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Tese de Doutoramento em Ciências
Área de conhecimento em Biologia

Trabalho realizado sob a orientação da
Professora Doutora Cristina Pereira-Wilson
E co-orientação da
Professora Doutora Maria Judite Almeida

Maio 2008

Acknowledgements

First of all, I would like to thank Dr. Cristina Pereira-Wilson, my main supervisor, for giving me the opportunity to do this work, for the scientific support and fruitful discussions we had. I am evenly grateful for her friendship and for giving me strength to go on.

To Dr. Maria Judite Almeida, my co-supervisor, for motivating me to accept this project and for introducing me to the scientific environment.

To Dr. Jonathan Wilson, a big thanks for his availability, for receiving me well in your lab at CIIMAR, and for all of your prompt help, patience and especially for your teachings.

To Dr. Manuel Fernandes-Ferreira for supplying me the batches of medicinal plants.

To Dr. Rosa Seabra, Dr. Paula Andrade and Dr. Patrícia Valentão from the Faculty of Pharmacy, University of Porto, for having contributed with the plants phytochemical characterisation.

To Dr. Hermann Koepsell for kindly providing the antibody to SGLT1, at the moment when it was most necessary.

To Dr. Olga Coutinho, for her friendship.

To the support given by the project POCI/AGR/62040/2004 funded by Fundação para a Ciência e Tecnologia, inserted in the "Programa Operacional Ciência e Inovação 2010".

To all the people of the department of Biology including technicians, colleagues and friends, for their good work environment and friendship.

I particularly would like to thank Cristóvão and João Pedro. Cristóvão for being my "bench supervisor", for supporting me even at the weakness moments and for the fruitful discussions about my work. I am grateful for your patience and teachings. To João Pedro for your good and bad mood, and for your unconditional support with the English language. To both, for their friendship and sincerity. Of course I can not forget to thank all the people that made part of our "animal biology small corner", especially Alice, Cristina, Carla and Paulina, thanks for all the enjoying late afternoons that we shared. To all my other friends, thanks for everything.

And because a good disposal to work does not exist without a good familiar environment in the back, I want to thank my parents and my sister for their patience, enthusiasm, unmatched care and for always making me believe in my capacities.

To my husband Carlos, for your fellowship and huge patience in good and bad moments, always with a word of encouraging and affection.

To all my rats, for having given their lives for this work…

…without them, this work wouldn't also be possible.

Evaluation of the antidiabetic efficacy and safety of medicinal plants of the genus *Salvia*

Abstract

Type 2 diabetes mellitus is a disease that is assuming epidemic proportions worldwide. The interest for the medicinal plants with antidiabetic properties has increased with the demand for new active compounds with minor side effects than the currently available drugs. In the present work, two species of the genus *Salvia* (*S. fruticosa* and *S. officinalis*) that enjoy of antidiabetic reputation were studied with regard to their mechanisms of action.

In order to study the effects of *S. fruticosa* tea on plasma glucose and on the well known diabetes-induced expression of intestinal Na+/glucose-cotransporter (SGLT1), diabetes was induced with streptozotocin (STZ) in rats (chapter 2). We verified that, in diabetic rats, although sage tea treatment did not decrease blood glucose levels to near physiological values, it allowed their stabilisation, contrarily to its control group, where hyperglycaemia aggravated during the experiment. Sage tea treatment also reduced the induction of SGLT1 expression on the apical membrane of enterocytes by the diabetic condition. In healthy rats, *S. fruticosa* tea did not interfere neither with the expression of SGLT1, nor with the levels of blood glucose, indicating that it does not reveal hypoglycaemic properties and does not affect normal levels of SGLT1 expression.

In a subsequent study using dietary manipulation of SGLT1 expression (chapter 3), we aimed to confirm *in vivo* the *S. fruticosa* tea effects on the SGLT1 glucose transporter, when mechanisms of induction of its expression are present. A low carbohydrate (LC) diet reduced the expression of rat glucose transporter. With the reintroduction of the diet rich in carbohydrates (HC diet), SGLT1 levels on the apical membrane (BBM) of enterocytes were re-established. When sage tea (instead of water) was given at the same time that HC diet was reintroduced, the recovery of SGLT1 levels on BBM was not fully achieved, indicating that *S. fruticosa* interferes with mechanism of increased expression of SGLT1. We also observed that rosmarinic acid (the most abundant phenolic compound of *Salvia* spp.) given as an aqueous solution (instead of water) attained the same results than sage tea, indicating that this seems to be the active principle in the extract. With this work we also aimed to understand the mechanism(s) that underlies the effect of *S. fruticosa* on SGLT1 expression. We found that the expression of protein kinase C (PKC) and of heat shock protein 70 (Hsp70) were affected by sage tea in a similar manner to the one obtained for SGLT1. These proteins have been reported to be involved in the translocation of SGLT1 to the plasma membrane, which aware for future studies on those signalling pathways in order to understand the molecular mechanisms.

Taking into account that the previous observations on the expression of SGLT1 could indirectly result from a decrease in glucose available in the intestinal lumen induced by sage tea, due to inhibition of digestive enzymes, we further evaluated the effects of sage water extracts (teas), as well as some of their individual compounds on the activity of the digestive enzyme α-amylase (chapter 4). The different sage teas did not inhibited *in vitro* the α-amylase activity, which was only attained in some extent by the individual compound luteolin-7-glucoside (L7G). Therefore, the result of sage on the SGLT1 expression seems not to be due to secondary effects on the carbohydrate digestion. In chapter 4, we also studied some effects of L7G and ursolic acid (UA) in rats. Both sage compounds reduced postprandial glucose levels in the plasma, as well as improved the lipid profile of the animals. However, while L7G mechanisms of glycaemic control seem to involve a slight inhibition of carbohydrate digestion, UA seems to act on the stimulation of liver glycogen deposition. Both compounds showed beneficial effects on plasma cholesterol levels and (UA mainly) on lipoproteins, thus demonstrating potential for reducing the risk of cardiovascular complications.

In the chapter 5, we performed some studies regarding the antidiabetic activities of *S. officinalis* tea. Previous results showed that *S. officinalis* tea treatment diminished fasting plasma glucose levels of non-diabetic mice, and did not produce effects on plasma glucose clearance mechanisms, which suggested effects on hepatic gluconeogenesis. Taking this into account, a study with STZ-diabetic and non-diabetic rats treated with *S. officinalis* tea was performed. Subsequently hepatocytes were isolated and their response to pancreatic hormones – insulin and glucagon – was studied. We verified that in hepatocytes isolated from non-diabetic rats, sage tea increased glucose consumption and decreased gluconeogenesis. *S. officinalis* essential oil increased hepatocyte insulin sensitivity and contributed to gluconeogenesis inhibition. Also in chapter 5 are results of a pilot trial with *S. officinalis* tea treatment during four weeks in human volunteers. Tea consumption was particularly beneficial on the control of lipid profile, as well as on their antioxidant defences, indicating beneficial properties namely on the prevention of cardiovascular diseases.

