Joana Mensurado Cartaxo Índias Coelho

Licenciada em Biologia

Disclosing the caffeine dose to be administered that restores insulin sensitivity in prediabetes animal

Dissertação para obtenção do Grau de Mestre em
Genética Molecular e Biomedicina

Orientador: Doutora Sílvia Margarida Vilares Santos Conde, Professora Auxiliar da Faculdade de Ciências Médicas da Universidade Nova de Lisboa

Júri:

Presidente: Prof. Doutora Paula Maria Theriaga Mendes Bernardo Gonçalves

Arguente: Prof. Doutor Ricardo Alexandre da Silva Santos Afonso

Vogal: Prof. Doutora Sílvia Margarida Vilares Santos Conde

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

Novembro, 2014

Joana Mensurado Cartaxo Índias Coelho

Licenciada em Biologia

Disclosing the caffeine dose to be administered that restores insulin sensitivity in prediabetes animal

Dissertação para obtenção do Grau de Mestre em
Genética Molecular e Biomedicina

Orientador: Doutora Sílvia Margarida Vilares Santos Conde, Professora Auxiliar da Faculdade de Ciências Médicas da Universidade Nova de Lisboa

Júri:

Presidente: Prof. Doutora Paula Maria Theriaga Mendes Bernardo Gonçalves

Arguente: Prof. Doutor Ricardo Alexandre da Silva Santos Afonso

Vogal: Prof. Doutora Sílvia Margarida Vilares Santos Conde

FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

Novembro, 2014

"Disclosing the caffeine dose to be administered that restores insulin sensitivity in prediabetes animal models"

The results discussed in this thesis originated the following communications in International and National congresses:

Sacramento JF, **Coelho JC**, Melo BF, Guarino MP and Conde SV. (2014) Assessment of caffeine dose and time of administration required for resetting insulin sensitivity in high sucrose diet in rats. 50th Meeting of EASD (European Association for the study of Diabetes), 14-19 September, Vienna, Austria

Coelho JC, Melo BF, Sacramento JF, Guarino MP and Conde SV (2014). Establishing the caffeine dose that chronically restores insulin sensitivity in animal model of prediabetes**.** Fundação Astrazeneca Innovate Competition, iMed conference 6.0®, 10-12 October, Lisboa, Portugal

Also, during the last year I was involved in other ongoing projects that originated the following communications:

Coelho JC, Melo BF, Sacramento JF, Ribeiro MJ, Guarino MP and Conde SV (2014). Are the effects of carotid sinus nerve resection on insulin sensitivity mediated by an increase in Glut4 expression in skeletal muscle?. XLIV Reunião Anual da Sociedade Portuguesa de Farmacologia, XXXII Reunião de Farmacologia Clínica e XIII Reunião de Toxicologia, 5-7 February, Coimbra, Portugal

Sacramento JF, Rodrigues T, **Coelho JC**, Matafome P, Ribeiro MJ, Seiça RM, Guarino MP, Conde SV (2014). Elucidating the mechanism by which carotid sinus nerve resection restores insulin sensitivity in pre-diabetes animal models. International Society for Arterial Chemoreception (ISAC) XIX University of Leeds, 29th June - 3rd July, Leeds, United Kingdom

Acknowledgement

Resumo

A diabetes tipo 2 é uma doença que afeta milhões de indivíduos contribuindo significativamente para a morbilidade e mortalidade no mundo. Nas últimas décadas tem sido descrito um efeito benéfico do consumo crónico de café na diabetes e na síndrome metabólica. O nosso grupo demonstrou que o consumo crónico de cafeína (1g/l) em ratos previne a insulino-resistência induzida por dietas hipercalóricas. Assim, o objetivo deste trabalho foi investigar qual a dose terapêutica de cafeína que restaura a sensibilidade à insulina num modelo de pré-diabetes. As experiências foram levadas a cabo em ratos *Wistar*. O modelo patológico utilizado foi o rato HSu, que se obtém através do consumo de sacarose na água (35%) durante 28 dias. Foi testado o efeito de várias doses de cafeína (0.5, 0.75 e 1g/l) em animais controlo e HSu durante 12 semanas na: sensibilidade à insulina, glicémia basal, tolerância à glucose, massa adiposa, expressão de transportadores Glut4 no músculo-esquelético e na concentração de nitric oxide (NO) no músculo-esquelético e no fígado. Demonstrou-se que a administração crónica de cafeína restaura a sensibilidade à insulina e a tolerância à glucose no modelo HSu, sendo o período de reversão inversamente proporcional à concentração de cafeína Observou-se que os animais HSu não aumentam de peso em relação aos controlos, embora possuam um aumento de massa gorda. A ingestão de cafeína não alterou o aumento de peso/dia e massa gorda quer nos animais controlo quer nos HSu. A ingestão de cafeina (1g/l) reverteu os níveis de expressão de Glut4 no músculo nos animais HSu. Os níveis de NO diminuíram no músculo-esquelético, e não no fígado, com a deita HSu e a cafeína foi incapaz de os alterar. Em conclusão, a cafeína pode ser utilizada como uma ferramenta terapêutica para o tratamento da prédiabetes e para a prevenção das doenças metabólicas.

Palavras-Chave: Cafeína, prediabetes, resistência à insulina, tolerância à glucose, Glut4

Abstract

Type 2 diabetes mellitus (T2DM) is a chronic disease affecting millions of individuals, contributing to significant morbidity and mortality worldwide. Caffeine is the most widely consumed psychoactive substance in the world and recently several epidemiological studies described beneficial effects of chronic coffee intake on T2DM and metabolic syndrome. Our group has shown that chronic caffeine intake (1g/l) prevents the development of insulin resistance and hypertension in diet-induced insulin resistante rats. Therefore, the main objective of this work was to investigate the therapeutic dose of caffeine that restores insulin sensitivity in a prediabetic animal model. Experiments were performed in *Wistar* rats. The prediabetic animal model used was the high sucrose (HSu) model, which is obtained by submitting the animals to 35% of sucrose in drinking water during 28 days. The effect of chronic caffeine (0.5, 0.75 and 1g/l) was tested in control rats and in HSu model during 12 weeks assessing: insulin sensitivity, basal glycemia, glucose tolerance, adipose tissue mass, Glut4 transporters and nitric oxide (NO) content in skeletal muscle and in the liver. We have seen that chronic caffeine intake restores insulin sensitivity and glucose tolerance in HSu rats, being the latency time inversely correlated with caffeine concentration. Also, HSu diet did not change weight gain comparing with controls but increased fat mass. Caffeine intake did not alter weight gain/day and fat mass. Caffeine (1g/l) restores Glut4 expression levels in skeletal muscle in HSu animals. NO levels decrease in skeletal muscle in HSu animals, but not in the liver, and caffeine did not modify these levels. These results suggest that caffeine can be used as a therapeutical tool for the treatment of prediabetes and prevention of T2DM.

Keywords: Caffeine, prediabetes, insulin resistance, glucose tolerance, Glut4

Table of contents

Figures

Tables

Abbreviations

AMP – Adenosine 5'-monophosphate

AMPK – AMP-activated protein kinase

ANOVA – Analysis of variance

ATP – Adenosine triphosphate

AUC – Area Under the Curve

cAMP – 3'-5'-cyclic adenosine monophosphate

FFA – Free fatty acids

FGP – Fasting plasma glucose

Glut – Glucose transporters

Glut 1 – Glucose transporters

Glut 2 – Glucose transporters type 2

Glut 4 – Glucose transporters type 4

HDL – High-density lipoprotein

HGP – Hepatic glucose production

HOMA - Homeostasis model assessment

HPLC - High-performance liquid chromatography

HSu – High-Sucrose

IL-6 – Interleukin 6

IRS – Insulin receptor substrates

ITT – Insulin tolerance test

K_{ITT} – Insulin tolerance constant

LDL – Low-density lipoprotein

MAPK – Mitogenic activated protein kinase

NO – Nitric Oxide

NOS – Nitric Oxide synthases

OGTT – Oral glucose tolerance test

PI3K – phosphatidylinositol

PKB/Akt – Protein kinase B

SAH – s-adenasyl-homocysteine

SH2 – *src*-homology 2

T2DM – Type 2 diabetes mellitus

TBS – Tris-buffered saline

TTBS – Tris-buffered saline with tween

1. Introduction

1.1 Type 2 Diabetes mellitus

Metabolic diseases, like type 2 diabetes mellitus (T2DM) and metabolic syndrome affects millions of individuals globally, contributing to significant morbidity and mortality worldwide. Diabetes affects over 8.3% of the world population and it is expected that in 2035 affects over than 592 millions of people worldwide (IDF, 2013). This escalate represents an alarming health problem with severe economic and social repercussions being imperative to better define the biological mechanisms causing metabolic diseases and to identify prevention strategies and treatment interventions that would help stem this epidemic.

From all types of diabetes the one that have been increasing worldwide is T2DM due to economic development, obesity, changes in diets and increased sedentary lifestyles. The criteria for the T2DM defined by the American Diabetes Association in 2014 is A1C ≥6.5% or fasting plasma glucose (FPG) ≥126 mg/dl or 2-h plasma glucose ≥200 mg/dl during an oral glucose tolerance test (OGTT) or in a patient with classic symptoms of hyperglycemia or hyperglycemic crisis, a random plasma glucose ≥200mg/dl (ADA, 2014).Insulin resistance and pancreatic β-cell dysfunction are the main abnormalities that play a central role in T2DM pathogenesis (figure 1.1). Insulin resistance can be defined as a state of reduced responsiveness by insulin target organs (the liver, the muscle and adipose tissue) to normal circulating levels of insulin. In fact, the imbalance between insulin secretion and action lead to hyperglycemia (Kahn, 2003; Wein and Florez, 2009).
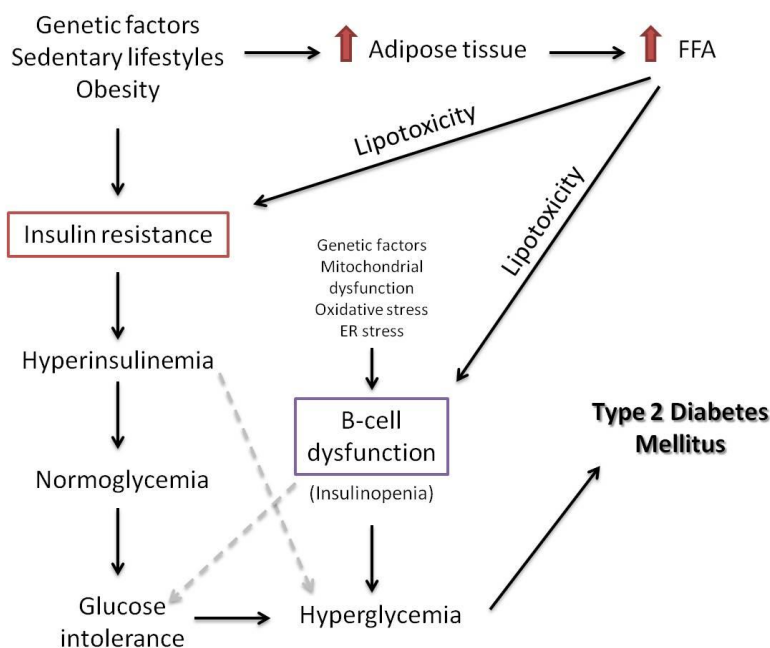
Figure 1.1 – Mechanism involved in Type 2 Diabetes Mellitus (T2DM) Pathogenesis. Insulin resistance and β-cell dysfunction are the main abnormalities responsible for hyperglycemia and subsequent T2DM development. Genetic factors, sedentary lifestyles and obesity are the main risk factors for the appearance of insulin resistance. When established, insulin resistance will lead to glucose intolerance. β-cell dysfunction lead to an impairment on insulin release (insulinopenia). Together (insulin resistance and β-cell failure) originate to hyperglycemia.

The response of the β-cell to a chronic hypercaloric ingestion and insulin resistance is a compensatory insulin secretion to maintain normoglycemia. Individuals that develop T2DM show an increase in insulin levels in the normoglycemic and prediabetes phases that maintain normal glycemia despite the insulin resistance due to β-cell compensation (DeFronzo, 2004) (figure 1.2). β-cell compensation involves the expansion of cell mass, enhanced insulin biosynthesis and increased nutrient-secretion coupling (Prentki and Nolan, 2006). Over time, β-cell compensation for insulin resistance starts to fail, resulting in a decline of β-cell function associated with a loss of β-cell mass, that progressively leads to late phase of T2DM (Leahy et al., 2005; Butler et al., 2003).

Several mechanisms have been proposed to be involved in the early dysfunction of β-cell including mitochondrial dysfunction, oxidative stress, endoplasmatic reticulum stress, dysfunctional triglyceride/free fatty acid (FFA) cycling and glucolipotoxicity (Ríos and Fuentes, 2009; Poitout and Robertson, 2002) (figure 1.1). Once hyperglycemia has been developed, other mechanisms as islet inflammation and O-linked glycosilation accelerate the damage, resulting in severe β-cell alterations and loss of β-cell mass by apoptosis (Ríos and Fuentes, 2009).
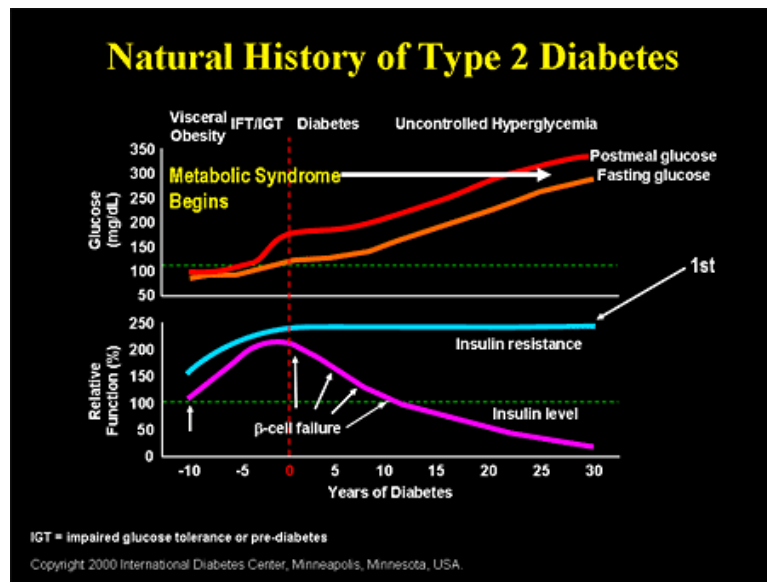
Figure 1.2 – Schematic representation of natural history of type 2 diabetes mellitus (T2DM). Relationship between glucose concentration (mg/dl) and the relative function (%) of insulin resistance and insulin level throughout the development of T2DM. In the beginning is observed an increase in insulin resistance which is compensated with an augment in insulin levels (increase in β-cell function), that maintain normal glucose levels. When the β-cell starts to fail, the insulin levels decreased and glucose (both fasting and postmeal) begins to rise leading to T2DM.

