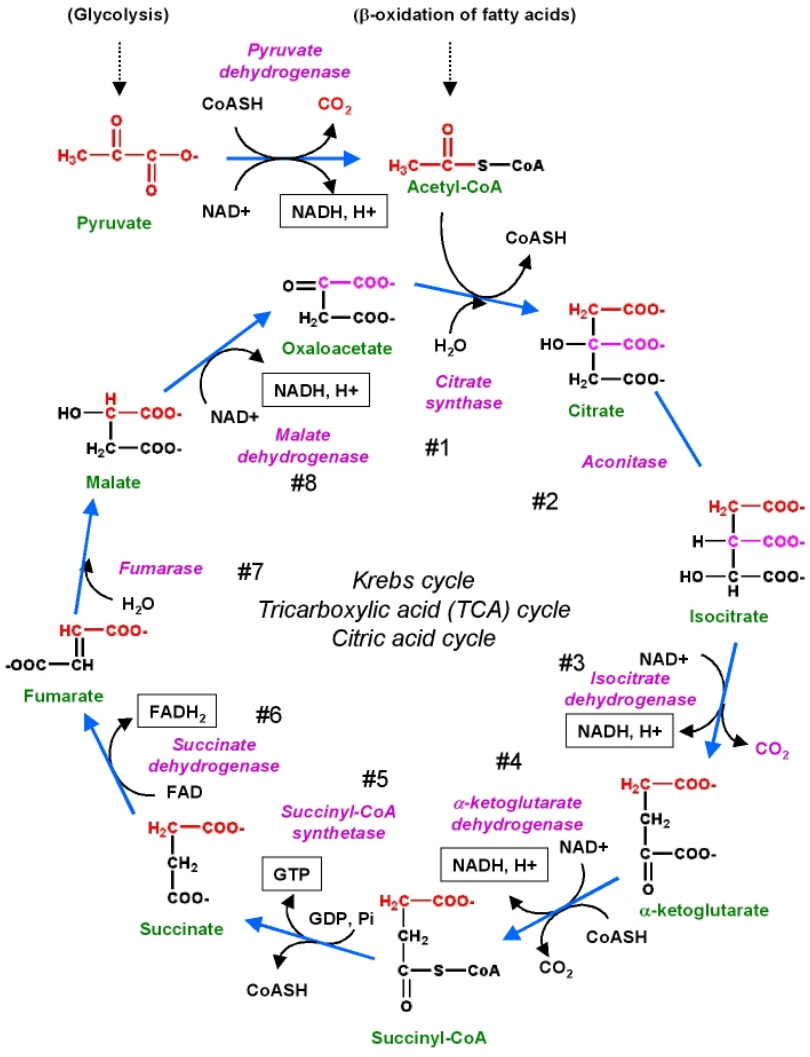

Figure A2. The Tricarboxylic Acid Cycle and chemical reactions with the enzymes. Adapted from http://www.mikeblaber.org/oldwine/BCH4053/Lecture36/krebs_02.jpg

Annex IV: Glycogenesis

Glycogenesis is the process by which occurs glycogen synthesis from glucose, depending on the demand of ATP and glucose (Nordlie *et al*., 1999). In presence of high levels of ATP and glucose, insulin promotes glucose convertion into glycogen to the stored in the liver and muscle cells. During glycogen synthesis, one molecule of ATP is required per each molecule of glucose that is incorporated into polymeric branched structure of glycogen. Glycogenesis is regulated by a balance of the enzymatic activities of glycogen synthase (GS) and glycogen phosphorylase (GP) (Bollen *et a*l., 1998).

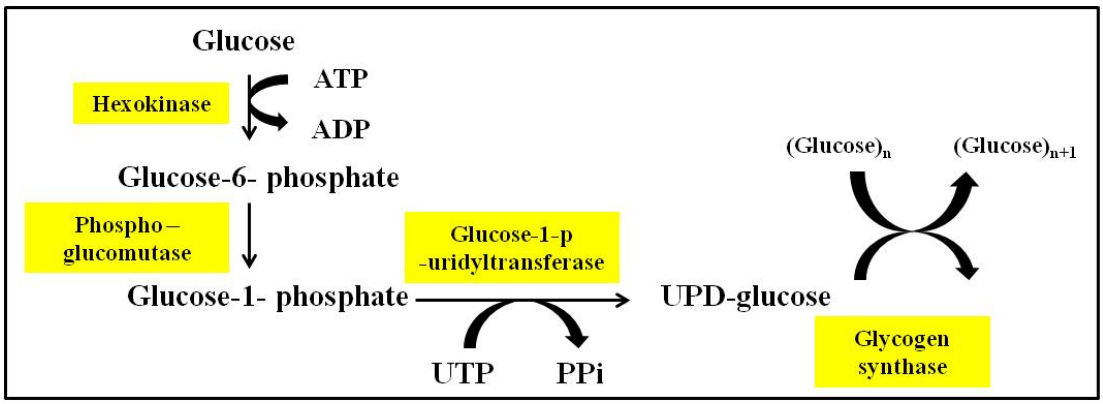

Figure A3. Glycogenesis reaction.