We concluded with this work that the antidiabetic properties of *S. fruticosa* seem to be at the intestinal level, particularly on the reduction of the diet/diabetes-induced increases in SGLT1 expression. Both Hsp70 and PKC seem to be involved in this effect, and the active principle of this tea seems to be rosmarinic acid. On the other hand *S. officinalis* is more effective on the regulation of hepatic gluconeogenesis and on control of lipid profile. In both cases the drinking of sage teas can be considered safe.

Avaliação da eficácia antidiabética e segurança de plantas medicinais do género *Salvia*

Resumo

Diabetes *mellitus* tipo 2 é uma doença que está a assumir proporções epidémicas a nível mundial. O interesse pelas plantas medicinais com propriedades antidiabéticas tem aumentado bem como a procura de novos compostos activos com efeitos secundários menores do que os dos fármacos actualmente utilizados. No presente trabalho foram estudadas duas espécies do género *Salvia* (*S. fruticosa* Mill. e *S. officinalis* L.) que gozam de reputação como antidiabéticas, no que respeita aos seus mecanismos de acção.

Em primeiro lugar, com o intuito de estudar os efeitos do chá de *S. fruticosa* na glucose plasmática e no aumento característico da expressão do transportador de glucose (SGLT1) numa situação de diabetes, esta foi induzida com estreptozotocina em ratos (capítulo 2). Verificou-se que em ratos diabéticos, embora o tratamento com o chá não tivesse diminuído os valores da glucose no sangue para valores próximos dos fisiológicos, permitiu a sua estabilização, contrariamente ao respectivo grupo controlo, no qual a hiperglicémia se agravou ao longo do ensaio. O tratamento com chá também reduziu o característico aumento da expressão do transportador de glucose (SGLT1) ao nível da membrana apical (BBM) dos enterócitos, associado à situação de diabetes. Em ratos não-diabéticos o chá de *S. fruticosa* não interferiu com a expressão do SGLT1, nem com os níveis de glucose no sangue, indicando que este chá não revela propriedades hipoglicemiantes, nem altera valores basais de expressão do SGLT1.

Num estudo posterior, de modo a confirmar *in vivo* os efeitos do chá de *S. fruticosa* sobre o SGLT1, recorreu-se à manipulação da dieta para a indução da expressão do SGLT1 (capítulo 3). Uma dieta pobre em hidratos de carbono (LC) reduziu a expressão dos transportadores de glucose dos ratos. Com a reintrodução da dieta rica em hidratos de carbono (HC) os níveis normais de SGLT1 foram retomados na BBM dos enterócitos. Contudo, quando administrado o chá de *S. fruticosa* aquando da reintrodução da dieta HC, a normalização dos níveis de SGLT1 na BBM foi bastante inibida, indicando uma vez mais que o chá interfere no mecanismo do aumento da expressão do SGLT1. Também se observou que o ácido rosmarínico (composto fenólico maioritário de espécies de *Salvia*) administrado em solução aquosa produziu efeitos semelhantes aos do chá, mas de uma forma mais significativa, indicando-o como um princípio activo do extracto. Com este trabalho procurou-se também perceber o(s) mecanismo(s) que estaria na base do efeito da *S. fruticosa* sobre o SGLT1. Verificou-se que a expressão da proteína cinase C (PKC) e da de choque térmico (Hsp70) foram afectadas de forma semelhante ao SGLT1. Uma vez que estas proteínas parecem estar envolvidas na

translocação do SGLT1 para a membrana, mais estudos são necessários para compreender efeitos nessas vias de sinalização.

Atendendo ao facto de os resultados obtidos sobre a expressão do SGLT1 poderem indirectamente resultar de uma diminuição da glucose no lúmen, induzida pelo chá devido à inibição de enzimas digestivas, avaliamos posteriormente os efeitos dos chás, bem como de alguns dos seus compostos individuais na actividade da enzima digestiva α-amilase (capítulo 4). Os diferentes chás de salva não inibiram *in vitro* a actividade da α-amilase, e apenas a luteolina-7-glicosídeo (L7G) o conseguiu em alguma extensão. Desta forma, o efeito do chá sobre a expressão do SGLT1 não parece ser devido a efeitos secundários sobre a digestão dos hidratos de carbono. No capítulo 4 também foram estudados alguns efeitos da L7G e do ácido ursólico (UA) em ratos. Ambos os compostos de salva diminuíram os níveis de glucose pós-prandial no plasma e melhoraram o perfil lipídico dos animais. Contudo, enquanto que os mecanismos de controlo da glicemia da L7G poderão envolver uma moderada inibição da digestão dos hidratos de carbono, o UA parece actuar na estimulação da deposição de glicogénio no fígado. Ambos os compostos tiveram efeitos benéficos sobre os níveis de colesterol no plasma, e o UA em particular sobre as lipoproteínas, mostrando assim potencial para reduzir o risco de complicações cardiovasculares.

No capítulo 5 são apresentados estudos relativos à avaliação de actividades antidiabéticas do chá de *S. officinalis*. Resultados anteriores mostraram que o chá de *S. officinalis* diminuía os níveis de glucose plasmática em jejum de ratinhos não-diabéticos, não produzindo efeitos sobre os mecanismos de remoção da glucose plasmática, o que sugeriu efeitos sobre a gluconeogénese. Atendendo a estes resultados, foi realizado um estudo com ratos diabéticos e não-diabéticos tratados com chá de *S. officinalis*. Posteriormente foram isolados hepatócitos e estudada a sua resposta às hormonas insulina e glucagon. Em hepatócitos isolados de ratos não-diabéticos, o chá administrado *in vivo* aumentou o consumo de glucose *in vitro* e reduziu a gluconeogénese. O óleo essencial de *S. officinalis* aumentou a sensibilidade dos hepatócitos à insulina e contribuiu para a inibição da gluconeogénese. Ainda no capítulo 5 são apresentados resultados de um estudo piloto com voluntários humanos tratados com chá de *S. officinalis*. O chá apresentou sobretudo efeitos benéficos no controlo do perfil lipídico, bem como nas defesas antioxidantes, indicando propriedades benéficas em particular na prevenção de complicações cardiovasculares.

Com este trabalho conclui-se que as propriedades antidiabéticas da *S. fruticosa* se situam sobretudo ao nível do intestino, em particular na redução da indução da expressão do SGLT1. Ambas as proteínas Hsp70 e PKC parecem estar envolvidas neste efeito e o princípio activo deste chá parece ser o ácido rosmarínico. Por outro lado, a *S. officinalis* é mais eficaz na regulação da gluconeogénese e no controlo do perfil lipídico. Em ambos os casos, a toma do chá de salva pode ser considerada segura.