There are two main risk factors for T2DM development, one is based on genetic characteristics (age, gender, race, T2DM family history, genetic predisposition) and other based on lifestyle (obesity, physical inactivity, metabolic syndrome, pre-diabetes, diet) (Leu and Zonszein, 2010; Zimmet and Shaw, 2009) (figure 1.1).

The first therapeutic approach for T2DM is based on alterations in lifestyle, as encourage weight loss and physical activity with the objective of reducing glycemia and insulin resistance in order to delay drug therapeutic intervention (Ajala *et al.*, 2013; Morsink *et al.*, 2013; Zanuso *et al.*, 2010).

1.1.1. Insulin signalling pathways

Insulin is a polypeptide hormone secreted by β-cells of pancreatic islets of Langerhans, which maintains normal glucose blood levels by promoting glucose uptake by cells. Insulin secretion is induced mainly by glucose in healthy individuals, and the pancreatic response to glucose is biphasic: the first phase representing the release of insulin already synthesised and stored, and the second one representing both stored and newly synthesised insulin (Wilcox, 2005).

Insulin mediates its action through the binding to insulin receptor. Insulin receptor is a glycoprotein constituted by an extracellular α-subunit and an extracellular, transmembranar and intracellular β-subunit that express tyrosine kinase activity. When insulin binds to the insulin binding domain, in α-subunit, promotes the dimerization of the receptor to form the $\alpha_2\beta_2$ complex and induces the autophosphorylation of β-subunit activating the insulin receptor tyrosine kinase (Gou, 2014).

When activated, insulin receptor tyrosine kinase phosphorylates specific intracellular proteins including insulin receptor substrates (IRS) 1 and 2, in muscle and liver, respectively. IRS proteins, when phosphorylated, function as recognition sites for proteins containing *src*-homology 2 (SH2) domains like 85-kDa regulatory subunit of phosphatidylinositol (PI3K) activating the PI3K→Protein Kinase B (PKB/Akt) signalling cascade (see for a review see Gou, 2014). PI3K, through activation of Akt promotes glucose transport, glucose metabolism, glycogen, lipid and protein synthesis (figure 1.3) (Saltiel and Kahn, 2011; Wilcox, 2005). IRS phosphorylation also actives RAS→Mitogenic-Activated-Protein Kinase (MAPK) cascade which mediates the effect of insulin on mitogenesis and cell growth (figure 1.3) (Gou, 2014).

Insulin signalling pathways activation, namely PI3K→Akt pathway also promotes glucose transport thought a mechanism that involves translocation of glucose transporters to the plasma membrane (figure 1.3).
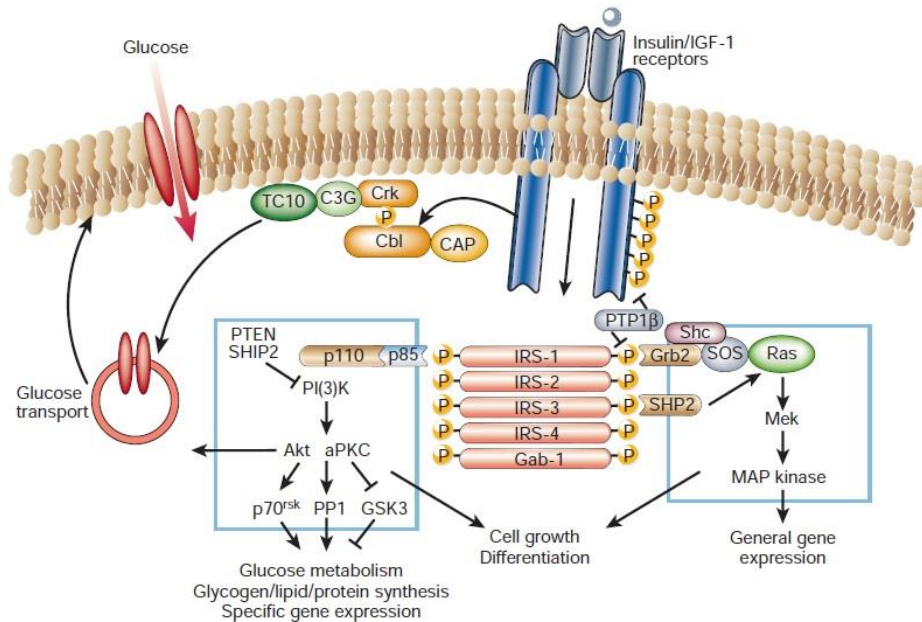
Figure 1.3 – Schematic representation of Insulin signalling pathways. Binding of insulin to its receptor leads to the phosphorylation of insulin receptor substract (IRS) originating the activation of several cascades like, phosphatidylinositol (PI3K), Protein Kinase B (Akt) Ras and Mitogenic-Activated-Protein Kinase (MAPK). Insulin action in cells is involved in general gene expression, cell growth differentiation, glucose metabolism, glycogen/lipid/protein synthesis, specific gene expression and glucose transport. (Saltiel and Khan, 2001).

1.1.2 Glucose homeostasis

Glucose is the obligatory energy substrate of the brain and because it cannot produce or storage glycogen it depends entirely of glucose in plasma (Owen *et al*., 1967). During basal or fasting state (12-16 h overnight fast) the majority of glucose is produced by the liver (gluconeogenesis and glycogenolysis) and only 15% is produced by gluconeogenesis in kidney. The brain is the major glucose consumer, using 50% of plasma glucose, followed by the liver and gastrointestinal tissues that use 25% and finally by insulin-dependent tissues like muscle (15-20%) and adipose tissue (2-4%) (DeFronzo, 2004; Gerich, 1993). Hypoglycaemia stimulates the release of glucagon and catecholamines which promotes glycogenolysis and prevents both insulin secretion and action, respectively (figure 1.4) (Muhammad and Gerich, 2010). After a meal the glucose plasma levels increased, triggering insulin release which stimulate glucose uptake by splandichnic (liver and gut) and peripheral (skeletal muscle and adipose tissue) tissues, (figure 1.4) and inhibit endogenous glucose production (DeFronzo and Ferrannini, 1987; DeFronzo, 2004; Marin *et al*., 1987).

Adapted from Wardlaw, et al, 2007.
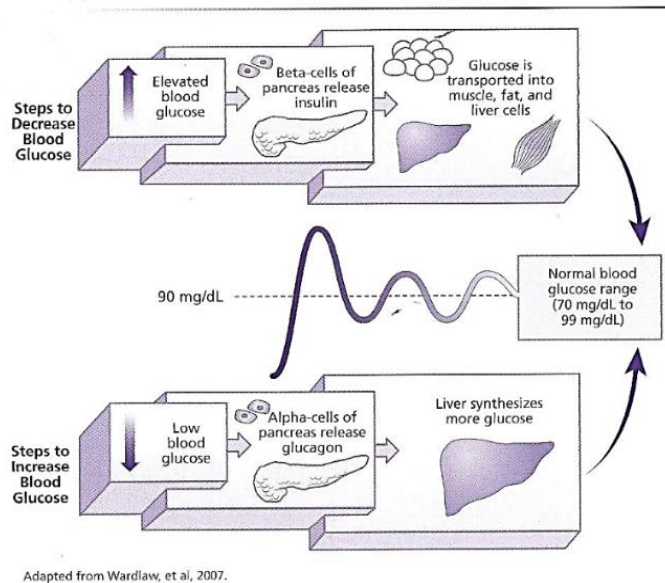
Figure 1.4 – Schematic representation of glucose homeostasis. Mechanisms that promote blood glucose increase/decrease. When blood glucose levels are elevated the pancreatic β-cells starts to produce and release insulin. The hyperinsulemia induce glucose uptake by peripheral tissues (skeletal muscle, adipose tissue and liver). On the other hand, hypoglycemia inhibits insulin release and promotes pancreatic glucagon release. Glucagon, which is the major counterpart of insulin, stimulates glucose production by the liver. (Adapted from Wardlaw and Hampl (2007).

Also glucagon secretion, which is the major counterpart of insulin in the regulation of plasma glucose, is inhibited by hyperinsulinemia leading to the suppression of hepatic glucose production (DeFronzo, 2004; Muhammad and Gerich, 2010). Skeletal muscle is the major site of glucose uptake in prostprandial state (DeFronzo and Tripathy, 2009) and glucose when captured by muscle can be store as glycogen or go to glycolysis where it can be totaly oxidated or release at glycolysis intermediates (lactate, alanine, pyruvate) (Kelley *et al.,* 1988).

Despite the major site of glucose uptake is the skeletal muscle, insulin plays an important role in adipose tissue by inhibiting lipolysis (Groop *et al.,* 1989). The inhibition of lipolysis prevents FFA release promoting glucose uptake by the skeletal muscle and inhibiting hepatic and renal gluconeogenesis. Hyperglycaemia also blocks glucagon release that will inhibit hepatic glycogenolysis (DeFronzo, 2004).

In mammals, most cells take up glucose from interstitial fluid by a passive, facilitative transport process, driven by the downward glucose concentration gradient across the plasma membrane Only in the epithelial cell brush border of the small intestine and the kidney proximal convoluted tubules glucose is absorbed or reabsorbed against its electrochemical gradient by a secondary active transport mechanism which uses the sodium concentration gradient established by Na+/K+/ATP pumps. (Bell *et al.,*1990). The passive, facilitative transport process is mediated by the family of facilitative glucose transporters (Glut)

(Bell *et al.,* 1990). Each glucose transporter isoform plays a specific role in glucose metabolism determined by its pattern of tissue expression, substrate specificity, transport kinetics, and regulated expression in different physiological conditions. There are 14 Glut proteins expressed in human (Thorens and Mueckler, 2010), but in the major peripheral insulin target tissues, skeletal muscle and adipose tissue, Glut 4 is the predominant transporter (DeFronzo, 2004). In these tissues, after exposure to insulin, Glut 4 concentrations on plasma membrane increased as the concentrations on intracellular pool decreased (DeFronzo, 2004). In liver and brain the predominant glucose transporters are Glut 2 and Glut 1, respectively.

1.1.3 Alterations in insulin action and glucose homeostasis in type 2 diabetes mellitus

T2DM is characterized by alterations in glucose homeostasis namely insulin resistance in liver, muscle and adipose tissue and impaired insulin secretion (DeFronzo, 2004).

In the fasting state, T2DM subjects shows an increased hepatic glucose production (HGP) due to enhanced gluconeogenesis (Magnusson *et al.,* 1992), which is higher with the severity of fasting hyperglycaemia (Jeng *et al.,* 1994). Also, these subjects exhibit increased insulin plasma levels when compared with normal subjects, and liver insulin resistance, as hyperglycemia and hyperinsulinemia are inhibitors of HGP (Campbell *et al.,* 1988; DeFronzo, 2004; Magnusson *et al.,* 1992). In fact many mechanisms have been shown to increased hepatic gluconeogenesis, like: hyperglucagonemia, enhanced sensitivity to glucagon, increased FFA oxidation and insulin resistance (DeFronzo, 2004).

Peripheral tissues, like adipose tissue and the liver and in particular skeletal muscle, are the most important sites of insulin resistance in T2DM (DeFronzo and Tripathy, 2009). Insulin resistance in skeletal muscle is characterized by a decreased glucose uptake as insulin induced-glucose uptake is blunted during an insulin clamp, and after 180min of hyperinsulinemia glucose disposal is reduced in 45% (DeFronzo *et al.,* 1985). In addition, it was shown in T2DM subjects that glycogen synthesis is impaired (Cline *et al.,* 1999; Shulman *et al.,* 1990) being this defect due to an diminished glucose transport into skeletal muscle cells (Cline *et al.,* 1999). In fact, it was previously described in subjects with insulin resistance and/or T2DM and in several animal species that the translocation of Glut4 glucose transporters to plasma membrane is reduced in skeletal muscle being this the possible cause of insulin resistance in this tissue (Garvey *et al.,* 1998). Also, since translocation of Glut4 transporters to plasma membrane is the end-step initiated by the binding of insulin to its receptor, is expected that insulin receptor expression to be decreased in this disease condition. In fact, several authors have described a decreased insulin action at receptor level, both in the expression of its receptor as well as its phosphorylation (DeFronzo, 2004;Saltiel and Khan, 2001). Also, defects in insulin signalling pathways have been observed, namely in PI3K

pathway, as IRS-1 and 2 tyrosine phosphorylation and subsequent PI3K association and activity are altered (DeFronzo, 2004; Fröjdö *et al.,* 2009). The genetic loss of IRS-1 and 2 leads to the inactivation of PI3K→Akt signalling pathway and this inactivation can elucidate the prevalence of insulin resistance and its association with T2DM (see review:Gou, 2014). Thereby, defects on PI3K→Akt signalling pathway, both in the liver and skeletal muscle, will lead to an increase in gluconeogenesis, defects on glucose metabolism and transport (figure 1.5) contributing to insulin resistance.

Free fatty acids (FFA) are another mediator that is known to be involved in the development of insulin resistance (Boden, 1997). FFA are stored as triglycerides in adipocytes, and during fasting conditions are used as an energy source (DeFronzo, 2004). Triglycerides are hydrolysed by adipose tissue lipases, and FFA released to the circulation. Insulin is an anti-lipolytic hormone and prevents FFA release through the inhibition of triglyceride hydrolyse. In patients with T2DM the concentrations of FFA in plasma are high, showing an impaired insulin action on lipolysis (Reaven *et al.,* 1988; Swislocki *et al.,* 1987). It is also known, that increased FFA concentrations inhibit glucose uptake by skeletal muscle, decreasing insulin secretion in the pancreas and promoting hepatic glucose production. In fact, in the postprandial state, lipolysis rate is increased despite the increased in insulin levels (DeFronzo, 2004; Eriksson *et al.,* 1999). It was also seen that exogenous insulin in T2DM subjects fail to reduce lipolysis failing in decrease FFA plasma levels (Groop *el tal.,* 1989).
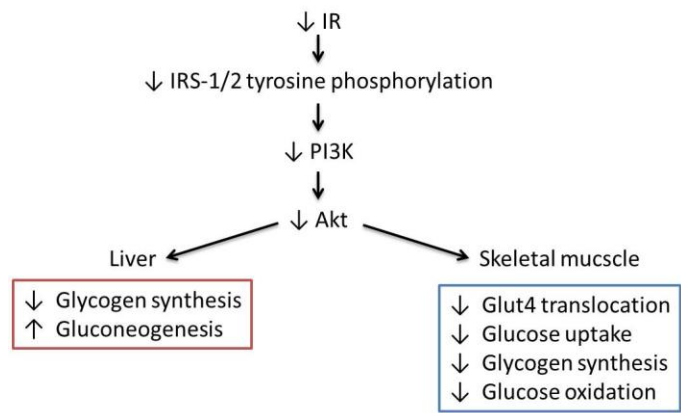
Figure 1.5 – Alterations in insulin signaling pathways that contribute to insulin resistance in the insulin-sensitive tissues, liver and skeletal muscle. Here is represented some of the molecules involved in insulin action as insulin receptor (IR), insulin receptor substract (IRS) proteins, the phosphatidylinositol (PI3K) and Akt.

