Carotid sinus nerve resection: therapeutic tool for the treatment of type 2 diabetes

Bernardete Sofia de Freitas Melo

Dissertação para obtenção de grau de Mestre em Biologia Molecular e Genética

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Resumo

Nas últimas décadas, tem ocorrido um aumento na prevalência de doenças metabólicas e cardiovasculares, o que representa um problema de saúde alarmante. Assim, é necessário esclarecer os mecanismos fisiopatológicos subjacentes a estas, de forma a desenvolver novas intervenções terapêuticas.

Os corpos carotídeos (CBs) são quimiorrecetores periféricos que têm como principal estímulo, a hipóxia, tendo sido propostos como sensores de glucose, implicados no controlo da homeostasia energética. Recentemente, foi descrito que a disfunção destes está envolvida na génese da resistência à insulina e da hipertensão arterial.

Assim, o principal objetivo deste trabalho foi estudar a abolição da atividade do CB como uma possível intervenção terapêutica para a reversão da resistência à insulina e da hipertensão. Para isso, foram utilizados dois modelos animais, submetidos a dietas hipercaolóricas, o modelo de dieta rica em sacarose (HSu), que é um modelo magro de resistência à insulina e hipertensão, e o modelo de dieta rica em lípidos (HF), que é um modelo combinado de obesidade, resistência à insulina e hipertensão, e que foi mantido numa dieta rica em lípidos.

A abolição da atividade do corpo carotídeo foi realizada através da ressecção do nervo do seio carotídeo (CSN). Parâmetros como sensibilidade à insulina, glicemia em jejum, níveis plasmáticos de insulina, variação de peso e gordura, perfil lipídico, níveis de ácidos gordos livres e expressão do recetor de insulina e dos transportadores de glucose Glut4 e Glut2, foram avaliadas.

Observou-se que as dietas hipercaolóricas alteraram os parâmetros acima mencionados. No entanto, a ressecção do CSN restaurou completamente a sensibilidade à insulina, a glicemia em jejum e os níveis de insulina plasmática. Para além disso, também impediu o ganho de peso nos animais submetidos às dietas hipercaolóricas, levando a uma diminuição da gordura total e visceral e a um melhor perfil lipídico. Além disso, a desnervação do CB levou, também, a um restauro dos níveis de recetor de insulina e de Glut4 no músculo-esquelético, e dos níveis de Glut2 no fígado.

Em conclusão, este estudo demonstrou que a abolição da atividade do CB pode ser utilizada como uma potencial intervenção terapêutica, destacando a importância do desenvolvimento de estratégias para reduzir a sobreactivação deste em doenças metabólicas.

Palavras-chave: corpo carotídeo, ressecção do nervo do seio carotídeo, diabetes mellitus tipo 2, resistência à insulina, dislipidémia
In the last decades there has been a dramatic increase in the prevalence of metabolic and cardiovascular diseases. This increase represents an alarming health problem urging quick clarification of the pathophysiological mechanisms that underlie these diseases in an attempt to develop therapeutic interventions. Carotid bodies (CB) are peripheral chemoreceptors whose main stimulus is hypoxia, and it was proposed that they can act as glucose sensors implicated in energy homeostasis control. Recently we have described that CB dysfunction is involved in the genesis of insulin resistance (IR) and hypertension (HT).

Thus, the main goal of this project was to investigate if the abolishment of CB activity can be a potential therapeutic intervention to reverse IR and HT. For that, we have used 2 pathological animal models, the high fat (HF) and the high sucrose (HSu) model. Abolishment of CB activity was performed through the resection of CB sensitive nerve, the carotid sinus nerve (CSN). Insulin sensitivity, fasting plasma glucose and insulin levels, weight and fat variation, lipid profile, plasma free fatty acids (FFA) levels, insulin receptor expression and glucose transporters Glut4 and Glut2 expression, were evaluated in controls and in pathological animal models.

We have observed that hypercaloric diets altered the parameters above mentioned. However, CSN resection completely restored insulin sensitivity, fasting plasma glucose and plasma insulin levels. Additionally, it also prevented additional weight gain in rats submitted to hypercaloric diets, leading to a decrease in total and visceral fat and to a better lipid profile. Furthermore, CSN denervation also restored IR and Glut4 levels in skeletal muscle and Glut2 levels in liver.

In conclusion, this study demonstrated that the abolishment of CB activity can be used as a potential therapeutic intervention, highlighting the importance of developing strategies to reduce CB overactivity in metabolic diseases.

**Key words:** carotid body, carotid sinus nerve resection, type 2 diabetes, insulin resistance, dyslipidemia
A prevalência de doenças metabólicas como obesidade, resistência à insulina, hiperlipidemia, hiperlipidemia e hipertensão tem vindo a aumentar drasticamente. Nas últimas duas décadas verificou-se um aumento no número de pessoas diagnosticadas com diabetes. Aliás, é estimado que em 2030 o número de pessoas com diabetes aumente para 439 milhões. Em Portugal, estima-se que 34% da população, entre os 20 e os 79 anos, sofram de diabetes ou pré-diabetes. Assim, tendo em conta esta epidemia, é de grande interesse identificar e implementar intervenções de forma a impedir ou retardar o seu aparecimento.

A diabetes mellitus tipo 2 é uma patologia poligénica complexa, caracterizada pela presença de defeitos quer ao nível da secreção da insulina (disfunção as células β do pâncreas) quer ao nível da ação da insulina (resistência à insulina). Assim, a primeira anormalidade detetável na diabetes mellitus tipo 2 é uma redução da habilidade do organismo em responder à insulina, ou seja resistência à insulina. Nesta altura, o pâncreas consegue aumentar a secreção de insulina de forma a normalizar os níveis de glucose no plasma (pré-diabetes) sendo esta fase caracterizada por resistência à insulina, normoglicemia e hiperinsulinemia. Contudo, com o tempo, as células β não conseguem manter a alta secreção de insulina, o que resulta no desenvolvimento de intolerância à glucose e eventualmente, diabetes.

Os corpos carotídeos são pequenos órgãos, localizados bilateralmente na bifurcação da artéria carótida. Estes órgãos são constituídos por dois tipos de células, as células tipo I (também chamadas de células quimiorrecetores) e as células tipo II (também chamadas de células de sustentação). Os corpos carotídeos são quimiossensores arteriais que detetam alterações nas pressões de O₂, CO₂ e Ph. Quando estes órgãos detetam hipóxia, hipercápnia ou acidose, as células quimorreceptoras libertam neurotransmissores que atuam nos seus recetores no nervo do seio carotídeo aumentando a frequência dos seus potenciais de ação. Esta atividade elétrica do nervo do seio carotídeo é integrada no tronco cerebral de forma a induzir um conjunto de reflexos respiratórios que irão normalizar, por hiperventilação, os gases sanguíneos alterados e que irão também regular a pressão sanguínea e a performance cardíaca, através da ativação do sistema nervoso simpático.

Para além do seu papel no controlo cardiorespiratório, foi mais recentemente proposto que o corpo carotídeo possa ser um sensor de glucose e que possa estar implicado no controlo da homeostasia da glucose e no controlo energético. Mais recentemente, foi também demonstrado o papel do corpo carotídeo na regulação da sensibilidade à insulina. Observou-se que a atividade dos quimiorrecetores periféricos do corpo carotídeo está aumentada em modelos animais hipercaleíricos de resistência à insulina e hipertensão e que a desnervação
crónica do nervo do seio carotídeo previne o desenvolvimento de resistência à insulina e hipertensão, o que significa que este órgão está envolvido na etiologia destas características patológicas presentes nas doenças metabólicas. Observou-se também que nestes modelos animais está presente uma sobreativação do sistema nervoso simpático e que a desnervação previne também este aumento, o que sugere que sobreativação do corpo carotídeo leva a um aumento da atividade do sistema nervoso simpático, iniciando assim um ciclo que levará a uma diminuição da sensibilidade à insulina e ao surgimento de hipertensão.

Assim, o objetivo principal deste trabalho foi estudar a abolição da atividade do corpo carotídeo como uma possível intervenção terapêutica para a reversão da resistência à insulina e da hipertensão. Os objetivos específicos foram investigar em modelos animais de pré-diabetes e síndrome metabólica: 1) se a ressecção do nervo do seio carotídeo restaura a sensibilidade à insulina e a hiperinsulinemia; 2) o efeito da ressecção do nervo do seio carotídeo no aumento de peso, na distribuição de gordura e no perfil lipídico e por último; 3) estudar os mecanismos moleculares pelos quais a ressecção do nervo induz um efeito benéfico nos parâmetros metabólicos e cardiovasculares.