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Abbreviations

AC	Adenylate Cyclase
AGE	Advanced Glycation End-products
ALT	Alanina aminotransferase
AMPK	AMP-activated Protein Kinase
AST	Aspartate aminotransferase
BBM	Brush-Border Membrane
BLM	Basolateral Membrane
DPP-IV	Dipeptidyl Peptidase-IV
EO	Essential Oil
G6Pase	Glucose-6-Phosphatase
GIP	Glucose-dependent Insulinotropic Polypeptide
GK	Glucokinase
GLP-1	Glucagon-Like Peptide-1
GLP-2	Glucagon-Like Peptide 2
GLUT	Facilitative Glucose Transporter (GLUT2, GLUT4, GLUT5)
GP	Glycogen Phosphorylase
GS	Glycogen Synthase
GSK3	Glycogen Synthase Kinase-3
GTT	Glucose Tolerance Test
HbA1c	Glycated Haemoglobin
HC	High-Carbohydrate
HDL	High-Density Lipoprotein
Hsp70	Heat Shock Protein 70
IFG	Impaired Fasting Glycaemia
IGT	Impaired Glucose Tolerance
IR	Insulin Receptor
IRS	Insulin Receptor Substrate
LC	Low-carbohydrate
L7G	Luteolin-7-Glucoside
LDL	Low-Density-Lipoprotein
OGTT	Oral Glucose Tolerance Test
PCNA	Proliferating Cell Nuclear Antigen
PEPCK	Phosphoenolpyruvate Carboxykinase
PHZ	Phlorizin

PI3K	Phosphatidylinositol 3-Kinase
PKA	Protein Kinase A
PKC	Protein Kinase C
PPARs	Peroxisome Proliferator-activated Receptors
RA	Rosmarinic Acid
SGLT1	Sodium Glucose Cotransporter-1
SREBP-1c	Sterol regulatory element-binding protein-1c
STZ	Streptozotocin
T1DM	Type 1 Diabetes Mellitus
T1R	Type 1 Taste G-protein-coupled Receptors (T1R2, T1R3)
T2DM	Type 2 Diabetes Mellitus
TZDs	Thiazolidinediones
UA	Ursolic Acid
WEs	Water Extracts
WHO	World Health Organisation

Chapter 1

General Introduction

1.1 Mechanisms of plasma glucose regulation

1.1.1. Glucose homeostasis – an overview

Among all of the nutrients that we obtain by feeding, glucose represents a large proportion of carbohydrates present in diet, and plays a central role in energy metabolism. It is a monosaccharide that results from an enzymatic digestion of more complex carbohydrates (starch, lactose, sucrose) in the gastrointestinal tract. The maintenance of blood glucose concentration in a narrow range of 3.9 to 5.6 mM (70.2 - 100.8 mg/dl; Roden & Bernroider, 2003) is of great physiological importance, particularly because the central nervous system depends on a continuous glucose supply, and high blood glucose levels (hyperglycaemia) are deleterious for the cells, due to glucotoxicity. Plasma glucose concentration depends on the dynamic equilibrium between intestinal glucose absorption, endogenous glucose production by the liver (glucose appearance) and glucose utilisation by the tissues (glucose disappearance). There is a coordination and integration of several physiological systems (such as sympathetic nervous system and endocrine system) in order to maintain glucose homeostasis (Roden & Bernroider, 2003). In fact, several hormones (in particular the pancreatic hormones insulin and glucagon), enzymes, substrates, transporters are involved in the regulation of plasma glucose levels (Aronoff *et al*., 2004). Insulin is the key regulatory hormone of glucose removal from the circulation whereas glucagon is the major regulator of glucose production.

The rapid sensing of elevated plasma glucose levels after a meal (above 5.6 mM) lead to a subsequent increase in insulin secretion by pancreatic β-cells. Although glucose is the necessary stimulus for insulin secretion this process is enhanced by increased levels of intestinal incretin hormones glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), released by enteroendocrine cells after a meal. High levels of insulin in circulation will increase glucose uptake and disposal into peripheral tissues (primarily in skeletal muscle), stimulate glucose utilisation and storage particularly by the liver (glycolysis and glycogenesis, respectively), and inhibits hepatic gluconeogenesis by suppression of glucagon secretion from pancreatic α-cells (**Fig. 1**; Aronoff *et al*., 2004). In addition, insulin secretion may also be stimulated by amino acids, such as arginine, and promotes protein and fat synthesis. Through these insulin-dependent mechanisms, physiological plasma glucose levels are reached, re-establishing glucose homeostasis. After a glucose load, the liver takes up approximately one-third of glucose, skeletal muscle and adipose tissue take up one-third and the non-insulin-dependent tissues (brain, blood elements and pancreatic β-cells) take up the other one-third (Moore *et al*., 2003). In contrast, lower levels of glucose (below 3.9 mM) stop the stimulation of insulin release and low insulin in the plasma (between meals or in fasting

conditions) allows glucagon secretion by pancreatic α-cells. Higher levels of glucagon in circulation will act particularly on the liver, stimulating hepatic glucose production (by increasing gluconoegenesis and glycogenolysis) and glucose release to the circulation, thus contributing to maintain plasma glucose levels within the physiological range (Aronoff *et al.*, 2004). The actions of these two pancreatic hormones insulin and glucagon are fundamental for glucose homeostasis.