1.2 Caffeine

Caffeine (1, 3, 7-trimethylxantine) is the most widely consumed psychoactive substance in the world. It is found in varying quantities in the beans, leaves, and fruits of more than 60 plants and is present in common beverages including coffee, tea, soft drinks and in products containing cocoa. Among different beverages, coffee has in general the highest value of caffeine concentration compared to tea, soft drinks and energy drinks (Heckman *et al,* 2010). Caffeine consumption from all sources can be estimated to be about 70-76 mg/person/day, yet it can be higher in some countries like USA, Canada, UK Sweden and Finland (Fredholm *et al.,* 1999). In these countries, except UK, coffee accounts for the majority of the daily caffeine consumption, where in UK tea is the beverage of choice (Heckman *et al.,* 2010). Coffee has in their composition some substances that can affect glucose metabolism as caffeine, chlorogenic acid and magnesium (van Dam and Hu, 2005). It was seen that magnesium has an inverse association with risk of type 2 diabetes both in man and women (Lopez-Ridaura *et al.,* 2004). Chlorogenic acid has an inhibitory effect on the hydrolysis of the glucose-6-phosphate, which could decrease glucose production (for a review see Higdon and Frei, 2006). Concerning caffeine effects, acute and chronic intake seems to have different consequences in glucose metabolism and in insulin sensitivity, which will be discussed below.

1.2.1 Metabolism and cellular mechanism of action

Once ingested, caffeine is absorbed in the gastrointestinal tract and is extensively metabolized in the liver (99%) (Fredholm *et al.,* 1999) in three different metabolites: theophyline (1,3-dimethylxanthine); paraxanthine (1,7-dimethylxanthine); and theobromine (3,7-dimethyxanthine). Theophyline has a more potent effect that caffeine as an inhibitor of adenosine receptors and paraxanthine is also at least as potent as caffeine (Fredholm *et al.,* 1999).

Caffeine, at a cellular level, can act through different mechanisms depending on its concentration in humans (Figure 1.6): 1) at higher concentrations, in the milimolar range, caffeine act through the mobilization of intracellular Ca^{2+} depots; 2) at high micromolar doses, caffeine inhibit phosphodiesterases thereby increasing cyclic adenosine monophosphate (cAMP) levels; and finally 3) at nanomolar concentrations, achieved after a single cup of coffee, caffeine is capable of blocking adenosine action by antagonism of its receptors (figure 1.6). The effect on adenosine receptors is the only mechanism significantly affected by the relevant doses of caffeine in humans. Therefore this mechanism shall be the only one to be considered and further discussed.
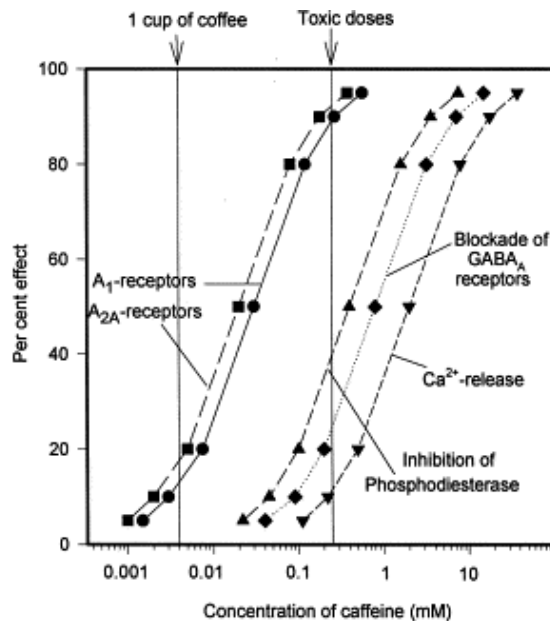
Figure 1.6 - Effect of caffeine on different biochemical targets in relation to its levels in humans (Fredholm *et al,* 1999).

1.2.2 Caffeine and adenosine

There are four known adenosine receptors subtypes named as A_1, A_{2A}, A_{2B} and A_3, which belong to the superfamily of G-protein-coupled receptors (Jacobson and Gao, 2006). Of the four subtypes, A_3 has been shown to be poorly affected by caffeine, as by other methylxanthines. The antagonist effect of caffeine is more potent at A_{2A} receptors, followed by A_1 and finally A_{2B} receptors (Table 1.1). During many years it was thought that adenosine, at physiological concentrations, only acted at A_1 and A_{2A} receptors and that A_{2B} (or A_3) was only activated under pathological concentrations of adenosine. Therefore, it was thought that the mechanism of action of caffeine was through the inhibition of the A_1 and A_{2A} receptors. However, nowadays it is known that the activation of the adenosine receptors not only depends of the adenosine levels but also on the density of the receptor subtypes existent in the site of action (Conde *et al.,* 2009).

Adenosine is a product of adenosine triphosphate (ATP) metabolism, being an ubiquitous substance in cells. It exerts its action through the binding to its four different types of adenosine receptors ($A_1 > A_{2A} > A_{2B} > A_3$). The four adenosine receptors have different affinity to endogenous adenosine, being activated by different amounts of endogenous adenosine (Conde *et al.,* 2009).

9

Table 1.1 – Potency of caffeine at rat and human adenosine receptors subtypes (Adapted from Fredholm *et al.,* 1999)

Receptor Subtype	Rat (K_D μM)	Human (K_D μM)
A_1	20	12
A_{2A}	8.1	2.4
A_{2B}	17	13
A_3	190	80

Adenosine regulates many physiological processes and the equilibrium between disposable of endogenous adenosine and the density of adenosine receptors at the site of action help to control the distinct physiological responses. The role of adenosine on glucose homeostasis and insulin sensitivity is not consensual. In adipose tissue adenosine has the ability to inhibit lipolysis thought the A_1 adenosine receptors, which results in enhanced insulin sensitivity (see review: Koupenova and Ravid, 2014). However, in skeletal muscle adenosine action on insulin sensitivity it's still controversial, as some authors have seem that the application of A_1 adenosine receptors antagonists enhanced muscle insulin sensitivity and reverses insulin resistance (Budohoski *et al.,* 1984; Challiss *et al.,* 1984) while others showed that A_1 adenosine receptors antagonists enhanced insulin resistance through the decreased of insulin-stimulated glucose uptake (Han *et al.,* 1998). In the liver, adenosine action through A_2 adenosine receptors enhanced gluconeogenesis and glycogenolysis (Buxton *et al.,* 1987; Bartrons *et al.,* 1989).

1.2.3 Caffeine, adenosine and Nitric Oxide

Nitric oxide (NO) is a physiological molecular messenger which mediates several basal functions and pathological states (Bredt and Snyder, 1994). NO is the major endogenous vasodilator, being currently used NO donors in clinical practice for the treatment of severe hypertension and congestive heart failure. NO is generated from L-arginine by constitutive NO synthases (eNOS and nNOS) and inducible NO synthase (iNOS) (Knowles and Moncada, 1994).

NO is another mediator that is known to be key in insulin sensitivity. Steinberg and his co-workers (1994) showed that insulin causes skeletal muscle vasodilatation through endothelium-derived NO, enhancing muscle-glucose uptake. Also they showed that inhibition of NO production by a NO synthetase inhibitor induces insulin resistance. In fact, it was seen that in T2DM patients the NO-mediated

vasodilatation is impaired (Williams *et al.,* 1996) as well as in obese man (Laakso *et al.,* 1989). Also the co-administration of glutathione and NO in rats enhanced insulin sensitivity (Guarino and Macedo, 2006).

It is well known that adenosine modulates NO release via A_1, A_{2A} and A_{2B} receptors (Olanrewaju and Mustafa, 2000; Ray and Marshall, 2006). Therefore, being caffeine, an adenosine antagonist, it is probably that caffeine administration alters NO synthesis and/or NO effects on insulin sensitivity. In fact, Bruce et al. (2002) showed that acute caffeine intake decreased exhaled NO. In this study the authors have measured exhaled NO during 4 hours after submit normal subjects to coffee (with an approximated caffeine content of 100mg per 200ml cup), decaffeinated coffee or decaffeinated coffee plus caffeine capsule (200mg) administration, and observe an absence of effects on exhaled NO only in decaffeinated coffee administration, meaning that caffeine reduces exhaled NO (Bruce et al. 2002). Also acute caffeine administration in rats reduces NOS expression in myocardial fibres (Corsetti *et al.,* 2008).

1.2.4 Acute vs chronic caffeine action on insulin sensitivity

Acute and chronic caffeine consumption has different actions in insulin sensitivity. During many years one of the lifestyle modifications, among subjects with metabolic and cardiovascular diseases, was the reduction of coffee consumption, based on studies that showed that caffeine when consumed acutely impairs insulin sensitivity (Keijzers *et al.,* 2002) and increased blood pressure (for a review see Rieksen *et al.,*(2009). However, recently several studies have shown a beneficial relationship between long-term coffee consumption and T2DM (Lopez-Garcia *et al.,* 2006; Van Dam *et al.,* 2002; Van Dam *et al.,* 2004; Van Dam *et al.,* 2006). Besides the discussion on the protective versus deleterious effects of coffee in T2DM, there is also controversy concerning the nature of the compound involved in it. Some authors claim that the beneficial effects of coffee are mediated by caffeine (Lopez Garcia *et al.,* 2006, Van Dam *et al.,* 2006, Zheng *et al.,* 2004) while others support that coffee components, apart from caffeine, are responsible for the protection against T2DM (Noordzij *et al.,* 2005, Greenberg *et al.,* 2005, Wu *et al.,* 2005).

Acute caffeine administration impaired insulin sensitivity in healthy subjects (Beaudoin *et al.,* 2013, Greer *et al.,* 2001; Keijzers *et al.,* 2002) and in T2DM patients (Lane *et al.,* 2004; Robinson *et al.,* 2004). In the Greer *et al* (2001) study, oral acute caffeine administration (5mg/kg body weight), after an overnight fast and 48h abstained from all methylxanthine-products containing in healthy subjects, result in decreased glucose disposal and carbohydrate storage. Also in this study the epinephrine levels were increase after oral caffeine administration. Confirming this study, Keijzers *et al* (2002) showed that after a caffeine administration (3mg/kg body weight), an increase in epinephrine levels, an increase in FFA levels and a decrease in insulin sensibility. More recently, in a study conducted by Beaudoin *et al.,* (2013) twenty-four healthy volunteers (12 males, 12 females) participated in 4 trials, in a crossover, randomized,

and double-blind fashion and it was showed that caffeine ingestion disrupted insulin sensitivity in a dose-dependent fashion beginning at very low doses (0-1 mg/kg body weight) in both healthy men and women.

Also, in T2DM and obese patients after acute caffeine administration (5mg/kg body weight) the FFA levels increased compared with placebo, however insulin and glucose levels were not significantly modified. Although the authors observed an increase in insulin and glucose levels after the oral glucose tolerance test (Robertson *et al.,* 2004). Likewise, Lane *et al* (2004) have showed an increase of glucose and insulin concentrations after an oral glucose tolerance test in T2DM patients preceded by an acute caffeine administration (375mg).

These effects of acute caffeine administration contrast with the long-term caffeine consumption, where beneficial actions have been reported. van Dam et al (2004) observed that in older and glucose intolerant subjects the higher caffeine consumption is associated to a lower incidence of glucose intolerance and a beneficial effect on insulin sensitivity. The same conclusions were taken in other cohort studies in healthy younger and middle-age women (van Dam *et al.,* 2006) and in healthy younger, middle-age women and men (Bhupathiraju *et al.,* 2014) where the risk of development T2DM is lower in the groups where the coffee consumption is higher. Although, coffee has many components able to modify insulin sensitivity, so it is necessary to evaluate the effects of caffeine itself. Recent studies of our group demonstrate that chronic caffeine intake (1g/L during 15 days) on *Wistar* rats prevent the development of insulin resistance induced by hypercaloric diets in pre-diabetes animal models, effect which appears to be mediated by a decrease in sympathetic nervous system activity (Conde *et al.*, 2012).

1.3 General and specific objectives

The main objective of this work is to investigate the therapeutic dose of caffeine that restores insulin sensitivity in a prediabetes animal model, hopping to found a therapy accessible, low-cost and without side effects therapy.

The specific aims of this work are:

1. Investigate if chronic administration of caffeine restores insulin sensitivity in a prediabetes animal model;

2. Investigate the time required for the reversion of insulin resistance;

3. Investigate possible mechanisms of action involved in the recovery of insulin sensitivity induced by caffeine.

2. Methods

2.1 Animals and experimental procedures

The experiments were performed in *Wistar* rats of both genders aged 8 -12 weeks old (250-450g), obtained from the animal house of the Faculdade de Ciências Médicas of Nova Medical School. The animals were maintained at control temperature and humidity (21 ± 1°C; 55 ± 10% humidity) with a 12h cycle of light and darkness.

Two groups of rats were used: a high-sucrose (HSu) group, which is a lean model of insulin resistance and hypertension (Conde *et al.,* 2012, Ribeiro *et al.,* 2013, Ribeiro *et al.,* 2005), and a control group. The HSu model was obtained by submitting the animals to 35% sucrose in drinking water during 28 days. The control group fed a standard chow (7.4% fat, 75% carbohydrate (4% sugar) and 17% protein; SDS diets RM1; Probiológica, Sintra, Portugal). Fasting glycemia, insulin sensitivity and glucose tolerance were assessed prior to submit the animals to the diets and after 28 days to confirm insulin resistance in the HSu animal model.

In order to investigate if chronic administration of caffeine restores insulin sensitivity in the HSu animal model, after 28 days caffeine was administered in drinking water to HSu and control groups. Three doses of caffeine were tested: 0.5g/L; 0.75 g/L and 1g/L. Rats without caffeine treatment were maintained in both control and HSu groups as controls. The animals were maintained under diets during 12 weeks after initiating chronic caffeine treatment. Insulin sensitivity and glucose tolerance were monitored each 2/3 weeks. Also, blood was collected from the tail vein after the oral glucose tolerance test (OGTT) in order to evaluate alterations in insulinemia and other mediators associated with insulin resistance.

Food and liquid intake in all groups of animals was monitored throughout the experimental protocol. Body weight and possible animal behavioural changes were monitored twice per week.

At the end of the experimental period and after glucose tolerance evaluation, rats were anaesthetized with pentobarbitone (60mg/Kg). Blood was collected by heart puncture and skeletal muscle and liver were collected for posterior analysis of mediators related with insulin sensitivity. Also visceral, perirenal and genital fat was weighted at the end of the experiment.

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.

2.2 Insulin Tolerance Test

The insulin tolerance test (ITT) was used to measure insulin sensitivity. The ITT is one of the earliest methods developed to assess insulin sensitivity *in vivo* and provides an estimate of overall insulin sensitivity, correlating well with the 'gold standard' hyperinsulinaemic–euglycaemic clamp (Monzillo and Hamdy, 2003).

ITT was performed always in conscious animals submitted to an overnight fasting of approximately twelve hours. The test consist in the measurement of basal glycaemia, followed by the administration of an insulin bolus (0.1 U/Kg) in the tail vein and the measure of the decline in plasma glucose concentration over 15 min at 1 min intervals. The constant rate for glucose disappearance (K_{ITT}) 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 (Conde *et al.,* 2012, Ribeiro *et al.,* 2013). Blood samples were collected by tail tipping and glucose levels were measured with a glucometer (Abbott Diabetes Care, Portugal) and test strips (Abbott Diabetes Care, Portugal).