As experiências foram realizadas em ratos Wistar, de ambos os sexos e com 3 meses de idade que foram submetidos a dietas hipercaalóricas de forma a induzir diabetes mellitus tipo 2 e síndrome metabólica. O modelo de dieta rica em sacarose (HSu), que é um modelo magro de resistência à insulina e hipertensão foi conseguido através da administração de 35% de sacarose na água de beber (durante 28 dias) enquanto que o modelo de dieta rica em lípidos (HF), que é um modelo combinado de obesidade, resistência à insulina e hipertensão, foi mantido numa dieta rica em lípidos (45% de gordura, 35% de carbohidratos e 20% de proteína) durante 21 dias). Paralelamente a estes grupos, um grupo de animais controlo foi mantido numa dieta standard.

Após as dietas (3 semanas para os animais HF e 4 semanas para os HSu), a resistência à insulina foi confirmada através de um teste de tolerância à insulina (ITT) e os grupos de animais foram divididos. Metade do grupo de animais foi submetido à ressecção bilateral do nervo do seio carotídeo e outra metade foi submetida ao mesmo procedimento cirúrgico, mas sem corte do nervo (grupo sham). Após estes procedimentos cirúrgicos, os animais foram mantidos nas suas dietas durante mais três semanas e vários parâmetros foram avaliados semanalmente (glicemia em jejum, sensibilidade à insulina e peso corporal). Para além disso, foi também monitorizada a ingestão calórica e de líquidos, diariamente, antes e após a cirurgia.

No final deste período, os animais foram anestesiados com pentobarbital (60mg/kg i.p.), e foi confirmada a ressecção do nervo do seio carotídeo através da ausência de respostas ventilatórias à hipoxia isquémica. De seguida foi recolhido sangue, por punção cardíaca, para quantificação da insulina plasmática e avaliação do perfil lipídico. Para além disso, também
foram colhidos tecidos como músculo-esquelético e fígado (para posterior homogeneização e análise de expressão de proteínas por Western Blot) e ainda gorduras totais e viscerais, que foram pesadas e posteriormente conservadas a -80°C.

A sensibilidade à insulina foi avaliada pelo teste de tolerância à insulina, o perfil lipídico foi avaliado pelo kit Randox, que avalia o colesterol total e os triglicéridos através ensaios enzimáticos colorimétricos, e o colesterol HDL e LDL através de métodos diretos de clearance. Por sua vez, os ácidos gordos livres foram avaliados através de teste colorimétrico. Para além disso foram também analisadas, por Western Blot, as expressões do recetor de insulina e do transportador de glucose Glut4 no músculo-esquelético e do transportador de glucose Glut2 no fígado.

Tal como anteriormente descrito pelo nosso grupo, apenas a dieta Hs induziu um aumento na glicémia basal. Observou-se também que após 3 semanas de HF e 4 semanas de Hs, a sensibilidade à insulina se encontrava significativamente diminuída. Quanto aos níveis de insulina plasmática, ambas as dietas hipercalóricas produziram um aumento dos mesmos (hiperinsulinemia).

Após as dietas, ambos os modelos animais de Hs e HF apresentaram um elevado aumento de peso por dia, em comparação com os controles. Contudo, apenas os animais submetidos à dieta HF apresentaram um aumento de gordura total e visceral.

Relativamente ao perfil lipídico, as dietas hipercalóricas não produziram alterações nos níveis de colesterol total ou de colesterol LDL. Contudo, após as dietas, os níveis de colesterol HDL encontravam-se diminuídos nos animais HF. Para além disso, ambas as dietas hipercalóricas provocaram um aumento nos níveis de triglicéridos, mas apenas a dieta rica em sacarose induziu um aumento nos ácidos gordos livres.

Tal como esperado, observou-se que em ambos os modelos patológicos de insulino-resistência ocorreu uma diminuição na expressão do recetor de insulina e na expressão de Glut4, no músculo-esquelético. O mesmo não se observou na expressão de Glut2 no fígado onde apenas a dieta HF levou a uma diminuição da mesma.

A ressecção do nervo do seio carótideo e consequente supressão da atividade do corpo carotídeo restaurou completamente a sensibilidade à insulina e também os níveis de glicémia em jejum e de insulina plasmática. Para além disso, impediu o aumento de peso, nos ratos submetidos às dietas hipercalóricas, levando a uma diminuição na quantidade de gordura visceral e total, e a uma melhoria do perfil lipídico. Adicionalmente, a desnervação do corpo carotídeo restaurou, também, os níveis de expressão do recetor de insulina e do transportador de glucose Glut4, no músculo-esquelético, e do transportador de glucose Glut2, no fígado.

Em conclusão, este estudo demonstra que a supressão da atividade do corpo carotídeo poderá ser utilizada como uma possível intervenção terapêutica, o que destaca a
importância de desenvolver estratégias de forma a reduzir a sobreativação do corpo carotídeo nas doenças metabólicas.

**Palavras-chave:** corpo carotídeo, diabetes mellitus tipo 2, resistência à insulina, ressecão do nervo do seio carotídeo, síndrome metabólica
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Abbreviation list

AKT/PKB – Protein Kinase B
Apo B – Apolipoprotein B
AS160 – Akt substrate of 160 kDa
Bad – BCL2-associated agonist of cell death
cAMP – Cyclic Adenosine Monophosphate
CB – Carotid Body
C/EBPs - CCAAT-enhancer-binding proteins
CHD – Coronary Heart Disease
CoA – Acetyl coenzyme A
CTL – Control
CTL Dn – Denervated control
CREB – cAMP response element-binding protein
CRTC2 – CREB regulated transcription coactivator 2
CSN – Carotid Sinus Nerve
DOCK1 – Dedicator of Cytokinesis 1
EDTA – Ethylenediamine Tetraacetic Acid
ELISA – Enzyme Linked Immunosorbent Assay
FFA – Free Fatty Acids
FOXO1 - Forkhead Box Protein O1
GLUT2 – Glucose Transporter Type 2
GLUT4 – Glucose Transporter Type 4
Grb-2 - Growth Factor Receptor-bound Protein 2
GSK3b – Glycogen Synthase Kinase 3 Beta
HDL-C – High Density Lipoprotein
HF – High Fat
HSu – High Sucrose
HT – Hypertension
IL- 6 – Interleukin 6
IGT – Impaired Glucose Tolerance
ITT – Insulin Tolerance Test
IR – Insulin Resistance
IRS 1-4 – insulin receptor substrate 1-4
LDL-C – Low Density Lipoprotein
MAPKS – Mitogen-activated Protein Kinase
mTORC1 – Mammalian Target of Rapamycin Complex 1
NFκB - Nuclear Factor Kappa-light-chain-enhancer of Activated B Cells
OGTT – Oral Glucose Tolerance Test
PIP3 – Phosphatidylinositol 3,4,5-trisphosphate
PDE3B – Phosphodiesterase 3B
PDK1 – Phosphoinositide-dependent Kinase-1
PDK2 – Pyruvate dehydrogenase kinase isoform 2
PPARγ – Peroxisome Proliferator-Activated Receptor Gamma
RhebGTPase – GTP-binding protein Rheb
SAT – Subcutaneous Adipose Tissue
SD LDL – Small Dense Low Density Lipoprotein
SOCS3 – Suppressor of cytokine signaling 3
Srepb1 – Sterol Regulatory Element-Binding Protein 1
T2DM – Type 2 Diabetes Mellitus
TG – Triglycerides
TNF-α – Tumour necrosis factor alpha
TSC2 – Tuberous Sclerosis Complex 2
VAT – Visceral Adipose Tissue
VLDL – Very Low Density Lipoprotein
1 Introdução

1.1 Diabetes Mellitus

Metabolic disorders like obesity, insulin resistance (IR), hyperglycemia, hyperlipidemia and hypertension (HT) clustered together have been described as the “insulin resistance syndrome” (Reaven, 1988; Moller and Kaufman, 2005). However, nowadays, the term “metabolic syndrome” has been adopted (Reaven, 1988; DeFronzo and Ferrannini, 1991; Kahn et al., 2005) and metabolic syndrome has become one of the major public-health challenges worldwide. Also, metabolic syndrome is a major risk factor for both cardiovascular dysfunction and diabetes mellitus (Albertini et al., 2005).

In the past two decades, there has been an increase in the number of people diagnosed with diabetes all over the world (Zimmet et al., 2001). In fact, it was estimated that in 2010 the number of people with diabetes was approximately 285 million and that in 2030 will increase to 439 million (Shaw et al., 2010). In the Portuguese population, it is estimated that about 34.5% (between the ages of 20 and 79 years) have diabetes or pre-diabetes and 44% of people with diabetes are unaware of their condition (Gardete-Correia et al., 2010). Taking into account this epidemic, there is of great interest identify and implement interventions to prevent or delay its onset.

According to the American Diabetes Association, the classification of diabetes includes four clinical classes: Type 1 Diabetes, Type 2 diabetes mellitus (T2DM), Gestacional Diabetes Mellitus and other specific types of diabetes due to other causes (Serrano-Ríos, Reviriego and Fuentes, 2010).