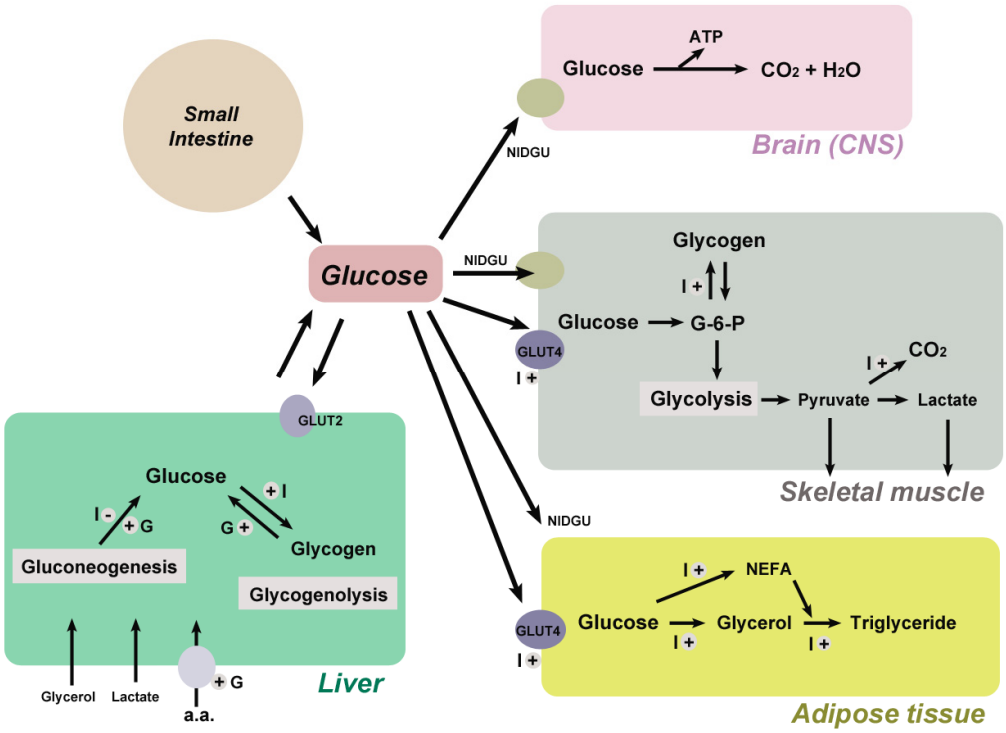

Fig. 1 – Overview of peripheral glucose disposal. NIDGU: non-insulin-dependent glucose uptake; G-6-P: glucose-6-.phosphate; NEFA: non-esterified fatty acids; a.a.: amino acids; I: insulin; G: glucagon; +: stimulation; CNS: central nervous system. [Adapted from Williams & Pickup, 2004]

However, when any of these mechanisms fail, the effective plasma glycaemic control is not achieved and multiple pathological conditions or diseases ensue, such as diabetes mellitus. A good understanding of the mechanisms of glucose homeostasis is central to the development of new pharmacological agents/ approaches to promote better clinical outcomes and quality of life for people with diabetes.

1.1.2. Diabetes mellitus – the result of defects in glucose homeostasis

Diabetes mellitus is a metabolic disease characterised by hyperglycaemia (chronically raised blood glucose concentration; Williams & Pickup, 2004). A diabetic condition occurs when there is a lack in insulin production by the endocrine pancreas and/ or a deficient response of target tissue cells to insulin (Klover & Mooney, 2004). The state of reduced responsiveness of target tissues (liver, muscle and adipose tissue) to normal insulin circulating concentrations is defined as insulin resistance, and is a feature of type 2 diabetes mellitus (T2DM, see section 1.1.2.1). Insulin resistance leads to a deregulation of carbohydrate and lipid metabolism, which results in exacerbation of its progression. Pancreatic β-cells that usually compensates for this situation, at some point, they can no longer compensate and do not respond efficiently to the high blood glucose levels (Bell & Polonsky, 2001). This situation result in a decline of glucose homeostasis followed by the development of glucose intolerance that in the majority of the cases progresses to diabetes.

Diabetic abnormalities include increased postprandial glucose production by the liver (in an unregulated fashion), diminished glucose uptake by the skeletal muscle and liver, and increased fatty acid generation (lipolysis) by the adipose tissue. In fact, when all of these disturbances are present due to destruction of β-cells, exogenous insulin is required (indicating late/ advanced stages of the disease) (Bell & Polonsky, 2001). Impaired insulin action together with hyperinsulinaemia leads to a wide range of other abnormalities that is usually referred as metabolic syndrome (also called insulin resistance syndrome or syndrome X). This syndrome includes high levels of glucose and triglycerides accompanied by low levels of high-density lipoproteins, enhanced secretion of very-low-density lipoproteins by the liver, increased risk of blood clotting and increased vascular resistance (Saltiel, 2000).

Diabetes is also accompanied by morphological and physiological adaptations of small intestinal mucosa that interfere with glucose metabolism. Increased number of absorptive cells (hyperplasia), increased number and activity of glucose transporters in enterocytes (increased absorption of sugars), and elevated levels of digestive enzymes (such as intestinal disaccharidases) are some of small intestinal adaptations to diabetes which contribute to postprandial hyperglycaemia (Ferraris and Diamond, 1997; Bhor *et al*., 2004; Ramaswamy & Flint, 1980).

All of these morphological and physiological alterations observed in diabetic intestine as well as in insulin target tissues contribute to increase plasma glucose levels and aggravate hyperglycaemia.

1.1.2.1 Diabetes mellitus – important considerations

Diabetes is the most common of all endocrine disorders and is a worldwide health problem. The prevalence of this disease is increasing alarmingly and recent estimates indicate that there were 171 million diabetics in the world in the year 2000 and this number is projected to rise to more than double (366 million) by 2030 (Wild *et al*., 2004). In Portugal, the number of diabetics in 2000 was 662,000 and it is predicted to rise to 882,000 in 2030 (according to the World Health Organisation).

Historical accounts of diabetes mellitus first appeared in the medical texts of several ancient cultures over 2000 years ago. The Egyptian Ebers papyri, the Greek Epidemics Book III of Hippocrates, and the Chinese Nei Ching described symptoms such as polyuria, polydypsia, polyphagia and unexplained weight loss (Cheng, 2000; Engelgau *et al*., 2004). Other frequent symptoms are dehydration (resulting from the loss of extra water in the urine), extreme tiredness and blurred vision or vision loss (Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2003). Additionally, the uncontrolled excessive ketone production (ketoacidosis) leads to lethargy, coma and death, if adequate treatment is absent. When these symptoms are clearly present and are accompanied with fasting blood glucose levels of 11.1 mM (200mg/dl) or greater, the diagnosis is usually unequivocal (Engelgau *et al*., 2004). The high persistent fasting and/ or postabsorptive blood glucose levels are called hyperglycaemia. Chronic hyperglycaemia is usually accompanied by a significant increase of glucose levels in urine as a result of saturation of glucose reabsorption by the kidney (glycosuria) and glycated haemoglobin (HbA_{1c}) levels in plasma. Glycosuria was a classical symptom that was considered since ancient times the diagnostic hallmark of diabetes. Was precisely the sweet taste of urine that allowed the discovery of this disease, thus being called “mellitus”. Today it is only used to indicate that the person needs to measure the blood glucose concentration, and not as a diagnostic parameter (Williams & Pickup, 2004). It is not considered a reliable method because, for example, glucose reabsorption varies within and between individuals and glucose levels in the urine also depends on the state of hydration of the subject (Williams & Pickup, 2004). The percentage of HbA_{1c} is used to assess glycaemic control and adjust treatment. Although it could be used in the diagnosis of diabetes, it can not be used alone (Williams & Pickup, 2004).