2.3 Oral Glucose Tolerance Test

For the OGTT the animals were submitted to an overnight fasting of approximately twelve hours. The test consisted of an oral administration of glucose (2g/kg) by gavage and the measurement of plasma glucose at 0, 15, 30, 60, 120 and 180 minutes. The blood samples were collected by tail tipping and glucose levels were measured with a glucometer (Abbott Diabetes Care, Portugal) and test strips (Abbott Diabetes Care, Portugal). Glucose excursion curves (plasma glucose vs time) were draw with the data obtained and the Area Under the Curve (AUC) was compared.

2.4 Collection of tissues

After an abdominal laparotomy, the liver and skeletal muscle were collected and cryopreserved at -80°C to posterior analysis of NO production and the expression of glucose transporters.

2.5 Glut4 Protein quantification by Western Blot

Skeletal muscle samples (50mg) were homogenized in 300µL of Zurich buffer (Tris-HCL 10mM; EDTA 1mM; NaCL 150mM; Triton X-100 1%; Sodium cholate 1%; SDS 1%) with proteases inhibitors (trypsin, leupeptin, pepstatin, aprotinin, sodium orthovanadate, PMSF). Then the homogenate was centrifuged *(Eppendorf*, Madrid, Spain) at 13000g during 20 minutes, and the supernatant collect, and

frozen at -80°C until further use. The protein quantification of the homogenate was determined by using the colorimetric assay *Micro-BCA* (*Pierce,* Madrid, Spain).

Samples of the homogenate (50μg) and markers (*Precision Plus Protein Duo Color*, Biorad, Madrid, Spain) were separated by SDS-PAGE (10%) in reductive conditions.

After protein sample separation by a SDS-PAGE technique, the proteins were transferred to a polyvinylidene fluoride membrane (*Merck Millipore*, Darmstadt, Germany). Once the transfer was concluded the membrane was blocked with a solution of milk powder (5%) in a Tris-Buffered saline, ph 7,6 (TBS), with tween 20 (TTBS 0,1%) (*BioRad*, Madrid, Spain) during 1h at room temperature. The membranes were incubated overnight at 4ºC with the primary antibody Goat anti-GLUT4 (1:200) (*SantaCruz Biotecnhology*, Dallas, EUA) and then washed 3 times during 15 minutes with TTBS 0,1%. Next the membranes were incubated with the secondary antibody Donkey anti-Goat (1:5000)(*SantaCruz Biotecnhology*, Dallas, EUA) during 90 minutes at room temperature and then washed 3 times during 15 minutes with TTBS 0,1%. The intensity of the signals was detected in a ChemiDoc molecular imager (Bio-Rad Laboratories) and quantified using Quantity One software (Bio-Rad Laboratories, Hercules, CA). The membranes were then reprobed and tested for GAPDH immunoreactivity (bands in the 37-kDa region) to compare and normalize the expression of proteins with the amount of protein loaded. Briefly, the membranes were incubated for the primary antibody mouse anti-GAPDH (1:250) (*SantaCruz Biotecnhology*, Dallas, EUA) overnight at 4ºC. After washed 3 times during 15 minutes the membranes with TTBS 0,1% they were incubated during 90 minutes at room temperature with the secondary anti-body goat anti-mouse (1:2000) (*SantaCruz Biotecnhology*, Dallas, EUA). After washing the membranes they were revealed.

2.6 Liver and skeletal muscle Nitric Oxide (NO) quantification

Skeletal muscle and liver samples (160 mg) were homogenized in 500 μl of homogenization buffer (Tris-HCL 100mM; EDTA 50mM; EGTA 50mM). Then the homogenate was centrifuged (*Eppendorf,* Madrid, Sapin) at 13000g, during 20 minutes at 4°C, and the supernatant was collected and stored at -80°C.

For the deproteinization of the samples, the homogenate samples were diluted 3x in absolute ethanol at 0°C and mixed in vortex. After resting during 30 minutes on ice, the samples were centrifuged at 12000g during 15 minutes at 4°C. Finally the supernatant was collected and stored at -80 until needed for NO quantification.

NO/NO_3^- concentration was determined by using a selective and sensitive NO/ozone chemiluminescence technique (*NO-Analyzer 280*; Sievers Research Inc., Boulder, CO, USA).

2.7 Drugs and chemicals

Aprotinin, caffeine, sodium cholate, EDTA, leupeptin, NaCl, sodium orthovanadate, pesptatin, PMSF, tripsin, Tris, Trito X-100 were all obtained from *Sigma-Aldrich* (Madrid, Spain). The insulin was commercially available as *Humulin® Regular* (Lilly, Portugal) in a concentration of 1000UI/ml.

2.8 Data Analysis

Data were analyzed with Graph Pad Prism Software, version 5 (*GraphPad Software Inc.*, San Diego, CA, EUA) and represented as mean ± SEM. The significance between means difference was estimate through *One* e *Two-Way Analysis of Variance* (ANOVA) with *Bonferroni*, and *Dunnett* and *Bonferroni multicomparison test*, respectively. Differences was significant when p>0.05.

3. Results

3.1 Effect of chronic caffeine intake in caloric intake and rats weight

Caloric intake was monitored twice a week and the effect of diet administration and chronic caffeine intake is summarized in table 3.1. Caffeine intake in the control group was only tested in the concentration of 1g/L, and this concentration did not modify caloric intake in this group of animals (table 3.1). As expected, HSu diet increased significantly caloric intake by 35.8%. Caffeine in all concentrations tested, except for the 0.75g/l concentration, did not change caloric intake within HSu group (table 3.1).

Table 3.1 – Effect of chronic caffeine administration on caloric Intake in control and High-Sucrose (HSu) diet animals.

	Caffeine concentration (g/L)	Caloric Intake (Kcal/Kg/day)
Control	0	196.4 ± 7.63
	1	183.6 ± 8.78
HSu	0	305.7 ± 14.58***
	0.5	292.84 ± 24.02***
	0.75	236.7 ± 22.97**, #
	1	319.95 ± 3.52***

Caloric intake was monitored twice a week. Data represent means ± SEM. ** $p < 0.01$ in comparison with controls; *** $p < 0.001$ in comparison with controls; # $p < 0.05$ in comparison with High-Sucrose (HSu) diet without caffeine. (One Way ANOVA with dunnet's or Bonferroni multicomparison test)

In table 3.2 is represented the effect of caffeine intake in weight increase (g/day) and fat weight (total, visceral and perirenal).

It can be seen, the HSu diet and chronic caffeine intake (in control and HSu groups) did not affect weight gain, both in male and female animals. Although, HSu diet promoted a significant increase in total, visceral and perirenal fat by 40, 35 and 54%, respectively, in comparison to controls. Caffeine intake did not modify fat mass in control animals and in Hsu animals when administered in the concentrations of 0.75 and 0.5g/l However, when administered in a 1g/l concentration produced a non-significant reduction in total, visceral and perirenal by 20, 26 and 21% on, respectively. Nevertheless, this decreased was not significant.

Table 3.2 - Effect of chronic caffeine administration on weight increase and total, visceral and perirenal fat in control and high-Sucrose (HSu) diet animals.

	Caffeine concentration (g/l)	weight increase (g/day)		Fat weight (g)		
		Male	Female	Total	Visceral	Perirenal
Control	0	1.867 ± 0.20	0.543 ± 0.10	16.59 ± 0.08	3.920 ± 0.28	4.877 ± 0.10
	1	1.581 ± 0.20	0.730 ± 0.14	19.46 ± 2.30	4.330 ± 0.36	7.223 ± 1.22
HSu	0	1.952 ± 0.16	0.682 ± 0.13	27.64 ± 4.80*	6.030 ± 0.83*	10.63 ± 3.34*
	0.5	1.547 ± 0,26	0.717 ± 0.09	31.67 ± 2.44**	5.827 ± 0.66*	12.66 ± 1.57*
	0.75	1.822 ± 0.16		31.02 ± 4.39*	6.597 ± 1.22*	13.33 ± 1.63*
	1	1.721 ± 0.02	0.437 ± 0.10	23.24 ± 0.55	4.455 ± 0.20	8.427 ± 0.85

Body weight increased, calculated as total weight variation during the experimental period, in control and high-sucrose (HSu) animals with and without caffeine (0.5, 0.75 and 1 g/l). Total, visceral and perirenal fat, weighed postmortem and corrected to body weight in control and HSu rats with and without caffeine (0.5, 0.75 and 1 g/l). Data are present as means of 4-6 values. *$p < 0.05$, **$p < 0.01$, compared with control values (One-way ANOVA with Dunnett multicomparison tests)

3.2 Effect of chronic caffeine intake on fasting glycemia and insulin sensitivity

Fasting glycemia was monitored in the beginning of the ITT and in OGTT, and the effect of chronic caffeine administration is represented in figure 3.1. HSu diet induced an increase in fasting glycemia at the 4th week of diet to 102 ± 5.72 mg/dl (control = 84.88 ± 4.09 mg/dl). Also in the following weeks, the fasting glycemia remained elevated, although the values were not statistical significant (figure 3.1C). Caffeine intake in control group did not change fasting glycemia (figure 3.1B). In the HSu group chronic caffeine intake in all concentrations tested, progressively restored fasting glycemia, being significantly different, at the 13th week in the caffeine concentration of 0,5g/l and in 16th week for the remaining caffeine concentrations, when compared to HSu group at the 4th week of diet (figure 3.1D-F).
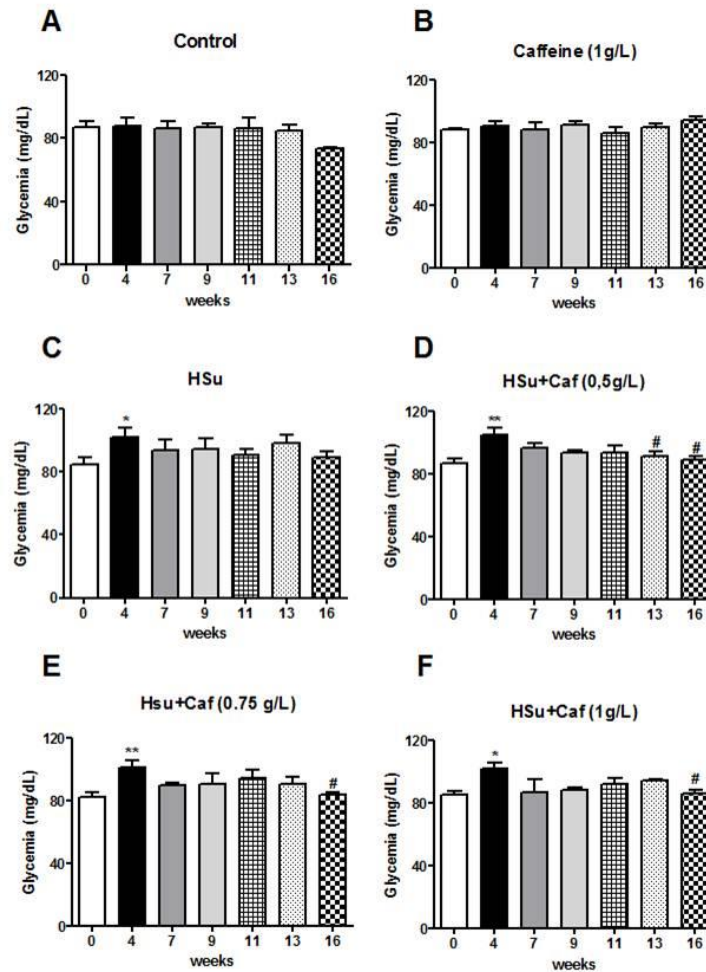
Figure 3.1 - Effect of chronic caffeine administration on fasting glycemia in control and high-sucrose (HSu) animals. Fasting glycemia was monitored in all insulin tolerance test (ITT) and oral glucose tolerance test (OGTT). Fasting glycemia in control (A), control with caffeine (1g/l) (B), Hsu (C), HSu with caffeine (0.5g/l) (D), HSu with caffeine (0.75g/l) (E) and HSu with caffeine (1g/l) (F) animals. Values are presented as the mean ± SEM. *p<0.05, **p<0.01, compared with control values (week 0); #p<0.05, compared with the values at the 4th week (One-way ANOVA with Dunnett multicomparison test).

The effect of chronic caffeine intake on insulin sensitivity in the control and HSu animals, with the different caffeine concentrations tested is represented in figure 3.2. In the HSu group, after 4 weeks of diet, the animals became insulin-resistant, since the constant of insulin tolerance test, KITT, decreased significantly to 2.355 %glucose/min (Kitt control = 4.7 ± 0.2 %glucose/min) (figure 3.2C). At the following weeks under diet the animals remained insulin resistant. In the control group, caffeine administration (1g/l) did not alter the insulin sensitivity (figure 3.2B). As we observe in figure 3.2D and E, the chronic caffeine intake in the concentrations of 0.5 and 0.75g/l progressively increases insulin sensitivity, in the HSu group, being completely restored at 9 and 5 weeks of caffeine administration, respectively. Also in the HSu

group, caffeine administration of 1g/l decrease insulin resistance faster than the other concentrations, being insulin sensitivity completely restored after 3 weeks of caffeine administration (Figure 3.2F).
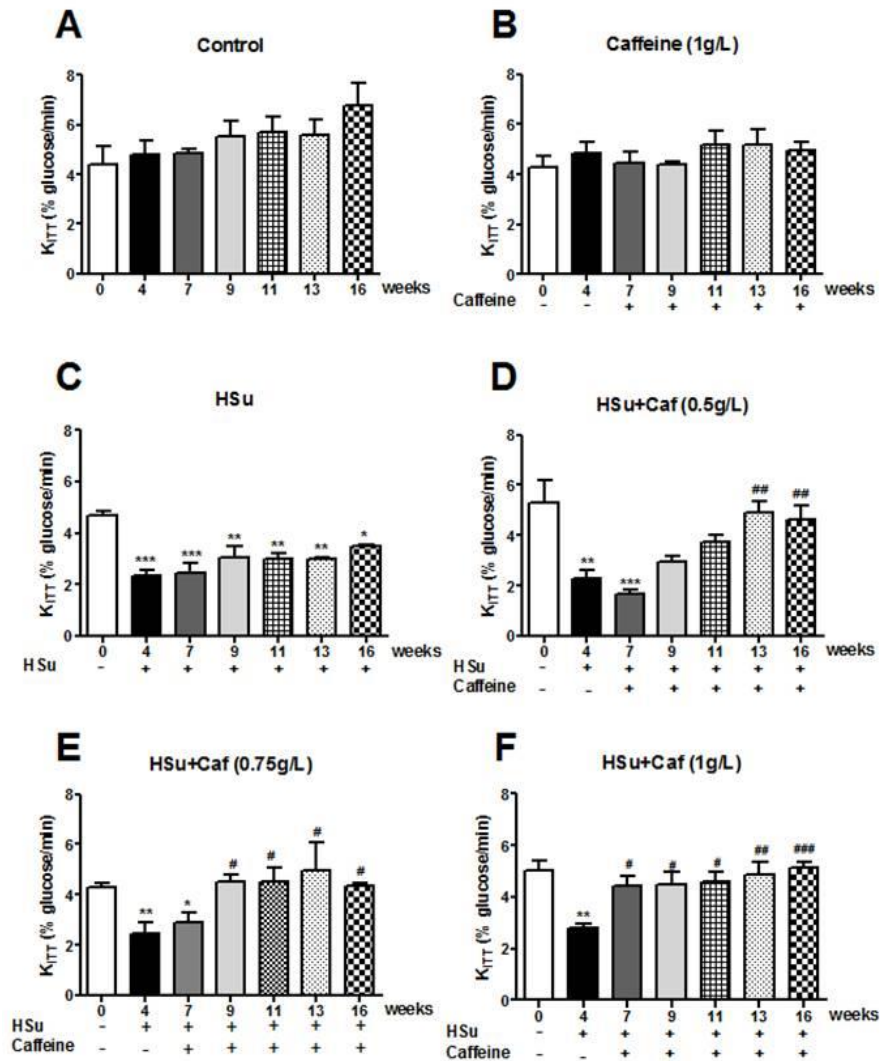
Figure 3.2 - Effect of chronic caffeine intake on insulin sensitivity determined by the insulin tolerance test and expressed as the constant rate for glucose disappearance (K_{ITT}) in control and high-sucrose (HSu) animals. Insulin sensitivity in control (A), caffeine (1 g/l) (B), HSu (C), HSu with caffeine (0.5 g/l) (D), HSu with caffeine (0.75 g/l) (E) and HSu with caffeine (1 g/l) (F) animals. Caffeine was administrated in the drinking water during 12 weeks together with control or Hsu diet after the animals became insulin resistant (after four weeks of HSu diet). Data are present as means of 4-6 values. *$p<0.05$, **$p<0.01$, ***$p<0.001$, compared with control values (week 0); #$p<0.05$, ##$p<0.01$, ###$p<0.001$, compared with the values at the 4th week (One-way ANOVA with Dunnett multicomparison test).