T2DM is a complex polygenic pathology characterized by the presence of defects in both insulin secretion (beta-cell dysfunction) and insulin action (Kashyap and DeFronzo, 2007).
1.2 Insulin and insulin action

Insulin is a 51 amino acids peptide hormone, organized in 2 polypeptide chains, A and B, of 21 and 30 amino acids, respectively, connected by disulphide bridges. Insulin is synthesized and release by β-pancreatic cells being central in regulation of carbohydrate and fat metabolism in the body. In insulin sensitive tissues, like skeletal muscle and fat tissue, insulin binds to its specific receptor, the insulin receptor inducing the uptake of glucose from the blood (Kahn and White, 1988).

The insulin receptor is a glycoprotein that belongs to a subfamily of receptor tyrosine kinases and consists of extracellular α- and transmembrane β-subunits. Due to its action as allosteric enzyme, the α-subunit inhibits tyrosine kinase activity of the β-subunits (Saltiel and Kahn, 2001; Guo, 2014). When insulin binds to the α-subunit of its receptor, the net effect is the dimerization of the receptor to form a complex, α2β2, in the cell membrane and autophosphorylation of the β-subunit at Tyr1158, Tyr1162 and Tyr1163 which leads to insulin receptor activation. Insulin receptor tyrosine kinase activation recruits and phosphorylates several substrates like insulin receptor substrates 1-4 (IRS1-4), SHC-adaptor protein, growth factor receptor-bound protein 2 (Grb-2-associated protein), dedicator of Cytokinesis 1 (DOCK1), CBL and APS adaptor proteins, providing specific docking sites for recruitment of downstream signalling proteins and leading to activation of both signalling cascades, Ras→ Mitogen-activated protein kinase (MAPKs) and phosphatidylinositol-3-kinase (PI3K)→Protein kinase B (Akt) (Guo, 2014). Ras→MAPKs activation mediates the effect of insulin on mitogenesis and cell growth whereas PI3K activation generates phosphatidylinositol (3,4,5)-triphosphate (PIP3) that will activate 3-phosphoinositide protein kinase 1 and 2 (PDK1 and PDK2), which mediate the insulin effect on metabolism and pro-survival (Guo, 2014). PDK1 and PDK2 are crucial for protein kinase B (Akt/PKB) activation, by inducing phosphorylation at T308 and S473, respectively (Guo, 2014).

Akt/PKB phosphorylates several downstream targets including: i) glycogen synthase kinase 3p (Gsk3b) which is phosphorylated and inhibited and, in turn, will dephosphorylate and activate glycogen synthase (GS); ii) tuberous sclerosis complex 2 (TSC2) which is inhibited, thereby activating GTP-binding protein Rheb (RhebGTPase) for the activation of mammalian target of Rapamycin complex 1 (mTORC1) and S6K, which promotes protein synthesis; (Guo, 2014) iii) Akt substrate of 160 kDa (AS160) which will activate Rab10GTPase, leading to glucose transporter type 4 (GLUT4) translocation to the plasma membrane allowing glucose uptake; iv) BCL2-associated agonist of cell death (Bad), for inhibition of apoptosis; and v) phosphodiesterase 3B (PDE3B) for cyclic adenosine monophosphate (cAMP) degradation. Also, Akt phosphorylates and inhibits cAMP response element-binding protein (CREB)-
regulated transcription coactivator 2 (CRTC2), that increases hepatic gluconeogenesis and phosphorylates sterol regulatory element-binding protein 1 (Srebp1), promoting liver lipogenesis (Wang et al., 2010). On the other hand, Akt/PKB phosphorylates Forkhead box protein O1 (Foxo1), inhibiting its transcriptional activity leading to a suppressed liver glucose production and promoting cell survival in the heart (Guo, 2014).

1.3 Glucose homeostasis

Among nutrients, glucose is the major source of fuel for energy metabolism thus, glucose homeostasis is ensured by a balance between glucose absorption from the intestine, production by liver, and uptake and metabolism by the peripheral tissues (Saltiel and Kahn, 2001).

In normal individuals, plasma glucose remains between values of 4 and 7 mM (despite periods of feeding and fasting). During fasting (10 to 12h overnight fast) the majority of total body glucose disposal takes place in insulin-independent tissues. Approximately 50% is used by the brain, 25% by the liver and gastrointestinal tissues, and the remaining 25% is used by insulin-dependent tissues, primarily muscle, and in a lesser extent by the adipose tissue. Approximately 85% of endogenous glucose production is derived from the liver, the remaining 15% is produced by the kidney and, both glycogenolysis and gluconeogenesis contribute equally to the basal rate of hepatic glucose production (DeFronzo, 2004).
After glucose ingestion, the increase in plasma glucose concentration stimulates insulin release by pancreatic β-cells. Increments in insulin plasma concentration inhibit lipolysis, leading to a decline in the plasma level of free fatty acids (FFA). This decline leads to an increase on muscle glucose uptake and contributes to the inhibition of hepatic glucose production. (Saltiel and Kahn, 2001; Boden and Shulman, 2002; DeFronzo, 2004).

Glucagon also plays a central role in glucose homeostasis regulation. During fasting conditions, approximately half of total hepatic glucose output is dependent on the maintenance of normal basal glucagon levels. On the other hand, after ingestion of a glucose-containing meal, glucagon secretion is inhibited contributing to hepatic glucose production repression (DeFronzo, 2004).

1.4 Alterations in insulin action and glucose homeostasis in metabolic disorders

1.4.1 Insulin resistance

Insulin resistance (IR) occurs when the insulin-sensitive tissue loss the response to insulin (Ye, 2013). Although the mechanisms that lead to the appearance of IR remain unknown, several factors have been proposed to explain this mechanism, among which are highlighted: obesity, inflammation, hyperinsulinemia, lipotoxicity/hyperlipidemia, stress, aging, fatty liver and hypoxia (Ye, 2013).

The vast majority of these factors are associated with obesity and aging, which are the major risk factors for IR in the population (Ye, 2013).

Obesity is characterized by a proinflammatory state that starts in adipose tissue and liver with elevated macrophage infiltration and expression of proinflammatory cytokines. Inflammation inhibits the insulin signalling activity in adipocytes and hepatocytes through several mechanisms: i) inhibition of IRS-1 and insulin receptor in the insulin signalling pathway (White, 2002; Ye, 2011); ii) inhibition of peroxisome proliferator-activated receptor gamma (PPARγ) function (PPARγ drives lipid synthesis and fat storage in cells) (Ye, 2008; Ye, 2011); iii) increase of FFA through lypolisys stimulation and blocking triglycerides (TG) synthesis (Ye, 2013).

Obese adipose tissue mainly releases proinflammatory cytokines among which are TNF-α, IL-6, leptin, and adiponectin. TNF-α induces IR directly (by IRS-1 phosphorylation)
(Kanety et al., 1995) and indirectly (by altering adipocyte differentiation and adipocyte lipid metabolism). Also, TNF-α promotes lipolysis and secretion of FFAs to the plasma, inhibits the conversion of pre-adipocytes to mature adipocytes (by downregulating adipogenic genes such as PPARγ and CCAAT/enhancer binding protein) (Xu et al., 1999; Makki et al., 2013), and can reduce GLUT4 gene expression (Kahn, 2000). IL-6 increases IR by upregulating SOCS3 which decreases insulin-induced IRS-1 phosphorylation (Senn et al., 2003). Also, it can promote dysregulation of white adipose tissue fatty acid metabolism inhibiting adipogenesis (Pricola et al., 2009) and it stimulates lipolysis, increasing FFA levels and body fat oxidation (van Hall et al., 2003). Leptin is primarily secreted by adipocytes being a key contributor to energy metabolism (Friedman, 1998). Leptin acts in the brain, directly or indirectly, by activation of specific centers in hypothalamus to decrease food intake, increase energy expenditure, influence glucose and lipid metabolism or alter neuroendocrine function (Makki et al., 2013). It is known that leptin levels are increased in obese subjects and preclinical and clinical studies showed that obese rodents and humans present leptin resistance that may contribute to lipid oxidation reduction in insulin-sensitive organs and thus contributing to lipids accumulation and IR. However, the mechanisms behind leptin resistance and their role it on IR is still not totally understood (van den Hoek et al., 2008; Makki et al., 2013). On the other hand adiponectin, an hormone secreted by adipose tissue, is reduced in obesity (Arita et al., 1999). This hormone has important insulin sensitizing effect, as showed by studies that have linked plasma adiponectin levels to insulin sensitivity in humans and in rodent models (Berg et al., 2002).