Annex V: Glycogenolysis

Glycogenolysis is the process of breaking down stored glycogen present in the liver and converter it into glucose by a series of hydrolysis reactions. The glycogen breakdown is accomplished by the coordinated action of two enzymes, glycogen phosphorylase, which releases glucose 1-phosphate by untangling the α-1,4-glycosidic linkages, and glycogen debranching enzyme that unfastens the branch points releasing free glucose Glucose 1-phosphate derived from glycogen in the cytosol may be isomerized into glucose 6-phosphate which is dephosphorylated to free glucose by glucose 6-phosphatase in order for glucose to leave the cell via glucose transporters (Adeya-Andany *et al*., 2016) (**Figure A4**).

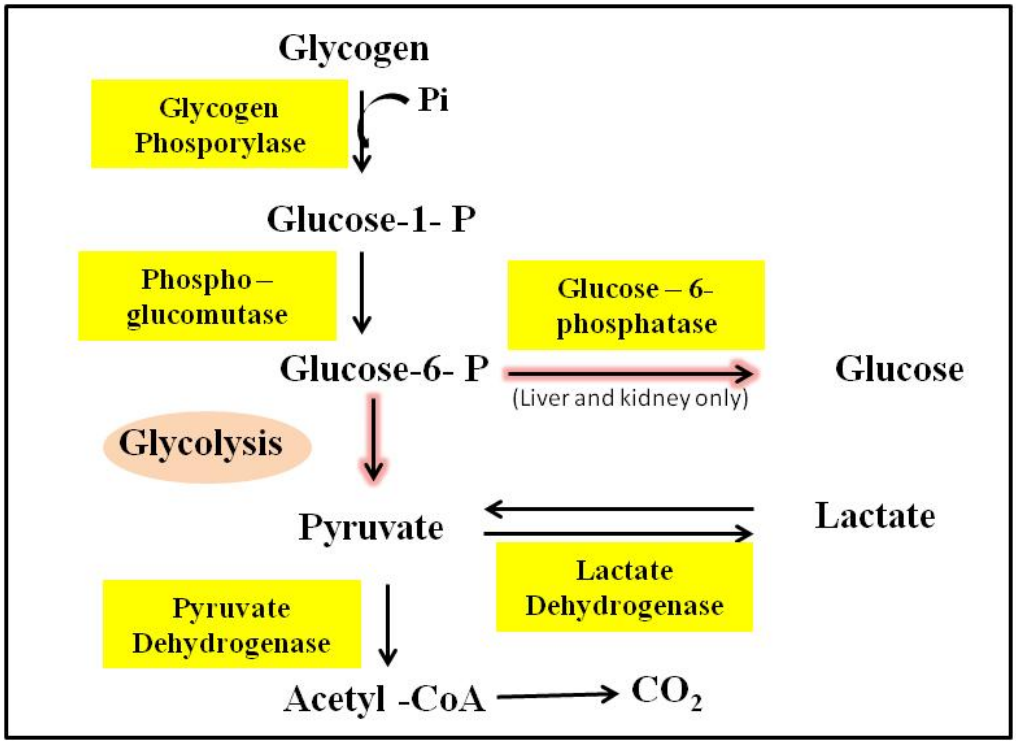

Figure A4. Reactions involved in glycogen breakdown.

Annex VI: Lipogenesis

Lipogenesis (fat synthesis) is the process that synthesizes fatty acids from excess carbohydrates, (such as glucose) and subsequent triglycerides synthesis that takes place in both liver and adipose tissue. Glucose is converted to pyruvate (by glycolysis) and in the mitochondria pyruvate is converted into citrate (by TCA cycle) **(Figure A5**). Citrate is exported to generate acetil-CoA, which is subsequent converted to malonyl-CoA by acetyl carboxylase and then to palmitic aid by fatty acid synthase followed by desaturation and the formation of triglycerides. The key rate limiting enzyme in the fatty acids synthesis is the fatty acid synthase. The triglycerides are packed in very low density proteins (VLDL) that enter in the circulation, and the triglycerides can undergo hydrolysis within capillaries of extrahepatic tissues, of which adipose and muscle are primary tissues. The FFAs may be reesterified and stored in triglycerides or can be oxidized for energy (Ameer *et al*., 2014; Strable & Ntambi, 2010).