3.3 Effect of chronic caffeine intake on glucose tolerance

The effect of caffeine intake on glucose tolerance is summarized in figure 3.3. In figure 3.3A it can be seen that caffeine administration (1g/l), after 16 weeks, does not modify glucose tolerance. On the other hand, HSu diet progressively impaired glucose tolerance over the weeks, becoming significantly different at the 16th week, in comparison with control (figure 3.3B).
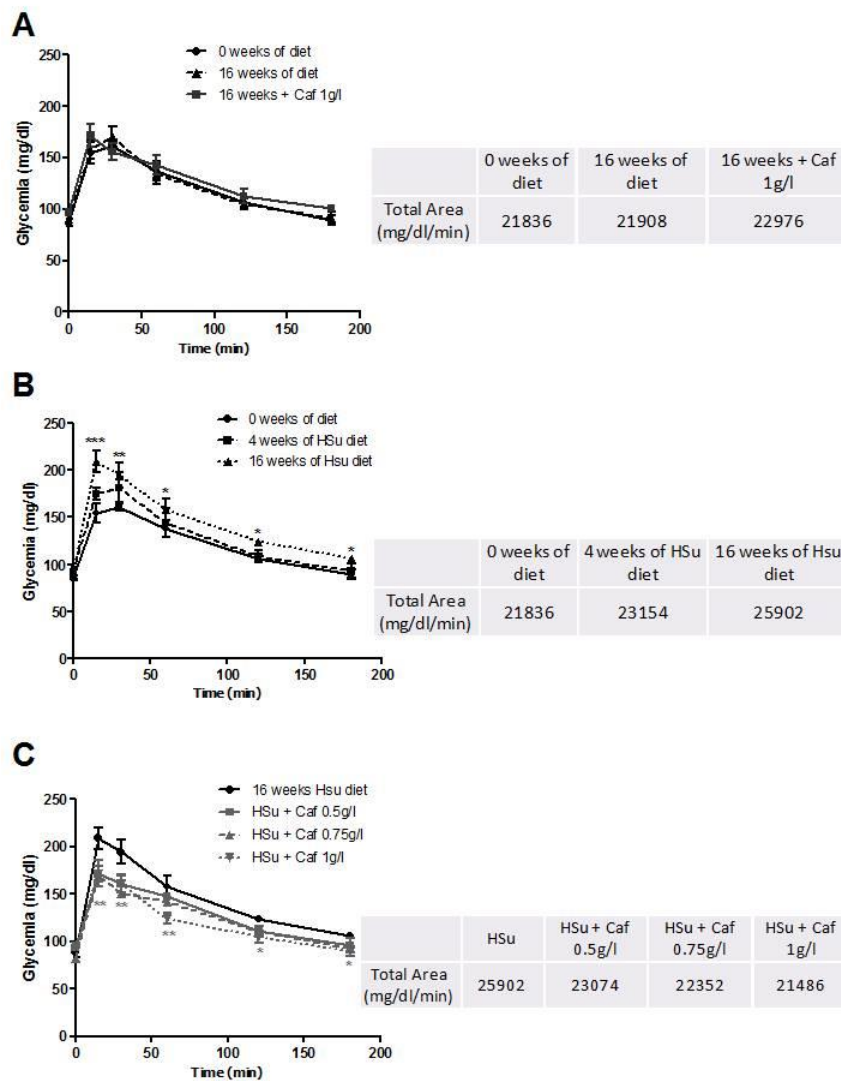
Figure 3.3 - Effect of chronic caffeine intake on glucose tolerance determined by the oral glucose tolerance test, in control and high-sucrose (HSu) animals. (A) Glucose tolerance in control (at 0 and 16 weeks of diet) and caffeine 1g/l (at 16 weeks of diet) animals. (B) Glucose tolerance in control (at 0 weeks) and HSu (at 4 and 16 weeks of diet) animals. (C) Glucose tolerance in HSu, HSu+Caf (0.5, 0.75 and 1g/l) animals (at 16 weeks of diet). Caffeine was administrated in the drinking water during 12 weeks together with control or Hsu diet after the animals became insulin resistant (after four weeks of HSu diet). Data are present as means of 4-6 values. *p<0.05, **p<0.01, ***p<0.001, compared with control values (One-way ANOVA with Dunnett multicomparison test).

Caffeine administration in the HSu group ameliorates glucose tolerance in comparison with the HSu group without caffeine intake (figure 3.3C). This effect of caffeine on glucose tolerance is more significant as higher is the concentration of caffeine administered, as we can see from the values of the AUC showed in the table and the glucose excursion curves presented in figure 3.3C.

3.4 Effect of chronic caffeine intake on Glut4 skeletal muscle protein expression

Glut4 transports are the main responsible for glucose uptake in skeletal muscle and therefore we have investigated if the reversion in insulin sensitivity and glucose tolerance can be associated with a recovery of Glut4 expression in this insulin-sensitive tissue. Chronic caffeine intake (1g/l) did not alter the Glut4 expression levels in skeletal muscle (figure 3.4). However, as expected, HSu diet decreased significantly Glut4 transporters expression by 59.2% (figure 3.4). Chronic caffeine intake in HSu group in a concentration of 0.5 g/l partially restored Glut4 expression, increasing its levels by 24.3%, whereas the concentration of 1g/l completely restored Glut4 expression levels (figure 3.4).
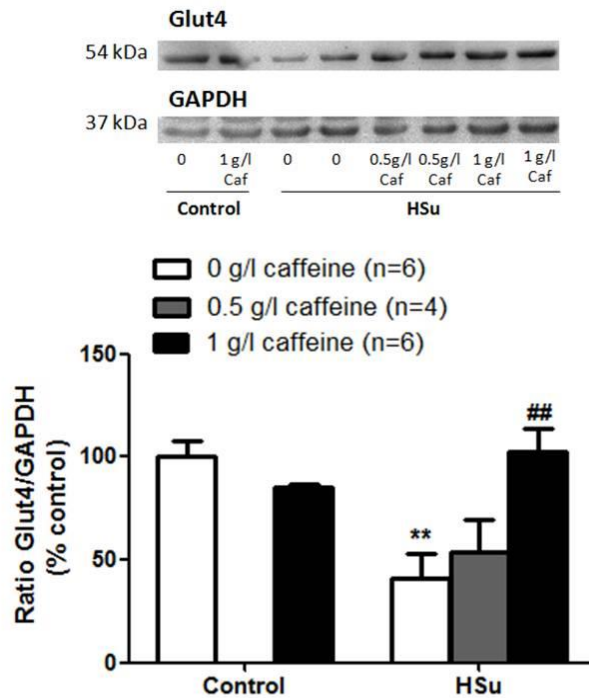
Figure 3.4 – Effect of chronic caffeine intake on Glut4 transporters expression in skeletal muscle in control and high-sucrose (HSu) animals. One-Way ANOVA with Dunnett multicomparison test, **p<0.01, compared with control; Two-Way ANOVA with Bonferroni multicomparison test, ##p<0.01 comparing values with and without caffeine.

3.5 Effect of chronic caffeine intake on NO production in skeletal muscle and liver

NO is known to be a key mediator in insulin sensitivity and therefore we have evaluated its content in the liver and in skeletal muscle. Figure 3.5A represents the effect of chronic caffeine administration on NO content in liver. Caffeine administration did not altered liver NO content in control group and, HSu diet as well as the combination of HSu plus caffeine in the concentrations of 0.5g/l and 1g/l did not change NO levels in the liver. Surprisingly, a chronic caffeine administration in a dose 0.75g/l in Hsu rats produced a significant increase in NO to 70.4 ± 5,7nmol/g tissue (control= 45.5 ± 1.8nmol/g tissue).
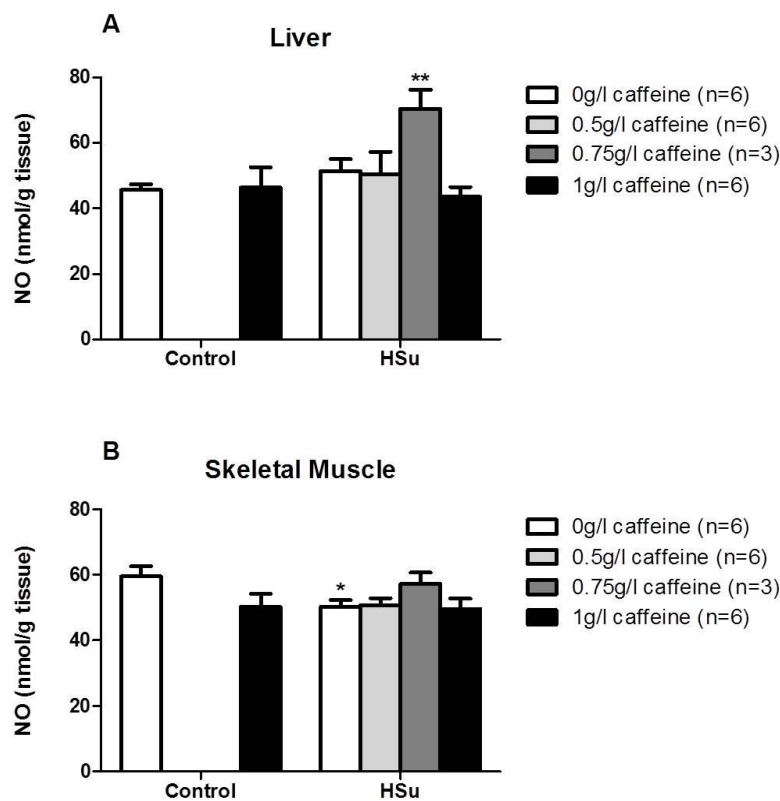
Figure 3.5 – Effect of chronic caffeine intake in nitric oxide (NO) content in the liver (A) and in skeletal muscle (B) in control and high-sucrose (HSu) animals. One-Way and Two-Way ANOVA with Dunnett and Bonferroni's multicomparison test, *p<0.05; **p<0.01, compared with control.

The effect of chronic caffeine intake on NO in skeletal muscle in both control and HSu groups is summarized in figure 3.5B. In control group caffeine administration produced a non-significant decrease of NO of 15.5%. HSu diet decreased significantly NO production in skeletal muscle by 16% when compared to controls (control = 59.5 ± 3.07nmol/g tissue). Chronic caffeine administration, in all concentrations tested, did not modify NO content in skeletal muscle in HSu group.

4. Discussion

The present work demonstrates, for the first time, that chronic caffeine administration restores insulin sensitivity, glucose tolerance and fasting hyperglycemia in prediabetic rats (HSu). In addition, we have shown that the latency time needed to restore insulin sensitivity and glucose tolerance is inversely correlated with caffeine concentration. Caloric intake, as well as weight increase per day and total fat mass, both in controls and in the prediabetes model, was not affected by caffeine consumption. Also, caffeine intake did not alter skeletal muscle Glut4 expression levels in control animals, but restored its levels in the HSu group when administered at concentration of 1g/l. Finally, we have shown that skeletal muscle NO levels were decreased in HSu animals and that caffeine did not modify these levels. All together, these results suggest that caffeine reversion of insulin resistance and glucose intolerance did not involve alterations in appetite, weight loss or in NO levels but can be due to a restore of insulin signaling pathways in insulin sensitive tissues, like skeletal muscle, and point forward to the use of caffeine for the treatment of prediabetes and prevention of T2DM.

At the onset of the discussion we want to state that the reasons that lead to the use of these concentrations of caffeine is the correspondence to the human coffee consumption. Caffeine concentration of 1g/l corresponds to a caffeine dose of 3/4 coffees per day, 0.75 g/L to 2/3 coffees per day and 0.5 g/L to 1/2 coffees per day (Fredholm *et al.,* 1999). Also, it should be noted that in these doses the only mechanism significantly affected by caffeine is the antagonism of adenosine receptors (Fredholm *et al.,* 1999).