As already mentioned, the relationship between IR and overall obesity is well established although it remains unclear which are the fat depots, visceral adipose tissue (VAT) or subcutaneous adipose tissue (SAT) that contribute more for this relationship. Several studies have demonstrated that there is a strong independent relationship between both VAT and SAT and IR, and that visceral fat accumulation decrease peripheral insulin sensitivity and enhance hepatic gluconeogenesis, being a more potent predictor of IR than SAT (Gastaldelli et al., 2002; Wagenknecht et al., 2003). On the other hand, Abate and colleagues found that SAT was more strongly associated with IR, in male patients with T2DM (Abate et al., 1996). It seems, however, that the deeper layer of SAT has an association with IR that is similar to the pattern for VAT whereas the superficial layer has a weaker association (Kelley et al., 2000). In addition to the accumulation of visceral fat and skeletal muscle IR, adipose tissue also presents IR, characterized by increased plasma FFA levels. This increased FFA levels are not supressed as normally because fat cells are resistant to the antilipolytic effect of insulin, resulting in chronically elevated plasma FFA (Bays et al., 2004). It is now consensual that plasma FFA are a major contributor of the IR syndrome according to the “overflow hypothesis”, which says that once the capacity of adipocyte to store TG is exceeded occurs an overflow
and, consequently, accumulation of FFA as long-chain fatty acyl CoAs in the muscle and liver, leading to NFkB activation from the nucleus, initiating inflammatory pathways including cytokines and growth factor production that interfere with insulin signalling, resulting in IR (Bays et al., 2004; Kashyap et al., 2007).

Another aspect that has been studied and related to IR is hyperinsulinemia. Hyperinsulinemia consists of increased levels of plasma insulin in fasting conditions being derived from the overproduction or decreased clearance of insulin in the body. Independently of which appears first, hyperinsulinemia by itself appears to contribute to perpetuate the IR (Shanik et al., 2008). In fact, in vivo, administration of high doses of insulin, reflecting, can cause IR (Shanik et al., 2008). Rizza et al., (1985) demonstrated that after 40 hours of insulin infusion, which elevated plasma insulin to levels similar to those found in states of IR, insulin-stimulated glucose utilization was significantly decreased. Also, one of the characteristics of hyperinsulinemic states is an increased level of inflammatory markers, including cytokines and C-reactive protein which, as already discussed early in this chapter, can induce IR (Shanik et al., 2008).

1.4.2 Impaired glucose tolerance

Impaired glucose tolerance (IGT) represents an intermediate state of abnormal glucose regulation, occurring between normal glucose homeostasis and diabetes (Nathan et al., 2007).

Studies have provided quantitative evidence that progression to glucose intolerance is associated with the development of IR. Therefore, early in the history of T2DM, IR is established but glucose tolerance remains normal (DeFronzo, 1992; DeFronzo 2007). As the fasting plasma glucose rises from 80 to 140mg/dL, the fasting plasma insulin concentration increases, reaching a value 2.0 to 2.5-fold higher than in non-diabetic individuals with normal weight and, when the fasting plasma glucose exceeds 140mg/dL, the beta cell is no longer able to maintain its elevated rate of insulin secretion and the fasting insulin concentration decreases progressively, resulting in IGT (DeFronzo., 2004). Therefore, IGT is defined as a 2-h serum glucose level between 140 and 199 mg/dl, during an oral glucose tolerance test (OGTT) (Krentz., 1996).

The progression from IGT to T2DM is heralded by β-cell failure to maintain its previously rate of insulin secretion in response to high values of glucose (DeFronzo., 2004).

1.4.3 Dyslipidemia

It is known that dyslipidemia is strongly associated with metabolic diseases, like T2DM being an elevated risk for coronary heart disease (CHD) (Mooradian, 2009; Klop et al., 2013)
The typical dyslipidaemia in T2DM can include the various types of dyslipidaemia identified in the general population. However, the core components of diabetic dyslipidaemia are increased fasting and postprandial plasma TG and FFA, low levels of high density lipoprotein cholesterol (HDL-C) (with HDL-C dysfunction) and normal or slightly increased low density lipoprotein cholesterol (LDL-C) with increased concentration of small dense LDL-cholesterol particles (SD LDL) (Mooradian, 2009; Wang and Peng, 2011; Klop et al., 2013).

The exact pathogenesis of T2DM dyslipidaemia is not known but there are evidences suggesting that IR has a central role in its development. The main cause of the features of diabetic dyslipidaemia is the increased FFA release from insulin-resistant fat cells (Frayn, 2001). The increased flux of FFA into the liver, in the presence of glycogen stores, promotes TG production leading to the secretion of apolipoprotein B (ApoB) and very low-density lipoprotein (VLDL) cholesterol. The decreased ability of insulin to inhibit FFA release leads to enhanced hepatic VLDL cholesterol production (Frayn, 2001; Taskinen, 2003; Mooradian, 2009). Therefore, the increased number of VLDL cholesterol particles and the increased levels of TG decrease the HDL-C levels and increase the concentration of SD LDL particles through multiple processes (See figure 4) (Mooradian et al., 2004; Mooradian et al., 2008).

![Figure 4](image-url)

**Figure 4** - Role of insulin resistance (IR) in type 2 diabetes mellitus (T2DM) dyslipidaemia. Insulin resistant cells release free fatty acids (FFA) leading to an increased flux of FFA into the liver which promotes triglyceride (TG) production and secretion of apolipoprotein B (ApoB) and very low-density lipoprotein cholesterol (VLDL). Cholesteryl ester transfer protein (CETP) promotes the exchange from VLDL-transported triglyceride to high-density lipoprotein (HDL)-transported cholesteryl ester (CE), which results in increased amounts of both atherogenic cholesterol-rich VLDL remnant particles and triglyceride-rich, cholesterol-depleted HDL particles. (Adapted from Mooradian, 2009)
1.5 Carotid Body

In cases of acute hypoxia, mammals need rapid respiratory and cardiocirculatory adjustments to guarantee that oxygenated blood can reach their tissues and their brain cells (López-Barneo, 2003; Peers et al., 2010). The main chemoreceptors that are able to detect alterations in blood gases and pH are the carotid bodies (CB) (López-Barneo, 2003).

The CB’s are small paired organs located at the bifurcation of the common carotid arteries that are innervated by nerve fibres from the glossopharyngeal (carotid sinus nerve), vagal, and the sympathetic nerve of the nearby superior cervical ganglion (González et al., 1995; Kumar, 2007; Paton et al., 2013). The CB’s receive blood via an arterial branch arising from internal or external carotid artery and are composed by two type of cells, type I cells (also called chemoreceptor cells or glomus cells) and type II cells (also called sustentacular cells) (Iturriaga, 2004). Type I cells are of neural crest origin and possess dark, as well as clear, cored vesicles and type II cells are similar to the glial cells of the nervous system (Prabhakar, 2000; Paton et al., 2013). (see figure 5).

When CB’s are activated by hypoxia and or by acidosis or hypercapnia they respond by increasing the action potential frequency in their sensory nerve, the carotid sinus nerve (CSN). Then, 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 and to regulate blood pressure and cardiac performance via sympathetic nervous system activation (Gonzalez et al., 1994). Also, the CB directly activates the adrenals via an increased sympathetic drive and also increases sympathetic vasoconstrictor outflow to muscle, splanchnic, and renal beds (Marshall et al., 1994). Apart from its role in the control of ventilation, the CB has been
proposed as a glucose sensor being implicated in the control of energy homeostasis (Koyama et al., 2000; Pardal and Lopez-Barneo, 2002; Zhang et al., 2007). However, the effect of hypoglycaemia on the CB is not consensual, as several authors have reported that low glucose did not affect the release of catecholamines from the intact CB and CSN frequency of discharges (Bin-jaliah et al., 2004; Conde et al., 2007, Gallego-Martin et al., 2012). Also recently, it has been demonstrated that insulin activates the peripheral chemoreceptors located at CB (Ribeiro et al., 2013) and probably hyperinsulinemia will originate the overactivation of the CB contributing to the increased sympathetic nervous system activity starting a cycle that leads to decreased peripheral insulin action and hypertension (Ribeiro et al., 2013). In fact, Ribeiro et al., 2013 have demonstrated that CB overactivity is present in animal models of IR and HT and that a bilateral resection of CSN prevents the development, not only of IR and HT induced by diet, but also of increased fasting plasma glucose, fasting plasma insulin, FFA, and systemic sympathoadrenal overactivity, meaning that CB plays a role in the regulation of whole body insulin sensitivity.
2 Aims

Taking into account the urgent need to find new therapeutic targets that can be used to metabolic disturbances, as T2DM and metabolic syndrome and knowing that this disturbances are associated with an increased CB activity and consequent increase in the sympathetic nervous system activity, the main goal of this project was to investigate the abolishment of CB activity as a potential therapeutic intervention to reverse IR and HT.

The specific aims of this project are:

- Investigate if CSN resection restores insulin sensitivity and hyperinsulinemia in animal models of prediabetes and metabolic syndrome

- Investigate the effect of CSN resection on weight gain, fat distribution and lipid profile in models of prediabetes and metabolic syndrome

- Study the molecular mechanisms by which CSN resection have a beneficial effect on metabolic and cardiovascular parameters.
3 Methods

3.1 Animals and experiments

Experiments were performed in Wistar rats (200–420 g) of both sexes, aged 3 months and obtained from the vivarium of Faculty of Medical Sciences.