However, in the majority of the cases, the development of diabetes is gradual and the subject does not realise its condition. Therefore, there is a necessity to identify people at risk, and the oral glucose tolerance test (OGTT) is the most reliable diagnostic method. It is commonly clinically used to diagnose intolerant and diabetic people, throughout the evaluation of the efficacy of their glucose clearance (to metabolise glucose). For the OGTT, the subject is tested in the morning (after 8-14 hours fasting) for the determination of basal blood glucose

levels. After fasting blood sample collection a set amount of glucose is given by mouth (recommended 75g glucose/300 ml of water). Further blood samples are collected (usually after two hours), and then the fasting and OGTT glucose values are compared (Williams & Pickup, 2004; Ravel, 1989). The diagnostic criteria for diabetes were updated and the reference values for blood glucose concentration in fasting and OGTT are presented on **Table 1** (Conget, 2002).

Table 1 – Diagnostic values of diabetes mellitus (adapted from Conget, 2002 and WHO, 2006)

	Fasting plasma glycemia (mmol/l [mg/dl])	2 hours after OGTT (mmol/l [mg/dl])
DM	≥ 7.0 [126]	≥ 11.1 [200]
IGT	< 7.0 [126]	7.8 – 11.0 [140-199]
IFG	6.1 – 6.9 [110-125]	< 7.8 [140]*
N	< 6.1 [110]	< 7.8**

DM – diabetes mellitus; IGT – Impaired Glucose Tolerance; IFG – Impaired Fasting Glucose; N – normal; OGTT – Oral Glucose Tolerance Test; *- If measured; ** - Not specified (implied).

The National Diabetes Data Group of the USA and the second World Health Organization Expert Committee on Diabetes Mellitus in 1979 and 1980 established for the first time the classification of diabetes mellitus and only minor modifications were made by WHO in 1985 (Alberti & Zimmet, 1998; Engelgau *et al*., 2004). This classification of diabetes mellitus recognises two major forms of diabetes: (1) Type 1 (previously known as insulin-dependent diabetes mellitus (IDDM) or juvenile-onset diabetes; autoimmune (type 1a) and idiopathic (type 1b), with beta-cell destruction) and (2) Type 2 (previously known as non-insulin-dependent diabetes mellitus (NIDDM) or adult-onset diabetes; with varying degrees of insulin resistance and insulin hyposecretion). Less frequently (accounting for 1 to 5% of all diagnosed cases) there have been identified and characterised other types of diabetes such as gestational diabetes mellitus (GDM) and maturity onset diabetes of the young (MODY) (Alberti & Zimmet, 1998). Although all of the types of diabetes are etiologically and clinically different, they share hyperglycaemia. In this thesis we will particularly address T2DM, since it is dramatically increasing and the one for which nutritional therapeutical and preventive strategies can be developed.

Type 2 diabetes mellitus (T2DM), previously known as non-insulin-dependent diabetes mellitus (NIDDM) or adult-onset diabetes, results from complex interactions of multiple factors, namely genetic and environmental components, such as high-energy Western diets and sedentary lifestyle (Conget, 2002; **Fig. 2**).

T2DM is the most common type of diabetes and accounts for 90 to 95% of all diagnosed cases of diabetes, affecting 10 to 20% of adults in many developed countries

(Engelgau *et al*., 2004; Bell & Polonsky, 2001). This type of diabetes occurs mainly in adults over age 40, although its prevalence has been increasing in children and young adults. Today, about 30% of new cases of diabetes in the second decade of life (particularly at 12-14 years) are T2DM (Marcovecchio *et al*., 2005).

This type of diabetes is characterised by resistance to the action of insulin (in liver, muscle and adipose tissue) that results in an impairment of glucose uptake, leading to insulin hyperstimulation and subsequently to a dysfunction of pancreatic β-cells (decreasing secretion of insulin) (Yki-Jarvinen, 1995; Cheng & Fantus, 2005; **Fig. 2**). Causes of overstimulation with the contribution of progressive β-cell dysfunction appear to be due to multiple factors, such as genetic determinants, chronic inflammation, glucotoxicity and lipotoxicity (deleterious effects of high levels of glucose and free fatty acids on β-cell, respectively) (Cheng & Fantus, 2005).

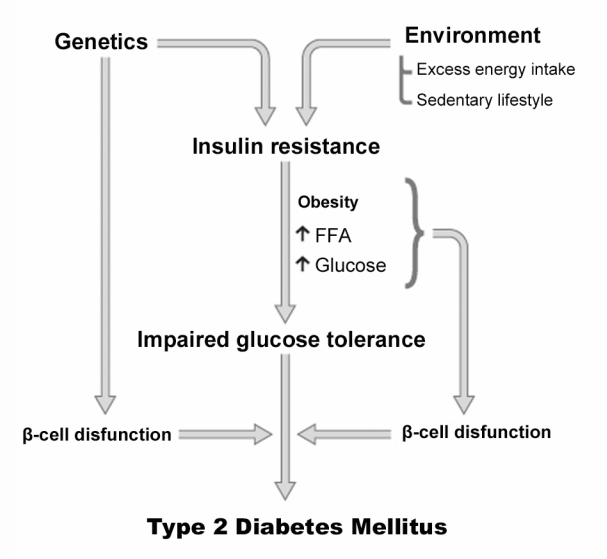

Fig. 2 – Overview of the pathogenesis of T2DM. FFA: free fatty acids. [Adapted from Cheng & Fantus, 2005]

Various risk factors are associated with this type of diabetes, such as increasing age, family history of diabetes, sedentary lifestyle, obesity, race and ethnicity (Williams & Pickup, 2004; Marcovecchio *et al*., 2005). In fact, obesity and T2DM are so closely related that the term "diabesity" has been suggested. Statistics demonstrate that 60-90% of all T2DM patients are or have been obese (Golay & Ybarra, 2005). Obesity (particularly abdominal obesity) is genetically determined and creates a progressive defect in insulin secretion coupled with increase in insulin resistance, contributing for the transition to diabetes (Golay & Ybarra, 2005). Although obesity is considered a strong risk factor for the development of T2DM, this type of diabetes can also be diagnosed in non-obese subjects, particularly elderly people (Conget, 2002). The ketoacidosis observed in T1DM is not frequent in T2DM. When it occurs, it is generally associated with stress (e.g. stress that result from an infection). This type of diabetes is also frequent in hypertensive and dislipidemic subjects, and in women with gestational diabetes antecedents. The risk to develop macro- and microvascular complications in people with T2DM is very high, since in addition to hyperglycaemia and hypertension, there is a abnormal lipid profile, that is characterised by high levels of triglycerides and low-density lipoprotein cholesterol (LDL), and low levels of high-density lipoprotein cholesterol (HDL) (Smith, 2007; Boden & Pearson, 2000).