HSu diet increase fasting glycemia and decrease insulin sensitivity after 4 weeks (28 days) of diet which remains throughout the 16 weeks in control animals. This increase in fasting glycemia and the decrease in insulin resistance are consistent with the previous studies from our laboratory indicating reproducibility in the animal models (Conde *et al.,* 2012; Ribeiro *et al.,* 2013), although these parameters were only analyzed up to four weeks in those studies. Also, the results obtained in our laboratory are in agreement with the findings obtained by others in animals and in humans. In a study taken by Ribeiro *et al.,* 2005 it was seen that liquid HSu diet (35%) intake during 6 weeks also decrease insulin sensitivity in *Wistar* and *Sprague-Dawley* rats. In humans it was shown that diets rich in sugar (glucose/fructose) decrease insulin sensitivity approximately in 25% (Beck-Nielsen *et al.,* 1980). In the present study, the increase in glycemia was only significant at the 4th week of HSu diet, and insulin resistance tends to diminish through time. Nevertheless, is important to highlight that these insulin sensitivity and fasting glycemia measurements were performed in conscious animals and therefore animal stress became a variable that must be taken into account. It is known that acute stress activates sympathetic nervous system and due to the close connection between sympathetic activation and insulin resistance, stress can alter metabolic parameters (Muhammad and Gerich, 2010; Lambert *et al.,* 2010). Chronic caffeine

administration in control animals did not alter fasting glycemia or insulin sensitivity, although when applied to HSu animals it restored completely fasting glycemia, being the effect significant for all the concentrations of caffeine tested at 12 weeks of diet. The beneficial/prejudicial effects of chronic coffee intake in the risk of T2DM in human were already commented. Recently a study performed in *Sprague-Dawley* rats showed that coffee administration during 12 weeks improves insulin sensitivity and glucose tolerance in 30% liquid HSu fed rats (Morakinyo *et al.,* 2013). On the other hand it was showed that decaffeinated coffee had beneficial effects on all body insulin sensitivity than decaffeinated plus alkaloid caffeine (20g/l) in high-fat fed *Sprague-Dawley* rats, indicating that caffeine antagonizes decaffeinated coffee action (Shearer *et al.,* 2007). However, these studies evaluated caffeinated/ decaffeinated coffee intake, and being coffee a mixed of many compounds, direct effect of caffeine was not evaluated. In the present study we have tested the direct effect of caffeine and our results are in concordance with recent studies where it was showed that chronic caffeine intake (caffeine 0.5 g/kg of food) during 8 weeks restores and ameliorates metabolic syndrome in diet-induced obese *Wistar* rats (Panchal *et al.,* 2012) and that chronic caffeine intake (15mg/kg/day) during 4 weeks improved insulin sensitivity in *Wistar-Kyoto* rats (Yeh *et al.,* 2014). In the present work, chronic caffeine consumption restored completely insulin sensitivity in a concentration dependent-manner, being the latency time necessary to restore this parameter inversely correlated with caffeine dose, suggesting that a low caffeine dose requires more time to induce substantial changes in order to recover the mechanisms. Recently, it was seen that acute caffeine ingestion impaired insulin sensitivity in a dose-dependent manner in humans (Beaudoin *et al.,* 2013). This caffeine induced-insulin resistance in humans is in agreement with the findings of our laboratory in rats, in where we have found that caffeine decreases in a dose-dependent manner insulin sensitivity (Sacramento *et al.,* 2014). The dose-dependent effect of chronic caffeine on insulin sensitivity herein described as well as the described for acute consumption (Beaudoin *et al.,* 2013; Sacramento *et al.,* 2014) suggest an effect mediated by adenosine receptors. In fact, Sacramento *et al.* (2014) have shown that insulin resistance induced by acute caffeine is mediated by A_1 and A_{2B} adenosine receptors.

As expected, we have shown that glucose tolerance gradually decreased with HSu diet consumption, showing a tendency to diminish at 4 weeks of diet and being significantly decreased at 16 weeks of diet. Some controversy exists regarding the effects of HSu diets in glucose tolerance. While some authors described that chronic HSu diets intake improves glucose tolerance in *Wistar* rats (Kergoat *et al.,* 1987) and in human (Anderson *et al.,* 1973), more recent studies described a reduction of glucose tolerance with this kind of diets (Kirino *et al.,* 2009; Sakamoto *et al.,* 2012), being these data in agreement with the present study. However, we must note that in some of these studies the authors have used solid HSu diets as well as different percentages of sucrose. Also, differences in animal species can also contribute for the distinct results, for example, in the Sakamoto *et al.* (2012) the study was performed in mice.

In the present study chronic caffeine intake in control animals did not alter glucose tolerance. Similar results were also observed by Úrzua *et al.*, (2012) and by Morakinyo *et al.,* (2013), since they didn't saw any effect of chronic coffee consumption on glucose tolerance in healthy rats. Also, Morakinyo *et al.,* (2013) shown that coffee administration to HSu rats improved glucose tolerance in these animals. Although, these authors have tested coffee and no caffeine as in the present study. In fact, herein we have observed a complete restore of glucose tolerance with the highest concentration of caffeine tested (1g/l). In a study taken by Ohnaka *et al.,* 2012 chronic caffeinated/decaffeinated coffee intake (5 cups/day), which corresponds to a caffeine dose similar to 1g/l, was tested in glucose metabolism during 16 weeks in overweight, middle-aged men with a mild-to-moderate elevation of fasting glucose. The authors have shown that glucose tolerance, assess through an OGTT, was not altered by caffeinated coffee at 8 weeks, yet after 16 weeks the 2-hour glucose and AUC glucose during OGTT were decreased (Ohnaka *et al.,* 2012). Also, in the same study the authors did not observe differences in fasting plasma glycemia with caffeinated chronic intake both in 8th and 16th week (Ohnaka *et al.,* 2012). In another previous study Wedick *et al.* (2011) demonstrated that chronic coffee intake (5 cups/day) during 8 weeks was also unable to alter glucose tolerance, glycemia and insulin sensitivity in overweight men and women. All together, these results suggest that for the amount of caffeine/coffee administered (1g/l, 4 to 5 cups of coffee per day) is necessary a longer-term intake (at least 12 weeks as in the present study) to see the caffeine beneficial effects on glucose tolerance.

The beneficial effect of coffee on glucose homeostasis described in several epidemiological studies have been attributed to weight loss due to an increased thermogenesis, lipolysis and fat oxidation induced by the drug (Cheung *et al.,* 1988, Choi *et al.,* 2011; van Dam *et al.,* 2006, Lopez-Garcia *et al.,* 2006, Zheng *et al.,* 2004). Nevertheless, this hypothesis stills debatable since it has also been observed that long-term coffee consumption does not cause significant weight reduction (Astrup *et al.,* 1992). One of the mechanisms that could contribute to weight loss are alterations in food intake, however the effects of caffeine/coffee administration in appetite and food/caloric intake are not consensual. Acute caffeine administration seems to reduce food intake both in rats (Racotta *et al.,* 1994) and in men, but not in women (Tremblay *et al.,* 1988). Also, acute coffee intake did not modify appetite in normal subjects (Gavrieli *et al.,* 2011) but reduced food intake in overweight/obese subjects (Gavrieli *et al.,* 2013). In contrast, in a study conducted by Pettenuzzo *et al* (2008), where it was evaluated the chronic administration of caffeine on feeding behavior of rats, it was reported that caffeine do not affected standard chow diet intake, although diminished food intake in relation to palatable food. The basis of these controversial may rely on the differences between acute and chronic caffeine administration, since the majority of the effects of caffeine on food intake was observed as a result of acute caffeine intake. Also we cannot forget that coffee is a mixture of many compounds, and perhaps when the effect of coffee is tested, the observed effect on food intake is not due to caffeine. Herein we have observed that caffeine did not alter caloric intake either in control group or in the HSu-treated group, except for the dose of 0.75

g/l tested in HSu animals, suggesting that is not the reduction in food intake that contribute to the beneficial effects of chronic caffeine intake in glucose homeostasis. At the moment we do not have a consistent explanation for the reduction of food intake observed for the dose of 0.75g/l, although since the number of animals tested in this group was lower (n = 3) than in the other groups (n = 6) we should increase the number of animals to verify this results before trying to achieve a plausible explanation.

The HSu model is described as being a lean model of insulin resistance (Conde *et al.,* 2012, Ribeiro *et al.,* 2005, Morakinyo *et al.,* 2013) therefore, and as expected, although caloric intake was increased in the HSu animals, the weight gain per day was not affected. Although after 16 weeks under HSu diet we have observed an increase in total, visceral and perirenal fat in HSu animals. In a study previously published by our group, it was found that HSu diet did not alter weight gain (g/day) yet, however no alterations were found in the percentage of visceral fat (Conde *et al.,* 2012). This absence of effect on visceral fat can be due to the fact that the HSu model tested in that study was submitted to 4 weeks of diet and not for 16 weeks as in the present one. Chronic caffeine intake did not alter weight gain and adipose tissue mass both in controls and HSu group, with the exception of 1g/l concentration that slightly decrease total fat, visceral and perirenal fat in HSu group, although these decreases were not statistical significant in relation to HSu group without caffeine. The absence of effects of caffeine on weight gain and in the adipose tissue mass in controls and HSu rats are in accordance with the previous results of our group (Conde *et al.,* 2012) and by others (Morakinyo *et al.,* 2013; Urzúa *et al.,* 2012) and suggest that is not the reduction on adipose mass or weight gain that is on the basis of caffeine effects on insulin sensitivity and glucose. Although it seems that at high doses of caffeine, the reduction of adipose tissue mass can be one of the mechanisms contributing to insulin sensitivity and glucose tolerance. In fact, recently it was seen that *in vitro* chronic caffeine decreases the expression of adipogenesis-related genes in a dose-dependent manner, inhibiting adipogenic differentiation in cell lines of rat adipose-derived stem cells (ADSCs) and a mouse bone marrow stromal cell line (M2-10B4) (Su *et al.,* 2013). Also Aoyagi *et al.,* (2014) showed that coffee decreased lipid accumulation in the adipose differentiation of mouse line cells (3T3-L1) in a dose-dependent manner and that reduction was correlated with a decrease in adipocyte marker genes. However, they do not show which coffee compounds are responsible for decreasing lipid accumulation. Therefore, we can suggest that one of the mechanisms of caffeine to restore insulin sensitivity and glucose tolerance can be the reduction of visceral fat by inhibiting adipocyte differentiation and that this happened at higher concentrations of caffeine.

Caffeine is a thermogenic agent, and therefore the consumption of caffeinated bevarages/caffeine is often associated to a body weight decrease due to increased thermogenesis. In fact some epidemiological studies show that an increase in caffeine intake was associated with a greater weight loss and relatively higher thermogenesis and fat oxidation in obese and postobese subjects (Lopez-Garcia *et al.,* 2006, Westerterp-Plantenga *et al.,* 2013). This correlation between caffeine-thermogenic effect and weight loss was also described in mice, as Zeng *et al.,* (2004) showed that chronic caffeine intake

reduced body weight through thermogenesis and fat oxidation. However the animal model is different from the used in the present study, meaning that caffeine can act through different mechanism of action. Although we haven't assess thermogenesis in the present study, we did not observe any caffeine-induced weight loss. Therefore we can exclude a thermogenic effect of caffeine as the responsible for the restore of insulin sensitivity and glucose tolerance.

Herein we have shown that animals submitted during 16 weeks of HSu diet present a decreased Glut4 expression in skeletal muscle, suggesting that an impairment of insulin signaling pathways in this organ contributes to insulin resistance. 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 (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. Chronic caffeine intake did not alter Glut 4 levels in control animals but increased in a concentration-dependent manner Glut4 expression in the skeletal muscle of HSu animals, suggesting that this is an important mechanism that contributes to insulin sensitivity and explain the return of glycemia to control levels. The effect of chronic caffeine intake on Glut4 expression described in the present thesis is in agreement with the partial reversal of the Glut4 expression induced by caffeine in the skeletal muscle of aged rats (Guarino *et al.,* 2013). Although, the reversion of Glut4 expression in HSu animals showed herein contrasts to what we have found in the study taken by Guarino *et al.* (2014) where chronic caffeine intake (1g/l) did not altered Glut4 expression in HSu group. Nevertheless, in this study caffeine was administered only for 15 days with the aim of evaluating if caffeine can prevent the development of diet-induced insulin resistance (Guarino *et al.,* 2014). All together these results suggest that is necessary more time of caffeine administration to see its effects on Glut4 expression levels. Egawa *et al.,* (2009, 2011a) have shown that acute caffeine did not alter Glut4 levels in skeletal muscle but impairs insulin signal transduction by inhibiting IRS-1 tyrosine phosphorylation and PI3K phosphorylation (Egawa *et al.* 2011b). However these studies evaluated acute caffeine effects and were performed with caffeine concentrations in the milimolar range that will affect other molecular targets than adenosine receptors (Fredholm *et al.* 1999), which may explain the difference compared to our results.

Herein, we have showed that NO levels in liver are not affected by HSu diet and by chronic caffeine administration. However, in the HSu group treated with 0.75g/l of caffeine it was observed an unexpected increase in NO concentration levels. At this moment we do not have any plausible explanation for this effect, however and at it was mentioned earlier in this discussion the HSu group treated with 0.75g/l possess a low number of animals (n = 3) than the others (n = 6), and this can affect the results.

NO content in the skeletal muscle was decreased after 16 weeks of HSu diet. This result is in concordance with others that saw impaired vascular responses to insulin, with decreased levels of eNOS

in skeletal muscle, in high-fat-sucrose rats (Bourgoin *et al.,* 2008) and in hypertensive HSu feeding rats (Mélançon *et al.*, 2006). Santuré *et al.* (2002) also showed that insulin-mediated skeletal muscle vasodilation, that is NO-dependent, was impaired in sucrose-fed rats. Also, the same authors have described a reduction in eNOS protein content in muscle, which could be responsible for this impairment (Santúre *et al.,* 2002). In the present study, chronic caffeine administration did not change skeletal muscle NO levels either in controls or in HSu animals, meaning that caffeine action on insulin sensitivity is not mediated by NO.

The insulin-sensitizing effect of chronic caffeine treatment may also be directly related to antagonism of adenosine receptors on insulin target tissues. The effect of adenosine on glucose homeostasis is currently a hot-topic area with controversial results: while positive effects of A_1 receptor partial agonists are well documented on adipose tissue glucose uptake (Dhala *et al.,* 2009), studies using adenosine receptor antagonists have shown increases (Espinal *et al.,* 1983), decreases (Han *et al.,* 1998) and no effect (Vergauwen *et al.,* 1994) of these drugs on skeletal muscle glucose uptake. More recently, Faulhaber-Walter *et al.* (2011) have demonstrated that A_1 receptor deficient mice have decreased glucose tolerance with pronounced IR, which suggests that these receptors contribute significantly to glucose homeostasis and insulin sensitivity. Also, A_{2B} receptor antagonists have been used to increase plasma insulin levels *in vivo* (Rusing *et al.,* 2006) and more recently to reduce insulin resistance (Figler *et al.,* 2011). More recently, a study from our lab tried to solve some of the controversy and Sacramento *el al.* (2014), as referred earlier in the discussion, showed that acute caffeine induces whole body-insulin resistance an effect that was mediated by A_1 and A_{2B} adenosine receptors, meaning that adenosine is an insulin-sensitizer substance. Also, it was shown in the same study that activation of A_1 adenosine receptors by an A_1 agonist increases glucose uptake skeletal muscle in a magnitude similar to the obtained with insulin, were A_{2B} agonist did not produced any effect on glucose uptake. Therefore, since adenosine is a sensitizer substance and since acute caffeine produces insulin resistance it is possible that the acute caffeine effect on insulin sensitivity will be lost with the chronic caffeine consumption due to a desensitization or an up-regulation of adenosine receptors thereby recovering adenosine action on insulin sensitivity. In the present work we did not evaluate adenosine receptor expression in insulin-sensitive tissues, although assessment of possible alterations in adenosine receptor expression as well as adenosine concentrations will be our next steps to understand the cellular mechanism of chronic caffeine effects.

5. Conclusion

We can conclude that chronic caffeine intake restores insulin sensitivity and glucose tolerance in an animal model of prediabetes in a concentration-dependent manner and that the latency time needed to restore these parameters is inversely correlated with caffeine concentration. Also, we have shown that the caffeine concentration that achieved the best results in metabolic performance was the higher concentration tested (1g/l). Moreover, we have demonstrated that the mechanism involves a restore of Glut4 expression in skeletal muscle and not alterations in the NO levels or weight gain. All together these results suggest that caffeine can be used therapeutically for the treatment of prediabetes and prevention of T2DM.