Two pathological animal models were used: the high-fat (HF) model which combines obesity, IR and HT (Shearer et al., 2009; Conde et al., 2012) and the high-sucrose (HSu) model which is a lean model of IR and HT (Ribeiro et al., 2013; Conde et al., 2012).

Rats were submitted to hypercaloric diets in order to induce T2D and metabolic syndrome. Therefore, the HSu model was achieved by administration of 35% sucrose (Panlab, Portugal) in drinking water during 28 days and the HF model was kept under a lipid rich diet (45% fat+35% carbohydrate+20% protein, Mucedola, Italy) during 21 days. The control group fed a standard diet (7.4% fat+75% carbohydrate (4% sugar)+17% protein, SDS diets RM1, Probiológica, Portugal).

After diets, IR was confirmed by an insulin tolerance test (ITT) and, after that, the animals were submitted to bilateral CSN resection under ketamine (30mg/kg)/xylazine (4mg/kg) anaesthesia and brupenorphine (10µg/kg) analgesia. The control groups were submitted to a sham procedure.

Caloric and liquid intake were monitored daily, before and after the surgical procedures in all groups of animals. Body weight and animal behavioural changes were assessed twice per week.

After the surgical procedure the animals were kept under the respective diets and fasting glucose, insulin sensitivity and body weight were evaluated weekly.

When insulin sensitivity was restored to control values in the Hsu and HF rats submitted to CSN cut, the animals were anaesthetised with pentobarbital (60mg/kg i.p.) and transferred to a heating pad to maintain body temperature at 37, 5 ± 0, 5ºC throughout the experiment. CSN resection was confirmed through the absence of ischemic hypoxia-induced hyperventilation assessed as occlusion of common carotid artery (Monteiro et al., 1989; Ribeiro et al., 2013).

Blood was collected and treated for quantification of plasma insulin and evaluation of lipid profile. Also, visceral fat and total fat were collected after an abdominal laparotomy and weighted, and tissues were collected and frozen at -80ºC.

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 Faculty of Medical Sciences.
3.2 Insulin tolerance test

The insulin tolerance test (ITT) provides an estimate of overall insulin sensitivity and consisted of the administration of an intravenous insulin bolus of 0.1 U (4.5 mg)/kg body weight, followed by the measure of the decline in plasma glucose concentration over 15 min at 1 min intervals. Before this test was performed, the animals were kept under an overnight fast and post-food-deprivation body weights were used to calculate insulin doses. Blood was drawn via tail tipping and glucose levels were measured with a Glucose Analyzer (Kinzig et al., 2010; Conde et al., 2012).

The constant rate for glucose disappearance (KITT) was calculated using the formula 0.693/t1/2. Glucose half-time (t1/2) was calculated from the slope of the least-square analysis of plasma glucose concentrations during the linear decay phase (Ribeiro et al., 2013; Conde et al., 2012).

3.3 Measurement of plasma insulin and circulating free fatty acids

After the experiments, blood and serum was collected, by cardiac puncture, to EDTA coated tubes and Eppendorfs respectively. Plasma samples were centrifuged (Sigma, Madrid, Spain) during 10 minutes at 3000g (4ºC) and serum samples were centrifuged (Eppendorf, Madrid, Spain) during 10 minutes at 13000g (4ºC).

Insulin concentrations were determined in plasma with an ELISA kit (Mercodia Ultrasensitive Rat Insulin ELISA kit; Mercodia AB, Uppsala, Sweden) and circulating FFA were determined in plasma, with an ELISA kit (96-well Serum/Plasma Fatty Acid kit for detection of non-esterified fatty acids; Zen-bio, North Carolina).

3.4 Lipid profile evaluation

Lipid profile was assessed in plasma using a RANDOX kit (RANDOX, Irlandox, Porto, Portugal) that evaluates total cholesterol and TG by Trinder-based colorimetric end-point assays, and HDL and LDL by a direct-HDL and direct-LDL clearance methods, respectively.

3.5 Western Blot

3.5.1 Western Blot analysis of insulin receptor and glucose transporter Glut4 protein expression in skeletal muscle

Samples of skeletal muscle (50 mg) were homogenized in Zurich (10mM Tris-HCl, 1mM EDTA, 150mM NaCl, 1% Triton X-100, 1% sodium cholate, 1% SDS) with a cocktail of protease inhibitors (trypsin, pepstatin, leupeptin, aprotinin, sodium orthovanadate PMSF), and centrifuged (Eppendorf, Madrid, Spain) at 13000g for 20minutes.
After centrifugation, the supernatant was collected and frozen at -80°C until further use. The protein concentration of the homogenates was determined by a Micro BCA colorimetric assay (Pierce, Madrid, Spain). Homogenate samples (50µg) and known molecular weight markers (Precision, Biorad, Madrid, Spain) were separated by SDS-PAGE (10%) under reductive conditions.

After samples separation by SDS-PAGE, they were electroblotted onto polyvinylidene difluoride membranes (PVDF) (0.45µM, Millipore, Spain). Then, nonspecific binding was blocked by incubating the membranes with a solution of 5% milk powder in Tris buffer saline (TBS), pH 7.6 containing Tween 20 (TTBS 0.1%) (BioRad, Spain), for 1 hour at room temperature. The membranes were then, incubated overnight at 4°C with primary polyclonal goat anti-Glut4 antibody (1:200) (Santa Cruz Biotechnology, USA) and primary polyclonal mouse anti-IR antibody (1:200) (Santa Cruz Biotechnology, USA).

After 3 washes of 15 min with TTBS (0.1%), membranes were incubated for 90 min with secondary mouse anti-goat antibody (1:5000) (Santa Cruz Biotechnology, USA) at room temperature. Following washing of secondary antibody, the membranes were incubated with a chemiluminescence reagent (Clarity™ Western ECL substrate, BioRad, United States) for detection of the protein of interest. Signal intensity was detected in Chemidoc Molecular Imager (BioRad Chemidoc, Spain).

Then IR membranes were washed and incubated for 2 hours at room temperature with polyclonal mouse anti-α-tubulin (1:200) and Glut4 membranes were washed and incubated for 2 hours at room temperature with mouse monoclonal anti-GAPDH antibody (1:250) (Santa Cruz Biotechnology, USA). After 3 washes of 15 min with TTBS (0.1%), membranes were incubated for 90 min with the secondary antibody goat anti-mouse (1:5000 and 1:2000) (Santa Cruz Biotechnology, USA), respectively for IR and Glut4. The incubation with α-Tubulin and GAPDH allow normalization of the bands and the comparison of the expression of insulin receptor and Glut4 proteins to the reference protein, α-Tubulin and GAPDH, respectively.

3.5.2 Western Blot analysis of glucose transporter Glut2 protein expression on liver

Samples of liver (100 mg) were homogenized in Blys solution (100 mM Tris-HCL pH7.5, 0.2mM EGTA pH8, 0.2mM EDTA pH8, 1% Triton X-100 and 0.27M Sucrose) with a cocktail of protease inhibitors (trypsin, pepstatin, leupeptin, aprotinin, sodium orthovanadate PMSF), and centrifuged (Eppendorf, Madrid, Spain) at 13000g for 20minutes.

After centrifugation, the supernatant was collected and frozen at -80°C until further use. The protein concentration of the homogenates was determined by a Micro BCA colorimetric assay (Pierce, Madrid, Spain). Homogenate samples (50µg) and known molecular weight
markers (Precision, Biorad, Madrid, Spain) were separated by SDS-PAGE (10%) under reductive conditions.

After samples separation by SDS-PAGE, they were electroblotted onto polyvinylidene difluoride membranes (PVDF) (0.45µM, Millipore, Spain). Then, nonspecific binding was blocked by incubating the membranes with a solution of 5% milk powder in Tris buffer saline (TBS), pH 7.6 containing Tween 20 (TTBS 0.1%) (BioRad, Spain), for 1 hour at room temperature. The membranes were, then, incubated overnight at 4 °C with primary polyclonal goat anti-Glut2 antibody (1: 200) (Santa Cruz Biotechnology, USA). After 3 washes of 15 min with TTBS (0.1%), membranes were incubated for 90 min with secondary donkey anti-goat antibody (1: 2000) (Santa Cruz Biotechnology, USA) at room temperature. Following washing of secondary antibody, the membranes were incubated with a chemiluminescence reagent (Clarity™ Western ECL substrate, BioRad, United States) for detection of the protein of interest. Signal intensity was detected in Chemidoc Molecular Imager (BioRad Chemidoc, Spain).

After detection, Glut2 membranes were washed and incubated for 2 hours at room temperature with mouse monoclonal anti-GAPDH antibody (1: 250) (Santa Cruz Biotechnology, USA). After 3 washes of 15 min with TTBS (0.1%), membranes were incubated for 90 min with the secondary antibody goat anti-mouse (1: 5000) (Santa Cruz Biotechnology, USA). GAPDH incubation allows normalization and comparison of the expression of Glut2 proteins to the reference protein.