Chronic complications of diabetes

Despite the availability of several classes of oral antidiabetic drugs and insulin, most patients fail to achieve satisfactory glycaemic control in the long term, and therefore the risk to develop cardiovascular complications is high. Around 3.2 million deaths every year are attributable to complications of diabetes (six deaths every minute). The incidence of cardiovascular disease (e.g. ischemic heart disease) in diabetic adults is 3 to 4 times higher in diabetic people than in the general population (Stamler *et al*., 1993; Jenkins *et al*., 2004).

All of the features that characterise the metabolic syndrome, such as abdominal obesity, hyperglycaemia, atherogenic dyslipidaemia, elevated blood pressure, prothrombotic state, and a proinflammatory state are linked to insulin resistance and lead to an increased risk of cardiovascular disease and T2DM (Grundy, 2005).

Chronic hyperglycaemia *per se* is responsible for the development of several microvascular (damages in peripheral vessels – retinopathy, nephropathy, and neuropathy) and macrovascular complications (damages in large vessels – ischemic heart disease, cerebrovascular disease and, peripheral vascular disease; Rubio Cabezas & Argente Oliver, 2007; Cheng, 2005). Diabetic complications are most likely the consequence of hyperglycaemia via oxidative stress, altering both metabolic pathways and nonenzymatic glycation of proteins. Elevated blood glucose concentration lead to a situation called glucotoxicity (Robertson & Harmon, 2006). However, because diabetic patients also show high levels of lipids in the plasma, several authors talk of glucolipotoxicity. When high plasma glucose concentration persists for a long time, the physiological pathways of glucose (glycolysis and oxidative phosphorylation) saturate and glucose is shunted to other pathways such as polyol pathway, diacylglycerol pathway and intracellular advanced glycation end-products (AGE) pathway (Nishikawa *et al*., 2007; Robertson & Harmon, 2006). All of these pathways lead to the increase in the production of reactive species (reactive oxygen species – ROS and reactive nitrogen species – RNS) which in turn increase oxidative stress and oxidative damage in proteins, lipids and DNA of the cells, particularly damaging pancreatic β-cells and endothelial cells (Robertson RP & Harmon JS, 2006; Jay *et al*., 2006). Hyperglycaemia via advanced glycation products pathway leads to the formation of advanced glycation end-products (AGEs). A covalent reaction between amino acids and sugars occur originating Amadori products (e.g. HbA_{1c} and fructosamine), and the products of their decomposition usually react with protein amino groups, forming AGEs (Jakus & Rietbrock, 2004). The increased accumulation, followed by the reaction with long-lived proteins (e.g. collagen), and the uptake of AGEs by the receptors on macrophages, endothelial cells, and platelets, are the major causes of the development of micro- and macrovascular complications in diabetes (Jay *et al*., 2006). The extent of hyperglycaemia is

the main factor for levels of glycation products in the body (Wu, 1993; Jakus & Rietbrock, 2004). AGEs accumulate throughout natural aging because they are resistant to enzymatic degradation and therefore are very stable. Accumulation of AGEs causes arterial stiffening in the vessel wall, glomerulosclerosis in the kidney, and vascular hyperpermeability in the retina. The pharmacological treatment used T2DM patients does not directly address the excess accumulation of AGEs. Several compounds that could inhibit AGE formation or cleave AGE cross-links are under research (Soro-Paavonen & Forbes, 2006).

Lipid abnormalities are common in T2DM patients, even if they show a reasonable glycaemic control (Williams & Pickup, 2004). This disturbance is called diabetic dyslipidaemia and is characterised by elevated plasma triglyceride and very low-density lipoproteins (VLDL), low high-density lipoprotein (HDL), with or without a raise in low-density lipoprotein (LDL) (Saxena *et al*., 2005; Williams & Pickup, 2004). The concentration and chemical modifications of lipoproteins in the plasma (particularly LDL and HDL levels) are positively correlated with the incidence of cardiovascular diseases. Glycation and glycoxidation of lipoproteins (mainly LDL) lends them pro-atherogenic properties and has been observed in the progression of diabetes (Wright *et al*., 2006). High levels of plasma glycated/glycoxidised-LDL contribute for the development of atherosclerosis (build up of cholesterol plaques that reduce the blood flow and increase blood pressure) (Wright *et al*., 2006; Jay *et al*., 2006). When this happens together with low-levels of HDL, there is an increased risk for heart disease or stroke. Moreover, several authors agree that if the HDL levels are low, even if LDL levels are well controlled, the risk for developing cardiovascular diseases is high (Toth, 2005). For that reason the maintenance of small levels of LDL and high levels of HDL is extremely important, but unfortunately in T2DM the scenery is opposite. Recently, a therapy that in addition to a reduction of LDL cholesterol increases HDL cholesterol has been shown to be more effective in diminishing the risk of cardiovascular diseases and associated morbidity and mortality (Boden & Pearson, 2000).

Diagnose and monitorisation of the severity of the various complications is of great importance. Autoantibodies against β-cells, AGEs, glycated haemoglobin (HbA_{1c}) and other blood glycated proteins are important markers that could be used for monitoring glycaemic control, giving information about the extent of micro- and macrovascular damage, contributing for the treatment of diabetic patients in the initial phase of their complications (Wu, 1993).

1.1.3. Organs and tissues involved in glucose homeostasis – main mechanisms

1.1.3.1. The endocrine pancreas and mechanisms of insulin secretion

As already mentioned, the pancreas has a central role in the control of glucose homeostasis (endocrine pancreas), as well as on the digestion (exocrine pancreas). There are two pancreatic hormones directly implicated with the metabolic pathways involved in the control of glycaemia – insulin (produced and secreted by pancreatic β-cells within the islets of Langerhans) and glucagon (produced and secreted by pancreatic α-cells within the islets of Langerhans). Although pancreatic β-cells are not normally considered insulin sensitive (contrarily to hepatocytes, myocytes and adipocytes), they are also important in glucose homeostasis. The secretion of these two hormones is reciprocally influenced by plasma glucose concentration, and insulin itself is responsible for the suppression of glucagon, when the levels of glucose in the plasma rise (Gerich, 1993; Aronoff *et al*., 2004).

Insulin is a protein that consists of two peptide chains (an A chain with 21 amino acids and a B chain with 30 amino acids), connected by two disulfide bridges. Human insulin differs only slightly from pig and beef mammalian insulins that have been extensively used for diabetes treatment (Karam, 1997; Williams & Pickup, 2004). Insulin is synthesised in pancreatic β cells from a single amino acid chain precursor molecule called proinsulin. Proinsulin is packaged into vesicles in the Golgi apparatus of the β-cell. Maturation of the secretory vesicles (granules) is associated with the conversion of proinsulin into insulin and connecting (C)-peptide (that exist in equimolar amounts inside these mature secretory granules; Karam, 1997). Granules containing insulin and C-peptide are translocated to the cell surface, fuse with the plasma membrane and the contents released by exocytosis.