6. Future work

The work presented in this Master thesis resulted from experimental work perfomed in the last year in order to obtain the degree of master in Genética Molecular e Biomedicina, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa. This year went to fast and in fact is not so much time to learn new techniques and to achieve results, therefore many questions related with the present thesis still unsolved and new ideas have come up. Our idea is to send the present work to be published in a peer review scientic journal and so, some experiments must be performed namely, the quantification of caffeine and its metabolites concentrations in the plasma rats by High-performance liquid chromatography (HPLC). In addition, we must continue to investigate the cellular mechanisms behind chronic caffeine action on insulin sensibility and glucose tolerance. In particular the effects of chronic caffeine in the expression of adenosine receptors both in skeletal muscle and in must be performed as well as effect of this xanthine in theexpression of insulin receptor and AMP-activated protein kinase (AMPK) activity in the same insulin-sensitive tissues.

In the present work we have demonstrated that chronic caffeine intake was capable of restore insulin sensitivity and glucose tolerance in a prediabetes animal model, however we did not know if the same will happens with a type 2 diabetes model, therefore as a future work we want to elucidate if caffeine is capable of restore these same metabolic parameters in a animal model of type 2 diabetes, which is obtained by a combined model of high sucrose plus high fat diet.

Finally, we want to translate this data to the clinic and for that we prompt us to start a clinical trial to evaluate the benefits of chronic caffeine intake in volunteer subjects with prediabetes, hypertension, metabolic syndrome and type 2 diabetes, also to assess the therapeutic dose of caffeine to be administered that restores insulin sensitivity, glucose tolerance and that normalizes blood pressure and finally to study the time frame necessary to restore insulin sensitivity, glucose tolerance and that normalizes blood pressure in these patients.

With this, we hope to find that chronic caffeine in Humans also restore some deregulated pathological features that are present in metabolic and cardiovascular diseases as it happens in animals and to establish the caffeine dose responsible for it. Positive results will allow us to develop cheap and low adverse reactions treatment for the most common diseases of the century.

7. References

Ajala O, English P and Pinkney J (2013). Systematic review and meta-analysis of different dietary approaches to the management of type 2 diabetes. Am J Clin Nutr, 97: 505-516

American Diabetes Association (2014). Diagnosis and Classification of Diabetes Mellitus. Diabetes Care, 36(1): S67-S74

Anderson JW, Herman RH and Zakiin D (1973). Effect of high glucose and high sucrose diets on glucose tolerance of normal men. Am J Clin Nutr, 26: 600-607

Aoyagi R, Funakoshi-Tago M, Fujiwara Y and Tamura H (2014). Coffee inhibits adipocyte differentiantion via inactivation of PPARγ. Biol Pharm Bull

Astrup A, Breum L, Toubro S, Hein P and Quaade F (1992). The effect and safety of an ephedrine/caffeine compound compared to ephedrine, caffeine and placebo in obese subjects on an energy restricted diet. A double blind trial. Int J Obes Relat Metab Disord, 16(4): 269-77

Astrup A1 Toubro S, Cannon S, Hein P, Breum L and Madsen J (1990). Caffeine: a double-blind, placebo-controlled study of its thermogenic, metabolic, and cardiovascular effects in healthy volunteers. Am J Clin Nutr., 51(5): 759-67

Bartrons R, Schaftingen EV and Hers HG. (1984) The ability of adenosine to decrease the concentration of fructose 2,6-bisphosphate in isolated hepatocytes. A cyclic AMP-mediated effect. Biochem J, 218(1): 157-163

Beaudoin MS, Allen B, Mazzetti G, Sullivan PJ and Graham TE (2013). Caffeine ingestion impairs insulin sensitivity in a dose-dependent manner in both men and women. Appl Physiol Nutr Metab, 38(2): 140-147

Beck-Nielsen H, Pedersen O and Lindskov HO (1980). Impaired cellular insulin binding and insulin sensitivity induced by high-fructose feeding in normal subjects. Am J ClinNutr, 33: 273-278

Beel GI, Kayano T, Buse JB, Burant CF, Takeda J, Lin D, Fukumoto H and Seino S (1990). Molecular Biology of Mammalian Glucose Transporters. Diabetes Care, 13(3): 198-208

Bhupathiraju SN, Pan A, Manson JE, Willett WC, van Dam RM and Hu FB (2014). Changes in coffee intake and subsequent risk of type 2 diabetes: three large cohorts of US men and women. Diabetologia, 57(7): 1346-1354

Boden G (1997). Role of Fatty Acids in the Pathogenesis of Insulin Resistance and NIDDM. Diabetes, 46: 3-10

Bourgoin F, Bachelard H, Badeau M, Mélançon S, Pitre M, Larivière R and Nadeau A (2008). Endothelial and vascular dysfunctions and insulin resistance in rats fed a high-fat, high-sucrose diet. Am J Physiol Heart Circ Physiol, 295(3): H1044-H1055

Bredt DS and Snyder SH (1994). Nitric Oxide: A Physiologic Messenger Molecule. Annu Rev Biochem, 63: 175-95

Bruce C, Yates DH and Thomas PS (2002). Caffeine decreases exhaled nitric oxide. Thorax, 57:361-363

Budohoski L, Challiss RA, Cooney GJ, McManus B and Newsholme EA. (1984) Reversal of dietary-induced insulin resistance in muscle of the rat by adenosine deaminase and an adenosine-receptor antagonist. Biochem J, 224(1): 327-330

Butler AE, Janson J, Bonner-Weir S, Ritzel R, Rizza RA and Butler PC (2003). β-Cell Deficit and Increased β-Cell Apoptosis in Human With Type 2 Diabetes. Diabetes, 52: 102-110

Buxton DB, Fisher RA, Robertson SM and Olson MS. (1987) Stimulation of glycogenolysis and vasoconstriction by adenosine and adenosine analogues in the perfused rat liver. Biochem J, 248(1): 35-41

Campbell PJ, Mandarino LJ and Gerich JE (1988). Quantification of the Relative Impairment in Action of Insulin on Hepatic glucose Production and Peripheral Glucose Uptake in Non-Insulin-Dependent Diabetes Mellitus. Metabolism, 37(1): 15-21

Challiss RA, Budohoski L, McManus B and Newsholme EA. (1984) Effects of an adenosine-receptor antagonist on insulin-resistance in soleus muscle from obese Zucker rats. Biochem J, 221(3): 915-917

Choi EY, Park SY and Cho YO (2011). Freeze-dried instant coffee can promote the activities of antioxidant enzymes and induce weight loss but also aggravate the plasma cholesterol profile in rats. Nutrition, 27: 1202-1205

Cline GW, Petersen KF, Krssak M, Shen J, Hundal RS, Trejanoski Z, Inzucchi S, Dresner A, Rothman DL and Sulman GI (1999). Impaired Glucose Transport as a Cause of Decreased Insulin-stimulated Muscle Glycogen Synthesis in Type 2 Diabetes. N Engl J Med, 341(4): 240-246

Conde SV, Silva TN, Gonzalez C, Carmo MM, Monteiro EC and Guarino MP. 2012. Chronic caffeine intake decreases circulating catecholamines and prevents diet-induced insulin resistance and hypertension in rats. Br J Nutr, 107: 86-95.

Corsetti G, Pasini E, Assanelli D and Bianchi R (2008). Effects of acute caffeine administration on NOS and Bax/Bcl2 expression in the myocardium of rat. Pharmacological Research, 57: 19-25

DeFronzo RA (2004). Pathogenesis of type 2 diabetes mellitus. Med Clin North Am, 88:787-835.

DeFronzo RA and Ferrannini E (1987). Regulation of Hepatic Glucose Metabolism in Humans. Diabetes/Metabolism Reviews, 3(2): 415-459

DeFronzo RA and Tripathy D (2009). Skeletal Muscle Insulin Resistance Is the Primary Defect in Type 2 Diabetes. Diabetes Care, 32(2): 157-163

DeFronzo RA, Gunnarsson R, Björkman O, Olsson M and Wahren J (1985). Effects of Insulin on Pheripheral and Splanchnic Glucose Metabolism In Noninsulin-dependent (Type II) Diabetes Mellitus. J Clin Invest, 76: 149-155

Dhalla AK, Chisholm JW, Reaven GM and Belardinelli L (2009). A1 adenosine receptor: role in diabetes and obesity. Handb Exp Pharmacol, 193: 271-295

Egawa T, Hamada T, Kameda N, Karaike K, Ma X, Masuda S, Iwanaka N and Hayashi T (2009). Caffeine acutely activates 5'adenosine monophosphate-activated protein kinase and increases insulin-independent glucose transport in rat skeletal muscles. Metabolism, 58(11): 1609-1617

Egawa T, Hamada T, Ma X, Karaike K, Kameda N, Masuda S, Iwanaka N and Hayashi T (2011a). Caffeine activates preferentially α1-isoform of 5'AMP-activated protein kinase in rat skeletal muscle. Acta Physiol (Oxf), 201(2): 227-238

Egawa T1, Tsuda S, Ma X, Hamada T and Hayashi T (2011b). Caffeine modulates phosphorylation of insulin receptor substrate-1 and impairs insulin signal transduction in rat skeletal muscle. J Appl Physiol., 111(6): 1629-1636

Eriksson JW, Smith U, Waagstein F, Wysocki M and Jansson P-A (1999). Glucose Turnover and Adipose Tissue Lipolysis Are Insulin-Resistant in Healthy Relatives of Type 2 Diabetes Patients - Is Cellular Insulin Resistance a Secondary Phenomenon?. Diabetes, 48: 1572-1578

Espinal J, Challiss RA and Newsholme EA (1983). Effect of adenosine deaminase and adenosine analogue on insulin sensitivity in soleus muscle of the rat. FEBS Lett, 158: 103-106

Faulhaber-Walter R, Jou W, Mizel D, Li L, Zhang J, Kim SM, Huang Y, Chen M, Briggs JP, Gavrilova O and Schnermann JB (2011). Impaired glucose tolerance in the absence of adenosine A1 receptor signaling. Diabetes, 60(10): 2578-2587

Figler RA, Wang G, Srinivasan S, Jung DY, Zhang Z, Pankow JS, Ravid K, Fredholm B, Hedrick CC, Rich SS, Kim JK, LaNoue KF and Linden J (2011). Links between insulin resistance, adenosine A2B receptors, and inflammatory markers in mice and humans. Diabetes, 60: 669-679

Fredholm BB, Bättig K, Holmén J, Nehlig A and Zvartau EE (1999). Actions of Caffeine in the Brain with Special Reference to Factors That Contribute to Its Widespread Use. Pharmacological Reviews, 51(1): 83-133

Fröjdö S, Vidal H and Pirola L (2009). Alterations of insulin signaling in type 2 diabetes: A review of the current evidence from humans. Biochim Biophys Acta, 1792: 83-92

Garvey WT, Maianu L, Zhu J-H, Brechtel-Hook G, Wallace P and Baron AD (1998). Evidence for Defects in the Trafficking and Translocation of Glut4 Glose Transporters in Skeletal Muscle as a Cause of Human Insulin Resistance. J Clin Invest, 101(11): 2377-2386

Gastaldelli A (2011). Role of beta-cell dysfunction, ectopic fat accumulation and insulin resistance in the pathogenesis of type 2 diabetes mellitus. Diabetes Res Clin Pract, 93S: S60-S65

Gavrieli A, Karfopoulou E, Kardatou E, Spyreli E, Fragopoulou E, Mantzoros CS and Yannakoulia M (2013). Effect of different amounts of coffee on dietary intake and appetite of normal-weight and overweight/obese individuals. Obesity (Silver Spring), 21(6): 1127-1132

Gavrieli A, Yannakoulia M, Fragopoulou E, Margaritopoulos D, Chamberland JP, Kaisari P, Kavouras SA and Mantzoros CS (2011). Caffeinated coffee does not acutely affect energy intake, appetite, or inflammation but prevents serum cortisol concentrations from falling in healthy men. J Nutr, 141(4): 703-707

Gerich JE (1993). Control of glycaemia. Baillieres Best Pract Res Clin Endocrinol Metab, 7(3): 551-586

Gou S (2014). Insulin signaling, resistance, and metabolic syndrome: insights from mouse models into disease mechanisms. J Endocrinol, 220: T1-T23

Greenberg JA, Axen KV, Schnoll R, et al. (2005) Coffee, tea and diabetes: the role of weight loss and caffeine. Int J Obes (Lond), 29: 1121-1129

Greer F, Hudson R, Ross R and Graham T (2001). Caffeine Ingestion Decreases Glucose Disposal During a Hyperinsulinemic-Euglycemic Clamp in Sedentary Humans. Diabetes, 50: 2349-2354

Groop LC, Bonadonna RC, DelPrato S, Ratheiser K, Zych K, Ferrannini E and DeFronzo RA (1989). Glucose and Free Fatty Acid Metabolism in Non-Insulin-Dependent Diabetes Mellitus. The American Society for clinical Investigation, 84: 205-213

Guarina MP and Macedo MP (2006). Co-administration of glutathione and nitric oxide enhances insulin sensitivity in Wistar rats. Br J Pharmacol, 147: 959–965

Guarino MP, Ribeiro MJ, Sacramento JF and Conde SV (2013). Chronic caffeine intake reverses age-induced insulin resistance in the rat: effect on skeletal muscle Glut4 transporters and AMPK activity. Age, 35: 1755-1765

Guarino MP, Sacaramento JF, Ribeiro MJ and Conde SV (2014). Caffeine, Insulin Resistance, and Hypertension. In *Coffee in Health and Disease Prevention*. Elsevier Inc.

Han DH, Hansen PA, Nolte LA and Holloszy JO. (1998) Removal of adenosine decreases the responsiveness of muscle glucose transport to insulin and contractions. Diabetes, 47(11): 1671-1675

Heckman MA, Weil J and de Meija EG (2010). Caffeine (1, 3, 7-trimethylxanthine) in Foods: A Comprehensive Review on Consumption, Functionality, Safety, and Regulatory Matters. J Food Sci, 75(3): 77-87

Higdon JV and Frei B (2006). Coffee and Health: A Review of Recent Human Research. Crit Rev Food Sci Nutr, 46:101-123

Hunter SJ and Garvey WT (1998). Insulin Action and Insulin Resistance: Diseases Involving Defects in Insulin Receptors, Signal Transduction, and the Glucose Transport Effector System. Am J Med, 105: 331-345

International Diabetes Federation. IDF Diabetes Atlas 6th ed. IDF. Brussels, 2013

Jacobson KA and Gao Z-G (2006). Adenosine receptors as therapeutic targets. Nat Rev Drug Discov., 5(3): 247-264

Jeng C-Y, Sheu WH-H, Fuh MM-T, Chen I and Reaven GM (1994). Relationship Between Hepatic Glucose Production and Fasting Plasma Glucose Concentration in Patients With NIDDM. Diabetes, 43: 1440-1444

Kahn SE (2003). The relative contributions of insulin resistance and beta-cell dysfunction to the pathophysiology of Type 2 diabetes. Diabetologia, 43: 3-19.