3.6 Data analysis

Data were assessed with Graph Pad Prism Software, version 4 (GraphPad Software Inc., San Diego, CA, USA) and presented as mean values with their standard errors. The significance of the differences between the mean values was calculated by one- and two-way ANOVA with Dunnett's and Bonferroni multiple comparison tests, respectively. Differences were considered significant at P<0.05.
4 Results

4.1 Effect of carotid sinus nerve resection on glycemia

Figure 6 shows the effect of chronic CSN denervation on fasting glycemia in hypercaloric animal models of IR, the HSu and HF model. CSN resection did not modify fasting glycemia in control and HF animals (Fig. 6A and C). However, HSu diet increased significantly by 48.7% fasting glycemia as previously described (Conde et al., 2012; Ribeiro et al., 2013), an effect that was totally reversed by CSN resection, as one week after the surgical procedure glycemia value was similar to control (control = 83.3±1.8 mg/dl; HSu one week after CSN resection = 83.1±4.3 mg/dl), remaining within two weeks later (Fig. 6B).

![Figure 6 - Effect of carotid sinus nerve (CSN) denervation on fasting plasma glucose in control (A), high-sucrose (HSu) (B) and high-fat (HF) (C) diet rats, respectively. White bars represent values of fasting plasma glucose in control animals without (CSN) denervation. Colour bars represent values of glycemia in rats before and after CSN resection.](image)

4.2 Effect of carotid sinus nerve resection on insulin sensitivity

In figure 7 is represented the effect of CSN resection on insulin sensitivity. Insulin sensitivity was determined by the insulin tolerance test and was not affected by CSN denervation in control animals (figure 7A). HSu and HF diets decreased insulin sensitivity by 47.0 and 40.1% from a control value of KITT = 4.39±0.94 % glucose/min, respectively (figure 7B and C).

IR was partially reversed one week after CSN denervation in HSu and HF animals. Two weeks after CSN denervation IR was totally reversed and this effect was maintained at the third week after CSN resection (figure 7B and C).
4.3 Effect of carotid sinus nerve resection on plasma insulin levels

Figure 8 illustrates the effect of CSN resection on plasma insulin levels. Both HSu and HF diets increased significantly plasma insulin levels by 55%, producing a markedly increase (hyperinsulinemia) from a control value of 1.98±1.31μg/L. Diet-induced hyperinsulinemia was totally reversed by CSN resection (figure 8).
4.4 Effect of carotid sinus nerve resection on weight variation, total fat, visceral fat and lipid profile in diet induced-insulin resistant rats

After 7 weeks of HSu diet (4 weeks to induce IR + 3 post-CB denervation) and 6 weeks of HF (3 weeks to induce IR + 3 post CB denervation), both animal models presented an increased weight gain per day than controls rats (Table 1). However, CSN resection prevented this weight gain induced by both hypercaloric diets (Table 1). Only HF exhibited an increase in total and visceral fat, which was partially reversed by CSN denervation (Table 1).

Table 1- Effect of CSN resection on weight, total fat and visceral fat in control, high fat and high sucrose diet rats.
Data with and without carotid sinus resection are means of 8-9 and 8-10 values, respectively.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Control</th>
<th>High-Sucrose Diet</th>
<th>High-Fat Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without CSN resection</td>
<td>With CSN resection</td>
<td>Without CSN resection</td>
</tr>
<tr>
<td><strong>Weight gain</strong> (g/day)</td>
<td>0.68±0.2</td>
<td>0.38±0.2</td>
<td>1.31±0.1*</td>
</tr>
<tr>
<td><strong>Total fat</strong> (g/kg)</td>
<td>51.7±2.4</td>
<td>47.4±6.3</td>
<td>47.2±3.0</td>
</tr>
<tr>
<td><strong>Visceral fat</strong> (g/kg)</td>
<td>9.9±0.4</td>
<td>9.5±0.6</td>
<td>10.2±0.5</td>
</tr>
</tbody>
</table>

Weight refers to the difference between weight measured 3 weeks after CSN denervation and weight immediately prior to denervation. One and Two-Way ANOVA with Dunnett’s and Bonferroni multicomparison tests, respectively; *p<0.05 and ***p<0.001 vs control; ##p<0.01 and ###p<0.001 comparing values with and without CSN resection.

In relation to lipid profile neither hypercaloric diets nor CSN resection significantly modified total cholesterol and LDL levels (fig. 8A and B). However, HDL levels were diminished by 27% in HF animals (control= 27.99±1.21mg/dL; HF=20.33±1.21 mg/dL), and CSN resection partially reversed this decrease (C). HSu and HF diets increased TG by 56.1 and 46.0%, respectively (control=31.065±3.49 mg/dL), and these values were brought back to control levels after CSN resection (D).
4.5 Effects of carotid sinus nerve resection in on plasma free fatty acids levels, in diet induced-insulin resistant rats

Figure 10 display the effect of CSN resection on plasma FFA. HSu diet increased plasma FFA by 32%, an effect that was absence in animals submitted to HF diet (control = 575.6±78.9µM; HF = 481.7±73.7 µM; HSu=852.05±141.62 µM). Surprisingly, CSN resection did not modified FFA in controls or in the pathological animal models.
4.6 Effect of carotid sinus nerve resection on insulin receptor and glucose transporter Glut4 protein expression in skeletal muscle

Figure 11 A and B show representative Western Blots that compare the expression of insulin receptor and glucose transporter Glut4 in skeletal muscle, which correspond to 97 and 54KDa, respectively, in control, HSu and HF animals.

As we can see in figure 11C and as expected, insulin receptor expression was significantly decreased by 51% and by 41%, in HSu and HF animals. Although, CSN resection totally restored insulin receptor expression in the HF model, it was incapable of modify insulin receptor expression in the HSu model (control = 100.00±8.56%; HSu = 62.68±10.58%; HF = 101.43±5.42%). Glut4 expression, presented in figure 10D, was decreased after HSu and HF diets (control = 100.00±7.25%; HSu =54.33±6.19%; HF = 92.68±6.37%). However, CSN resection totally restored Glut4 expression to control values (control = 100.00±7.25%; HSu with CSN resection=96.25±6.50%; HF with CSN resection = 101.57±16.63%).

Figure 11 – Effect of carotid sinus nerve (CSN) resection on insulin receptor and glucose transporter Glut4 protein expression in skeletal muscle. A) and B) are representative images of Western Blot comparing insulin receptor (97 kDa) and Glut4 (54 kDa) expression; C) presents the mean values of the expression of insulin receptor without CSN resection (n=5-10) and with CSN resection (n=2-4) in relation to a-Tubulin expression; D) presents the mean values of Glut4 expression without CSN resection (n=5-10) and with CSN resection (n=5-6) in relation to GAPDH expression. Bars represent mean ± S.E.M. One-Way ANOVA with Dunnett multicomparison test, **p<0.01, ***
4.7 Effect of carotid sinus nerve resection on glucose transporter Glut2 expression in the liver

Figure 12 depicts the effect of CSN resection on Glut2 expression in the liver in controls and in insulin-resistant animal models. Panel A shows representative Western Blots for Glut2, which corresponds to 62 kDa and for GAPDH, which corresponds to the 37 KDa band, in control, HSu and HF animals. As it can be seen HSu diet did not modify Glut2 expression and HF diet showed a tendency to decrease (control = 101.70±6.32%; HSu = 107.30±9.26%; HF = 93.8±12.42%). CSN resection did not altered Glut2 expression in controls or in HSu animals, although it increased significantly by 34% in HF animals.

Figure 12 – Effect of carotid sinus nerve (CSN) resection on Glut2 expression, in the liver in control, HSu and HF insulin-resistant animals. A) shows a representative Western Blot of Glut2 expression; B) present the mean values of Glut2 expression without CSN resection (n=5-10) and with CSN resection (n=2-4) in relation to GAPDH expression. Bars represent mean ± S.E.M. Two-Way ANOVA with Bonferroni multicomparison test, *p<0.05 comparing values with and without CSN resection.
5 Discussion

In this study it was demonstrated for the first time that CSN resection and the consequent abolishment of CB activity completely restores insulin sensitivity, fasting plasma glucose and plasma insulin levels in hypercaloric-animal models of IR and HT. Additionally, CSN resection was also capable of restore normal body weight preventing weight gain and leading to a decrease in total and visceral fat and to a better lipid profile. Furthermore, it was also showed that CB denervation restores insulin signalling pathways in skeletal muscle as it normalize insulin receptor and Glut4 levels and enhance Glut2 levels in the liver.