Although there are several stimuli for insulin secretion (e.g. amino acids: arginine, leucine and lysine; vagal stimulation), rising blood glucose concentration is the most potent stimulus (Aronoff *et al*., 2004). Glucose induced-insulin release from the β-cell happens in a biphasic pattern: an **acute first phase** (when glucose concentration in the plasma increases suddenly) that lasts only few minutes, followed by a **sustained second phase** (when glucose concentration is held at this level), the insulin release gradually falls and then rises again to achieve a steady sate level that lasts as long as plasma glucose is elevated (Karam, 1997; Williams & Pickup, 2004). In T2DM patients, first-phase insulin secretion is absent and in some cases a fall in plasma insulin concentration is found. A second-phase of insulin release can be found in this type of diabetic patients, although in most severe cases this second-phase response is also deficient (Weir & Bonner-Weir, 2004). Endogenous insulin has a circulatory half-life of 3-5 minutes (Karam, 1997).

After being transported by the facilitative glucose transporter GLUT2 (see section 1.2.1) into the β-cell, glucose is rapidly phosphorylated to glucose-6-phosphate by the enzyme glucokinase (GK), which like GLUT2, acts in these cells as a glucose sensor. The subsequent increase of ATP in the cytosol as consequence of glucose metabolism leads to plasma membrane depolarization and closure of K_{ATP}-sensitive channels, which in turn results in Ca^{2+} influx (and even Ca^{2+} mobilisation from intracellular stores) causing secretory granule translocation and insulin exocytosis (Bouché *et al*., 2004; **Fig. 3**). This pathway is known as K_{ATP}channel-dependent pathway and is particularly responsible for the fist phase of glucose-stimulated insulin release. However, a K_{ATP}-channel independent pathway exist and is reported to be responsible for the second phase of glucose-stimulated insulin release (although not well characterised seems to involve multiple mechanisms and second messengers).

Additionally, it has been shown that when glucose is orally loaded (after a meal), the enteroendocrine hormones GLP-1 and GIP enhance glucose-induced insulin-secretion, by facilitating closure of K_{ATP}- channels (Brubaker & Anini, 2003; Doyle & Egan, 2007). Upon binding to its receptor in the surface of β-cell, GLP-1 activates adenylate cyclase, causing an increase in cAMP levels, which results in the activation of PKA or cAMP-regulated guanine nucleotide exchange factor II (Doyle & Egan, 2007).

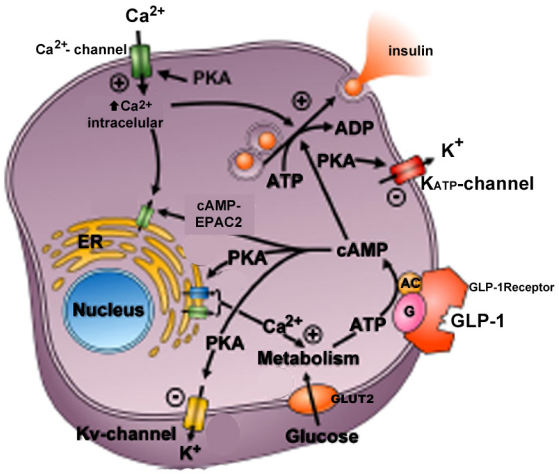

Fig. 3 – Schematic representation of insulin secretion mechanisms upon stimulation by GLP-1. GLP-1R: glucagon like peptide-1 receptor; AC: adenylate cyclase; G: G coupled protein; PKA: protein kinase A; cAMP-EPAC2: cAMP-regulated guanine nucleotide exchange factor II. [Adapted from Holst, 2007]

PKA activation is responsible for the closure of K_{ATP}-sensitive channels that results in an increase of Ca^{2+} concentration in the cytosol (Doyle & Egan, 2007; Holst, 2007), which in turn leads to the fusion of insulin-containing secretory granules with plasma membrane and insulin secretion (**Fig. 3**). This explains why rises in plasma glucose by intravenous injection has a lower effect on insulin secretion than does glucose taken orally (and is called the incretin effect).

In T2DM patients, the incretin effect is reduced to half, contributing for postprandial hyperglycaemia (Efendic & Portwood, 2004). This decrease in the incretin effect is mainly attributed to a loss of GIP-regulated insulin secretion and a decreased secretion of GLP-1 (Efendic & Portwood, 2004). At pancreatic level, besides its role in potentiating insulin secretion by the pancreatic β-cells, GLP-1 also exerts trofic effects on pancreatic β-cell (inducing antiapoptotic pathways and β-cell neogenesis and proliferation; Doyle & Egan, 2007; Drucker, 2007) and suppress glucagon secretion by α-cells of Langerhans islets (Drucker, 2007; Aronoff *et al*., 2004), contributing therefore for keeping glucose homeostasis (see section 1.2.1).

Insulin exerts its main biological effects by binding to its specific receptor (a glycoprotein comprised of two extracellular α-subunits and two β-subunits that span the cell membrane) present on cell membranes of many organs and tissues (liver, skeletal muscle and adipose tissue). Insulin is responsible for lowering the high blood glucose concentrations detected after a meal, by promoting glucose utilisation and/or storage in skeletal muscle and adipose tissue (see sections 1.1.3.3 and 1.1.3.4), by inhibiting hepatic glucose production (see section 1.1.3.2) and by controlling glucagon secretion (by the pancreatic α-cells) (Aronoff *et al*., 2004; Consoli, 1992). Additionally, other insulin actions are: inhibition of liver ketogenesis; stimulation of protein synthesis in muscle (thereby decreasing the amount of free gluconeogenic substrates available in circulation); increased synthesis of triglycerides in the liver and storage in adipose tissue and inhibits adipocyte lipolysis (Karam, 1997).

When the blood glucose concentrations decrease to levels below physiological (~3.9 mM; in a fasting state or postabsorptive state), glucose-induced insulin secretion does not occur. Under these conditions the secretion of another pancreatic hormone – **glucagon** is possible and is responsible for raising plasma glucose levels, thus contributing to maintain blood glucose homeostasis. Glucagon is characterised by opposing the effects of insulin therefore it is called a counterregulatory hormone. The effects of this pancreatic hormone are mainly produced on the liver. The increase in plasma glucose levels is the result of glucagon's stimulation of hepatic glucose production (via glycogenolysis and gluconeogenesis; Aronoff *et al*., 2004) (see section 1.1.3.2). As mentioned above, secretion of glucagon from pancreatic α-cells is inhibited by high levels of insulin. It was also been reported that high levels of glucose in the blood, the incretin hormone GLP-1 and amylin (polypeptide also secreted by β-cells) also inhibits the secretion of glucagon (Aronoff *et al*., 2004; Agius, 2007).