Keijzers GB, De Galan BE, Tack CJ and Smits P (2002). Caffeine Can Decrease Insulin Sensitivity in Humans. Diabetes Care, 25: 364-369

Kelley D, Mitrakou A, Marsh H, Schwenk F, Benn J, Sonnenberg G, Arcangell M, Aoki T, Soresen J, Berger M, Sonksen P and Gerich J (1988). Skeletal Muscle Glycolysis, Oxidation, and Storage of an Oral Glucose Load. J Clin Invest, 81: 1563-1571

Kergoat M, Bailbé D and Portha B (1987). Effect of high sucrose diet on insulin secretion and insulin action: a study in the normal rat. Diabetologia,30: 252-258

Kim JY, Nolte LA, Hansen PA, Han DH, Kawanaka K and Holloszy JO (1999). Insulin resistance of muscle glucose transport in male and female rats fed a high-sucrose diet. Am J Physiol, 276(3 Pt 2): R665-R672

Kirino Y, Kamimoto T, Sato Y, Kawazoe K, Minakuchi K and Nakahori Y (2009). Increased Plasma Dipeptidyl Peptidase IV (DPP IV) Activity and Decreased DPP IV Activity of Visceral But Not Subcutaneous Adipose Tissue in Impaired Glucose Tolerance Rats Induced by High-Fat or High-Sucrose Diet. Biol Pharm Bull, 32(3):463-467

Knowles RG and Moncada S (1994). Nitric oxide synthases in mammals. Biochem. J., 298: 249-258

Koupenova M and Ravid K (2013). Adenosine, Adenosine Receptors and Their Role in Glucose Homeostasis and Lipid Metabolism. J Cell Physiol

Laakso M, Edelman SV, Brechtel G and Baron AD (1990). Decreased Effect of Insulin To Stimulate Skeletal Muscle Blood Flow in Obese Man: A Novel Mechanism for Insulin Resistance. J Clin Invest, 85: 1844-1852

Lambert GW, Straznicky NE, Lambert EA, Dixon JB and Schlaich MP (2010). Sympathetic nervous activation in obesity and the metabolic syndrome - causes, consequences and therapeutic implications. Pharmacol. Ther, 126: 159-172

Lane JD, Barkauskas CE, Surwit RS and Feinglos MN (2004). Caffeine Impairs Glucose Metabolism in Type 2 Diabetes. Diabetes Care, 27(8): 2047-2048

Leahy JL (2005). Pathogenesis of Type 2 Diabetes Mellitus. Arch Med Res, 36: 197-209

Leu JP and Zonszein J. Diagnostic Criteria and Classification of Diabetes. In Poretsky L. *Principles of Diabetes Mellitus*. 2nd ed., USA, Springer, 2010, p.107-115

Lopez-Garcia E, van Dam RM, Qi L and Hu FB (2006). Coffee consumption and markers of inflammation and endothelial dysfunction in healthy and diabetic women. Am J Clin Nutr, 84: 888-93

Lopez-Garcia E, van Dam RM, Willett WC, Rimm EB, Manson JE, Stampfer MJ, Rexrode KM and Hu FB (2006). Coffee consumption and coronary heart disease in men and women: a prospective cohort study. Circulation, 113(17): 2045-2053

Lopez-Ridaura R, Willett WC, Rimm EB, Liu S, Stampfer MJ, Manson JE and Hu FB (2004). Magnesium Intake and Risk of Type 2 Diabetes in Men and Women. Diabetes Care, 27:134-140

Magnusson I, Rothman DL, Katz LD, Shulman RG and Shulman GI (1992). Increased Rate of Gluconeogenesis in Type II Diabetes Mellitus. The American Society for Clinical Investigation, 90: 1323-1327

Mårin P, Rebuffé-Scrive M, Smith U an Björntorp P (1987). Glucose Uptake in Human Adipose Tissue. Metabolism, 36(12): 1154-1160.

Mélançon S, Bachelard H, Badeau M, Bourgoin F, Pitre M, Larivière R and Nadeau A (2006). Effects of high-sucrose feeding on insulin resistance and hemodynamic responses to insulin in spontaneously hypertensive rats. Am J Physiol Heart Circ Physiol, 290(6): H2571-H2581

Monzillo LU and Hamdy O (2003). Evaluation of Insulin Sensitivity in Clinical Practice and in Research Settings. Nutrition Reviews, 61(12): 397-412

Morakinyo AO, Adekunbi DA, Dada KA and Adegoke AO (2013). Coffee Consumption Attenuates Insulin Resistance and Glucose Intolerance in Rats fed on High-Sucrose Diet. Niger J Physiol Sci, 28: 179-185

Morsink LM, Smits MM and Diamant M (2013). Advances in Pharmacologic Therapies for Type 2 Diabetes. Curr Atheroscler Rep, 15(302): 1-14

Muhammad ZS and Gerich JE. Normal Glucose Homeostasis. In Poretsky L. *Principles of Diabetes Mellitus.* 2nd ed., USA, Springer, 2010, p.19-35.

Ohnaka K, Ikeda M, Maki T, Okada T, Shimazoe T, Adachi M, Nomura M, Takayanagi R and Kono S (2012). Effects of 16-Week Consumption of Caffeinated and Decaffeinated Instant Coffee on Glucose Metabolism in a Randomized Controlled Trial. J Nutr and Metab, 2012:207426

Olanrewaju HA and Mustafa SJ (2000). Adenosine A2A and A2B receptors mediated nitric oxide production in coronary artery endothelial cells. General Pharmacology, 35: 171-177

Owen OE, Morgan AP, Kemp HG, Sullivan JM, Herrera MG and Cahil GF (1967). Brain Metabolism during Fasting. J Clin Invest, 46 (10): 1589-1595.

Panchal SK, Wong W-Y, Kauter K, Ward LC and Brown L (2012). Caffeine attenuates metabolic syndrome in diet-induced obese rats. Nutrition, 28: 1055-1062

Pettenuzzo LF, Noschang C, von Pozzer Toigo E, Fachin A, Vendite D and Dalmaz C (2008). Effects of chronic administration of caffeine and stress on feeding behavior of rats. Physiol Behav, 95(3): 295-301

Poitout V and Robertson P (2002). Minireview: Secondary β-Cell Failure in Type 2 Diabetes – A Convergence of Glucotoxicity and Lipotoxicity. Endocrinology, 143(2):339-342

Prentki M and Nolan CJ (2006). Islet b cell failure in type 2 diabetes. J Clin Invest, 116(7): 1802-1812

Racotta IS, Leblanc J and Richard D (1994). The effect of caffeine on food intake in rats: involvement of corticotropin-releasing factor and the sympatho-adrenal system. Pharmacol Biochem Behav, 48(4): 887-92

Ray CJ and Marshall JM (2006). The cellular mechanisms by which adenosine evokes release of nitric oxide from rat aortic endothelium. J Physiol, 570(1): 85-96

Reaven GM, Hollenbeck C, Jeng C-Y, Wu MS and Chen Y-DI (1988). Free Fatty Acid, Lactate, and Insulin for 24h in Patients With NIDDM. Diabetes, 37: 1020-1024

Ribeiro MJ, Sacramento JF, Gonzalez C, Guarino MP, Monteiro EC and Conde SV (2013). Carotid body denervation prevents the development of insulin resistance and hypertension induced by hypercaloric diets. Diabetes, 62: 2905-2916

Ribeiro RT, Lautt WW, Legare DJ and Macedo MP (2005). Insulin resistance induced by sucrose feeding in rats is due to an impairment of the hepatic parasympathetic nerves. Diabetologia, 48(5): 976-983

Riksen NP, Rongen GA and Smits P (2009) Acute and long-term cardiovascular effects of coffee: implications for coronary heart disease. Pharmacol Ther, 121: 185-191

Robinson LE, Savani S, Battram DS, McLaren DH, SathasivamP and Graham TE (2004). Caffeine Ingestion Before an Oral Glucose Tolerance Test Impairs Blood Glucose Management in Men with Type 2 Diabetes. J Nutr, 134(10): 2528-2533

Rusing D, Muller CE and Verspohl EJ (2006). The impact of adenosine and A(2B) receptors on glucose homoeostasis. J Pharm Pharmacol, 58: 1639-1645

Sacramento JF, Ribeiro MJ; Yubero S, Melo BF, Obeso A, Guarino MP, Gonzalez C and Conde SV (2014). Disclosing caffeine action on insulin sensitivity: effects on rat skeletal muscle. Eur J Pharm Sci (pree-submited)

Sakamoto E, Seino Y, Fukami A, Mizutani N, Tsunekawa S, Ishikawa K, Ogata H, Uenishi E, Kamiya H, Hamada Y, Sato H, Harada N, Toyoda Y, Miwa I, Nakamura J, Inagaki N, Oiso Y and Ozaki N (2012). Ingestion of a moderate high-sucrose diet results in glucose intolerance with reduced liver glucokinase activity and impaired glucagon-like peptide-1 secretion. J Diabetes Invest, 3(5): 432-440

Saltiel AR and Kahn R (2001). Insulin signalling and the regulation of glucose and lipid metabolism. Nature, 414: 799-806

Santuré M, Pitre M, Marette A, Deshaies Y, Lemieux C, Larivière R, Nadeau A and Bachelard H (2002). Induction of insulin resistance by high-sucrose feeding does not raise mean arterial blood pressure but impairs haemodynamic responses to insulin in rats. Br J Pharmacol, 137(2): 185-196

Shearer J, Sellars EA, Farah A, Graham TE and Wasserman DH (2007). Effects of chronic coffee consumption on glucose kinetics in the conscious rat. Can J Physiol Pharmacol, 85(8):823-30.

Shulman GI, Rothman DL, Jue T, Stein P, DeFronzo RA and Shulman RG (1990). Quantification of Muscle Glycogen Synthesis in Normal Subjects and Subjects With Non-insulin-depedent Diabetes by [13]C Nuclear Magnetic Resonance Spectroscopy. N Engl J Med, 322(4): 223-228

Steinberg HO, Brechtel G, Johnson A, Fineberg N and Baron AD (1994). Insulin-mediated Skeletal Muscle Vasodilation Is Nitric Oxide Dependent: A Novel Action of Insulin to Increase Nitric Oxide Release. J Clin Invest, Inc., 94: 1172-1179

Su SH, Shyu HW, Yeh YT, Chen KM, Yeh H and Su SJ (2013). Caffeine inhibits adipogenic differentiation of primary adipose-derived stem cells and bone marrow stromal cells. Toxicol In Vitro, 27(6):1830-1837

Swislocki ALM, Chen Y-DI, Golay A, Chang M-O and Reaven MG (1987). Insulin suppression of plasma-free fatty acid concentration in normal individuals and patients with Type 2 (non-insulin-dependent) diabetes. Diabetologia 30:622-626

Thorens B and Mueckler M (2010). Glucose transporters in the 21st Century. Am J Physiol Endocrinol Metab, 298: 141-145

Tremblay A, Masson E, Leduc S, Houde A and Després J-P (1988). Caffeine reduces spontaneous energy intake in men but not in women. Nutrition Research, 8(5): 553-558

Urzúa Z, Trujillo X, Huerta M, Trujillo-Hernández B, Ríos-Silva M, Onetti C, Ortiz-Mesina M and Sánchez-Pastor E (2012). Effects of Chronic Caffeine Administration on Blood Glucose Levels and on Glucose Tolerance in Healthy and Diabetic Rats. J Int Med Res, 40: 2220-2230

Van Dam RM and Feskens EJ (2002). Coffee consumption and risk of type 2 diabetes mellitus. Lancet, 360: 1477-8.

van Dam RM and Hu FB (2005). Coffee Consumption and Risk of Type 2 Diabetes. JAMA, 294(1): 97-104

Van Dam RM, Dekker JM, Nijpels G, Stehouwer CD, Bouter LM and Heine RJ (2004). Coffee consumption and incidence of impaired fasting glucose, impaired glucose tolerance, and Type 2 Diabetes: the Hoorn Study. Diabetologia, 47: 2152-9.

van Dam RM, Dekker JM, Nijpels G, Stehouwer CDA, Bouter LM and Heine RJ (2004). Coffee consumption and incidence of impaired fasting glucose, impaired glucose tolerance, and type 2 diabetes: the Hoorn Study. Diabetologia, 47: 2152-2159

Van Dam RM, Willett WC, Manson JE and Hu FB (2006). Coffee, caffeine, and risk of type 2 diabetes: a prospective cohort study in younger and middle-aged U.S. women. Diabetes Care, 29: 398-403.

van Dam RM, Willett WC, Manson JE and Hu FB(2006). Coffee, caffeine, and risk of type 2 diabetes: a prospective cohort study in younger and middle-aged U.S. women. Diabetes Care, 29(2): 398-403

Vergauwen L, Hespel P and Richter EA (1994). Adenosine receptors mediate synergistic stimulation of glucose uptake and transport by insulin and by contractions in rat skeletal muscle. J Clin Invest, 93: 974-981

Wardlaw GM and Hampl JS (2007). Perspectives in Nutrition. 7th ed. New York, NY: McGraw Hill

Wedick NM, Brennan AM, Sun Q, Hu FB, Mantzoros CS and van Dam RM (2011). Effects of caffeinated and decaffeinated coffee on biological risk factors for type 2 diabetes: a randomized controlled trial. Nutrition Journal, 10(93): 1-9

Wein NJ and Florez JC. Type 2 Diabetes Mellitus. In Ríos MS and Fuentes JAG. *The molecular and genetic basis of type 2 diabetes mellitus.* 1st ed., Spain, ELSEVIER, 2009, p. 53-70

Westerterp-Plantenga MS, Lejeune MP and Kovacs EM (2005). Body weight loss and weight maintenance in relation to habitual caffeine intake and green tea supplementation. Obes Res, 13(7): 1195-1204

Wilcox G (2005). Insulin and Insulin Resistance. Clin Biochem Rev, 26: 19-39

Williams SB, Cusco JA, Roddy M-A, Johnstone MT and Creager MA (1996). Impaired Nitric Oxide-Mediated Vasodilation in Patients With Non-Insulin-Dependent Diabetes Mellitus. JACC, 27(3): 567-574

Wu T, Willett WC, Hankinson SE, et al. (2005) Caffeinated coffee, decaffeinated coffee, and caffeine in relation to plasma C-peptide levels, a marker of insulin secretion, in U.S. women. Diabetes Care, 28: 1390-1396

Yeh T-C, Tseng C-J and Liou J-C (2014). Caffeine intake improves fructose-induced hypertension and insulin resistance by enhancing central insulin signalling. Hypertension, 63(3): 535-41

Zanuso S Jimenez A, Pugliese G, Corigliano G and Balducci S (2010). Exercise fot the management of type 2 diabetes: a review fo the evidence. Acta Diabetol, 47: 15-22

Zheng G, Sayama K, Okubo T, Juneja LR and Oguni I (2004). Anti-obesity effects of three major components of green tea, catechins, caffeine and theanine, in mice. In Vivo, 18(1): 55-62

Cheung WT, Lee CM and Ng TB (1988). Potentiation of the anti-lipolytic effect of 2-chloroadenosine after chronic caffeine treatment. Pharmacology, 36(5): 331-339