In the present work we have seen that HF diet did not modified significantly glycemia levels, whereas HSu diet did. These results are in agreement with the previous findings from our laboratory where it was observed that 3 weeks of HF diet were incapable of promoting hyperglycemia, although 4 weeks of HSu did increased fasting glycemia (Conde et al., 2012; Ribeiro et al., 2013). While the increase in fasting glycemia by HSu diet is consensual throughout the majority of the studies (Kamgang et al., 2005; Wilson and Hughes 1996; Ribeiro et al., 2005; Ebaid et al., 2006), the normoglycemia during HF diet is not. In Kamgang et al., (2005) Wistar rats were distributed into three dietary groups and received either a normal diet, a HSu diet (carbohydrates 65-70%, fat 25-30%, protein 10-15%) or a HF diet (carbohydrates 35-40%, fat 50-55%, protein 10-15%) during 4 months. They show that the fasting glycemia levels were significantly elevated in the HSu and HF groups, when compared to the control group and that it was highly correlated with the food intake in the HSu group (Kamgang et al., 2005), although in this study the increase in fasting glycemia promoted by HF diet could be due to high percentage of carbohydrates in the diet.

Also, in the present study it was observed that HF and HSu diet, as previously described, induced a decrease in insulin sensitivity (Conde et al., 2012; Ribeiro et al., 2013). This IR was also consistent with the results described by Nascimento et al., (2008) study, in which Wistar rats were distributed into two groups and received either normal or a cycle of hypercaloric diets over a 14-week period. Nascimento et al., (2008) observed that the hypercaloric pellet-diet cycle promoted IR. Furthermore, Santuré et al., (2002) conducted a study in Male Sprague-Dawley rats that were randomized to receive a sucrose- or regular rat chow-diet for 4 weeks, and they found that sucrose feeding induces IR which is consistent with the results described herein.

The present work demonstrates that both HSu and HF diets produced a markedly increase in plasma insulin levels (hyperinsulinemia), being these results consistent with our previous works (Conde et al., 2012; Ribeiro et al., 2013). All together, the results described in the present thesis concerning the effects of hypercaloric diets, both HF and HSu, in the
metabolic parameters, like fasting glycemia, insulinemia and insulin sensivity and the previous published (Conde et al., 2012, Ribeiro et al., 2013, Guarino et al., 2014) show that these metabolic features in these animals models are reproducible within groups in our laboratory. One of the first papers that described that increased caloric intake led to statistically significant increases in fasting plasma insulin (22%), glucose (5%) and triglyceride (30%) levels was the one by Olefsky et al., (1975). They also saw that, most of the changes returned to control levels during the succeeding 2 weeks of increased caloric intake, although statistically significant elevations of fasting plasma glucose (10%), insulin (8%), and cholesterol (15%) levels were still seen at the end of the hypercaloric dietary period (Olefsky et al., 1975). The increase in plasma insulin levels after hypercaloric diets was also observed by McDonald et al., (2011), in which male rats were fed an HF diet from weaning until 39 weeks, presenting hyperinsulinemia that was not sufficient to maintain normoglycemia, resulting in hyperglycemia. Also, Oliveira-Júnior et al., (2010) work showed that rats fed with HF diet present hyperinsulinemia. The same findings were observed by Khalkhal et al., (2012) in a work in which sand rats were exposed to either a low or a high caloric diet and after 9 months, pancreatic islets were isolated and incubated in the presence of increasing cytokine concentrations. At the end of the high-energy diet, animals were all over-weight, exhibiting IR, hyperinsulinemia and normoglycemia or a marked type-2 diabetic state.

It is known that obesity is strongly associated with IR (Ye, 2013) and that hypercaloric diets induce weight gain (Conde et al., 2012). Herein we have shown that the both hypercaloric diets induce an increase in weight gain, although the gain/day induced by HF diet was significantly higher than the obtained with the HSu diet. The increase in body weight with HF diet is not new, as several authors have already described it. Bravo et al., (2014) described an increase in body weight in rats kept under diet HF diet during 16 weeks. The same results were obtained by Burlamaqui et al., (2011), in a study in which 22 rats were distributed equally for two groups, GI (fed with standart diet) and GII (fed with hypercaloric and hyperlipid diet). After 23 weeks of experiment, weight was greater in GII. In Humans, this relation was studied by Koopman et al., (2014) in a work where 36 lean and healthy men were randomized to a 40% hypercaloric diet for 6 weeks or a eucaloric control diet. The caloric surplus consisted of fat and sugar (high-fat-high-sugar; HFHS) or sugar only (high-sugar; HSu) and was consumed together with, or between, the three main meals, thereby increasing meal size or meal frequency, resulting in increased body mass index (BMI). All these findings are coherent with the results presented in the present work.

Herein, we have also showed that although hypercaloric diets increased weight gain, only HF diet has induced an increase in total and visceral fat. This absence of effects of HSu
diet on total and visceral fat suggest that increased adipose tissue mass is not contributing to IR in this animal model, which is in accordance with previous results by Kanazawa’s group (Kanazawa et al., 2003). In addition, we have shown that none of the hypercaloric diets used in the present work significantly modified total cholesterol and LDL levels. However, both HSu and HF diets increased TG. This increase in the TG levels was already observed by Nascimento et al., (2008), where they found that hypercaloric diets promoted obesity and dyslipidemia, being the TG levels increased in the HF diet, when compared to controls (Nascimento et al., 2008). Akiyama et al., (1996) have also investigated the effects of a HF diet by infusing hypercaloric diet (360 kcal/kg body wt./day) or control diet (180 kcal/kg body wt./day), through a cannula directly into the stomach of animals, and they found that serum levels of triglyceride were significantly elevated in the HF group, when compared to those in the control group. Also in relation to lipid profile, in the present work we have observed that HDL levels were decreased in the animals fed with HF diet. These results were expected, taking into account the results obtained, in humans, by Laakso et al., (1985). Laakso et al., (1985), studied serum lipids and lipoproteins in 277 non-insulin-dependent diabetics (NIDDs) and in 124 non-diabetic control subjects. From the 277 NIDDs, 88 were treated with diet, 134 with oral drugs and 55 with insulin and they showed that the diabetics had lower levels of HDL and HDL2-c than non-diabetic control subjects and that body mass index had a significant negative correlation to HDL-C (Laakso et al., 1985). Although, the results obtained by us in the present work and the findings in humans contrast with the work by Kamgang et al., (2005) in which male Wistar rats maintained in a HF diet (carbohydrates 35-40% kcal, fat 50-55% kcal, protein 10-15% kcal) or in a HSu diet (carbohydrates 65-70% kcal, fat 25-30% kcal, protein 10-15% kcal), during 4 weeks showed total HDL and LDL cholesterol levels were significantly elevated in the HF diet group whereas the HDL level was significantly lower in the HSu diet group. This difference, in relation to the results obtained in the present work, may be due to the different times in which the animals were kept under diets, 4 weeks for both HF and HSu diet groups in Kamgang et al., (2005) study and 7 and 6 weeks for HSu and HF groups, respectively, in the present study.

In contrast to the HF diet, the HSu diet increased circulating FFA levels, indicating augmented lipolysis or decreased FFA oxidation in the sucrose-fed rats as previously described by our laboratory (Conde et al., 2012) and by others (Delarue and Magnan 2007, Cahova et al., 2012). These results may also be due to an increased de novo lipogenesis in adipocytes and in the liver in this model, which in combination with IR may lead to enhanced lipolysis and systemic FFA (Lewi et al., 2002, Cahova et al., 2012). In fact, Cahova et al., (2012) recently find that in male hereditary hypertriglyceridemic rats fed with either a HSu diet (70 cal% as sucrose), or a HF diet (70 cal% as saturated fat) that HSu diet was associated with the depression of FFA oxidation in parallel with increased TG secretion and de novo FFA
synthesis. In contrast, in the HF diet administered animals the intracellular TG-derived FFA were channelled predominantly to the oxidative utilization (ketogenesis) (Cahova et al., 2012). Different results were obtained by Sumiyoshi et al., (2006) in which C57BL/6J mice (4 week old) were divided into groups and fed with a low-fat low-sucrose diet (3% fat, 5% sucrose, wt/wt), a HF diet (45% fat, wt/wt), and a HSu diet (50% sucrose, wt/wt), for 55 weeks and they that the plasma FFA concentration was significantly greater in mice fed the HF diet than in those fed the HSu diet and greater in both of those groups than in mice fed the LL diet. The different results obtained in the different studies may rely on the time at which the animals were kept under their diets, the composition of diets or even in the different animal species in which the experiments have been performed.