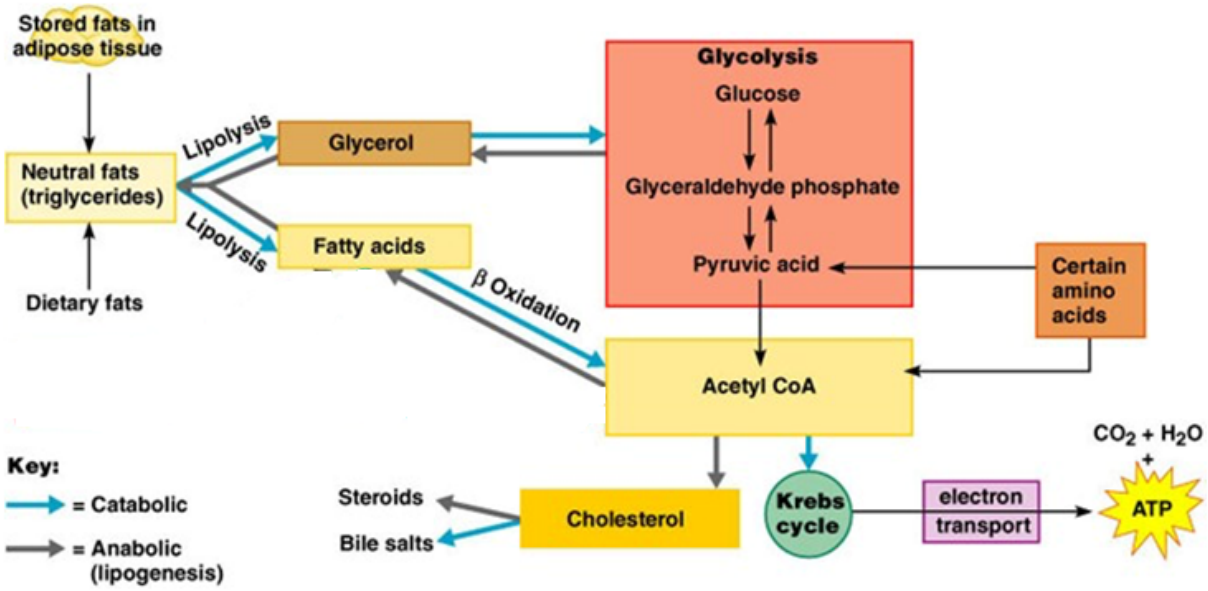

Figure A5. Free fatty acids (FFAs) metabolism. Adapted from (Benjamin Cummnigs 2001)

Annex VII: Lipolysis

Lipolysis is the catabolic process leading to the breakdown of triglycerides, when mobilization of endogenous energy stores is required (i.e exercise and fasting). Triglycerides are stored in fat cells (adipocytes) and when breakdown it is released fatty acids and glycerol (one triglyceride molecule into three FFAs and one glycerol) to the circulation to be used by peripheral tissues where they can serve as substrate for β-oxidation and ATP. In mammals, this process occurs through the sequential action of three lipases (**Figure A6**). Adipose triglyceride lipase converts triglycerides to diacyglycerol and is the rate limiting enzyme in the lipolytic pathway. Diaglycerol is hydrolyzed to monocylglyecerol by hormone sensitive lipase and monoglyceridelipase cleaves monocylglyecerol into glycerol and FFAs (**Figure A6**) (Duncan *et al*., 2007; Nielsen *et al*., 2014).

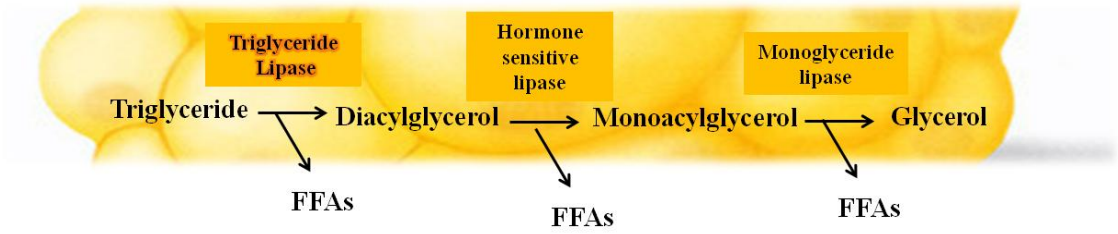

Figure A6. Hydrolysis of triglycerides into free fatty acids (FFAs) and glycerol. In the yellow squares the enzymes involved, being the triglycerides lipase (red color) the rate limiting enzyme of lypolysis.

Table A1. Resume of the metabolic pathways of carbohydrate metabolism and related tissues.

Metabolic Pathway	Description	Tissues
Glycogenesis	**Conversion of glucose to glycogen for storage**	**Liver, and skeletal and cardiac muscle, although most tissues store some glycogen**
Glycogenolysis	**Breakdown of glycogen into glucose for energy production or to be released into the blood stream (liver only for the last one**	**Liver, skeletal and cardiac muscle**
Glycolysis	**Oxidation of glucose**	**Most tissue types**
Gluconeogenesis	**Conversion of non-carbohydrate compounds to glucose**	**Liver**
Tricarboxylic acid cycle	**Oxidation of pyruvate and acetyl CoA**	**Most tissue types**
De novo lipogenesis	**Conversion of glucose metabolites to fatty acids**	**Liver and adipose tissue**
Lipolysis	**Conversion of triglycerides to glycerol and free fatty acids**	**Adipose tissue**

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