Insulin resistance is manifested by a loss of the response of the insulin-sensitive tissue, like skeletal muscle, adipose tissue and the liver, to insulin and it is usually characterized by deficiencies in insulin signalling pathways (Ye, 2013). In the present work we have evaluated the alterations in the expression of insulin receptor and Glut4 in the skeletal muscle, the major tissue responsible for peripheral glucose uptake. We have showed that both hypercaloric diets tested in the present work decreased insulin receptor expression, although only HSu diet induced a decrease in total Glut4 expression. Several authors have already reported a decreased insulin receptor expression in insulin-resistant states, like Iwashita et al., (2002) in where they found that male Sprague-Dawley rats submitted to HF diet (45% fat, 35% carbohydrate) and high-carbohydrate diet (5% fat, 75% carbohydrate) during 16 weeks present a diminished expression of insulin receptors in soleus and gastrocnemius muscles. Herein, we also have shown that Glut 4 expression is decreased in Hsu but not in HF animals. These results are in agreement with the previous findings of our lab, where animals submitted to 4 weeks of HSu diet exhibit a decrease in 58% in Glut4 expression but not HF animals submitted to 3 weeks of diet (Guarino et al., 2014). Also, Kim et al., (1999) have showed that glucose uptake in skeletal muscle is diminished in rats fed with HSu diet, and so the impaired Glut4 expression seen by us in the present study and in our previous findings (Guarino et al., 2014) could explain the impaired glucose uptake. In Chen et al., (2014) study, similar results were obtained in Glut4 expression in skeletal muscle and adipose tissue, in C57BL/6J mice fed with a HF diet (35% carbohydrate and 45% fat) for 12 weeks. Although, in Kong et al., (2013) work, muscle expression of Glut4 protein was markedly reduced in the Sprague-Dawley rats that were fed with HF diet (20,1% carbohydrate, 59,8% fat and 20,1% protein) for 8 weeks. Despite the different results between studies, the fact that, in the present work, the HF diet did not promoted changes on total Glut4 expression does not mean that the number of Glut4 transporters in plasma membrane, and therefore, responsible for glucose uptake, were not reduced. It is known that Glut4 continuously recycles through several membrane systems of
adipose and muscle cells and accumulates in sequestration vesicles (GSV) due to very slow exocytosis rates in the absence of insulin stimulation (Huang and Czech, 2007). Thus, it is possible that decreased insulin signalling in the muscle cells, as seen herein by a decreased insulin receptor expression in muscle, induced by the HF diet, prevents Glut4 exocytosis from GSV and subsequent translocation of Glut4 to plasma membrane. Furthermore, some studies indicated that insulin-resistant rodents exhibit downregulation of total Glut4 expression selectively in adipocytes and not in skeletal muscle, although insulin-stimulated glucose transport is also impaired in muscle (Shepherd and Kahn, 1999).

Herein we have also found that Glut 2 transporter expression was decreased modestly in the HF animal models, but not in the HSu animals, and so this reduction can be one of the mechanisms contributing to liver-insulin resistance. This decreased expression of Glut2 in the liver is consistent with the findings of other authors (Jwa et al., 2012; Gan et al., 2013, Yang et al., (2012) Choi et al., (2013)). In fact, Jwa et al., (2012) showed that HF diet during 10 weeks decreased plasma membrane Glut2 protein concentration. Also, Gan et al., (2013) observed that Glut2 expression in the liver was down-regulated markedly in animals submitted to HF diet group, during 4 and 8 weeks.

One of the most remarkable results of our work is that we have demonstrate here, for the first time, that carotid sinus nerve resection is capable of restore insulin sensitivity, fasting plasma glucose and plasma insulin levels in hypercaloric-animal models of IR and HT. This results are in line with Ribeiro et al., (2013) work, in where it was shown that CSN ressection prevents the development of IR and HT induced by hypercaloric diets. Also, we have shown herein that the alterations in insulin signalling pathways in insulin sensitive tissues induced by hypercaloric diets are restored after CSN resection, and therefore this could be one of the mechanims contributing to the restore of insulin sensitivity.

Additionally, we showed that CSN resection was also capable of restore normal body weight preventing weight gain and leading to a decrease in total and visceral fat and to a better lipid profile. In our previous publication by Ribeiro et al., (2013) we have showed that CSN resection prevents the increase in sympathetic nervous system activity seen in hypercaloric-induced IR states. Also, obesity is associated with increased sympathetic nervouse system activity accompanied by a blunted sympathetic nervouse system reactivity (Van Baak, 2001) and according to our results, bilateral CSN cut normalizes SNS reactivity allowing increased energy expenditure and weight loss, suggesting that the IR induced by CB overactivation is due to a direct stimulation of the sympathetic nervous system. Therefore, a complete restore of sympathetic activity would restore both insulin secretion and insulin sensitivity in insulin-sensitive tissues, like skeletal muscle, liver and adipose tissue. In fact, we have observed that CSN restore insulin receptor and Glut4 expression in skeletal muscle and increase Glut2
expression in the liver, these mechanisms contributing to a recovery of insulin sensitivity. Although, and since, we have observed extensive effects of hypercaloric diets on weight gain, adipose mass and lipid metabolism, the study of the molecular alterations in insulin signaling pathways in adipose tissue and the effect of CSN resection on these pathways would contribute enormously to the understand of the mechanism behind CSN resection effects.

In the last couple of years, several reports of non-classical roles of the carotid bodies on glucose homeostasis and metabolic regulation have been published, contributing to launch the CB as a putative therapeutic target for the treatment of endocrine diseases. Our group has been actively involved in the process and recently we described that chronic CB overstimulation is implicated in the etiology of diet-induced IR (Ribeiro et al, 2013). That results together with the data obtained in the present thesis contributed to strengthen that CB blockade/modulation represents a novel and unexploited therapeutic approach for the treatment of metabolic diseases.

Blocking CB activity, therapeutically, has been widely used to treat several diseases. Toorop et al., (2009) have reported the denervation of carotid artery as a therapeutic for carotid sinus syndrome. Also, in Toorop et al., (2007) study, it was reported carotid denervation as a therapeutic for a patient with carotid sinus syndrome accompanied by excessive vomiting. Apart for excessive vomiting and carotid sinus syndrome, unilateral CB resection was used to treat dyspnoea in asthmatic and chronic obstructive pulmonary disease patients (Nakayama, 1965; Holton and Wood, 1965) with documented reductions on systolic blood pressure (Nakayama, 1965). More recently, Paton and co-workers suggested that ablation of the CB or of its afferents can represent a powerful intervention to reduce excessive sympathetic activity in neurogenic hypertension and other sympathetic mediated diseases (Paton et al., 2013). In the same line of evidence Niewinski et al.(2013) has reported that unilateral carotid resection in a 56-year-old male patient with ischemic chronic heart failure (CHF) restored autonomic balance and improved exercise capacity and, therefore quality of life , effects that are maintained for at least 6 months.

Besides the surgical resection of the CB, another way of modulating CB activity would be to directly target its effector, the SNS. The SNS 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 fibres, 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 the CBs seem to play in both the metabolic and hemodynamic control, they represent a natural candidate for Bioelectronic Medicines to be tested in a not so distant future.
6 Conclusion

The prevalence of metabolic disorders such as IR and obesity has increased dramatically and, despite the efforts that have been made in order to change bad dietary habits and sedentary lifestyles, the number of people with T2DM continues to increase. Thus, it is urgent to study the pathophysiological mechanisms underlying these and identify strategies for prevention and treatment that can help stop this epidemic.

This study, demonstrates, for the first time, that the abolishment of CB activity can restore insulin sensitivity and normalize weight gain, suggesting that the modulation of CB activity can be used therapeutically for the treatment of metabolic disturbances.
7 Future work

Despite the novelty of the results here presented and after one year working on this study, there are questions that need to be further explored. Therefore as a future project, we still want to explore the mechanisms by which CSN resection avoids obesity, and for that we will answer to the following questions:

1) Do these rats lose weight after being denervated due to an increase in energy expenditure?
2) Do these animals have a decreased nutrient consumption and therefore even under hypercaloric diets, they lose weight?
3) Does CSN resection influence adipocyte differentiation?
4) Can white adipose tissue being converted in brown adipose tissue?

To answer to these questions, experiments will be performed in Wistar rats. Two animal models will be used: a prediabetes model obtained by ingestion of a 45% lipid-rich diet during 3 weeks and a T2DM model obtained by ingestion of 60% lipid-rich diet and 35% sucrose in drinking water during 20 weeks.

CSN resection will be performed in these animals. Insulin sensitivity and glucose tolerance will be evaluated; weight, fasting glycemia and insulinemia will be monitored. Energy expenditure will be also monitored by measurement of PCO2 and PO2 by gasimetry and by functional magnetic resonance of hypothalamic nuclei. After CB denervation, when insulin sensitivity and glucose tolerance were restored animals will be submitted to a terminal protocol to collect adipose tissue to perform adipocyte volume histology and to study the expression of several molecular mediators of brown adipose tissue like PGC1α and PRDM16 and adipogenesis (like C/EBP, SREBP1c and PPAR. Also, activities of lipoprotein lipase (LPL) and hormone-sensitive lipase (HSL) will be assessed as markers for adipose tissue metabolism.

The clarification of weight loss-associated mechanisms induced by CSN denervation will open new doors for intervention for the treatment of one of highest pandemics of this century: obesity.
8 Bibliographic references


Carotid sinus nerve resection: therapeutic tool for the treatment of type 2 diabetes


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