The opposite actions of these two pancreatic hormones (insulin and glucagon), contribute for the re-establishment of physiological glucose levels, by interfering with numerous pathways in peripheral organs. The glucagon-insulin ratio is important to determine the prevailing effect on blood glucose concentration.

In type 2 diabetes mellitus (T2DM), there is a lack in insulin secretion and/or action and the effect of glucagon prevails (postabsorptive hepatic glucose production does not decrease and

exhibits a positive correlation with fasting plasma glucose concentration). The impaired insulin action (lack on the insulin response by its target tissues) is called insulin resistance. Multiple defects have been associated with insulin resistance including defects in insulin receptor and in post-receptor signalling pathways (Bailey, 2007). This situation leads to hyperglycaemia and to permanent β-cell stimulation with continuous insulin secretion (hyperinsulinaemia). Hyperinsulinaemia, insulin resistance and mild hyperglycaemia lead to β-cell failure and hypoinsulinaemia, with aggravation of hyperglycaemia (type 2 diabetes condition) (Weir & Bonner-Weir, 2004). Hyperglycaemia becomes toxic (glucotoxicity) and gradually causes β-cell deterioration and accelerated apoptosis, through mechanisms that involve oxidative stress (Robertson & Harmon, 2006). Oxidative stress that increases the production of reactive oxygen species (ROS) is associated with chronic hyperglycaemia, because of metabolisation of excessive levels of glucose, which in turns lead to cellular damage and even cell death. Studies with rodent islets showed that pancreatic β-cells have lower levels of intrinsic antioxidant enzymes (Lenzen *et al*., 1996; Sigfrid *et al*., 2004), and this situation puts these cells at a greater risk of oxidative damage by the excessive ROS produced from glucose. According to Robertson & Harmon (2006), chronic exposure of β-cells to high levels of glucose (*in vivo* and *in vitro*), causes decreased levels of two insulin gene transcription factors (PDX-1 and MafA), which results in a reduction of the insulin promoter activity, decrease in the content of insulin mRNA and protein, and consequently in insulin secretion (Robertson & Harmon, 2006). Lower levels of plasma insulin in turn stimulates pancreatic α-cells to continue secreting glucagon (hyperglucagonemia) and consequently hepatic glucose production (mainly through gluconeogenesis), which exacerbates hyperglycaemia (Consoli, 1992; Aronoff *et al*., 2004).

1.1.3.2. The liver

The liver plays a major role in blood glucose homeostasis. It contributes to euglycaemia by rapid clearance of glucose from the portal vein in the postprandial state and through glucose production in the postabsorptive state (Postic *et al*., 2004; Agius, 2007). The cells involved in these mechanisms are the hepatocytes, where pathways such as glycolysis (glucose oxidation), gluconeogenesis (glucose production), glycogenesis (glycogen synthesis) and glycogenolysis (glycogen degradation) take place (Klover & Mooney, 2004; **Fig. 4**). Therefore the expression/activity of numerous enzymes in these cells are increased or decreased, depending on blood glucose concentration.

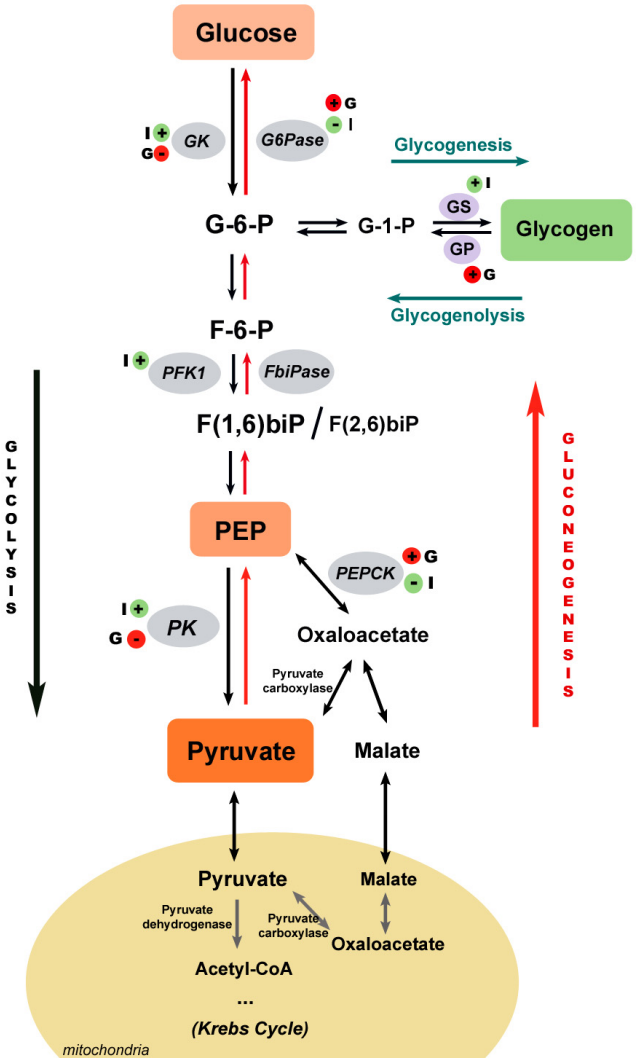

Fig. 4 – The fate of glucose in the hepatocyte and the influence of the pancreatic hormones insulin and glucagon. G-6-P: glucose-6-phosphate; F-6-P: fructose-6-phosphate; F(1,6)/(2,6)biP: fructose-(1,6)/(2,6)-bisphosphate; PEP: phosphoenolpyruvate; GK: glucokinase; PFK1: phosphofructo-1-kinase; PK: pyruvate kinase; G6Pase: glucose-6-phosphatase; FbiPase: fructose-(1,6)/(2,6)-bisphosphatase; PEPCK: phosphoenolpyruvate carboxykinase; G-1-P: glucose-1-phosphate; GS: glycogen synthase; GP: glycogen phosphorylase; I: insulin; G: glucagon; + : stimulation; - : inhibition. [Adapted from Nordlie *et al.*, 1999]

In the **postabsorptive state** (overnight fast), the concentration of glucose in the blood falls and the liver will normalise the fasting plasma glucose levels through hepatic net production of glucose, thus supplying the required amount of glucose to the brain (central nervous system). In this period, about 65-75% of basal hepatic glucose output is derived from glycogenolysis and the remaining 25-35% is from gluconeogenesis (Zierath & Kawano, 2003). The regulation of hepatic glucose production is mainly determined by the action of the pancreatic hormone **glucagon**. High levels of circulating glucagon cause inhibition of glycogen synthesis and glycolysis, and highly stimulate glycogenolysis and gluconeogenesis (Jiang & Zhang, 2003; **Fig. 5**).

Glucagon binds to its receptor in the plasma membrane of hepatocytes and activates adenylate cyclase leading to an increase in cAMP levels that in turn activates protein kinase A (PKA). The activation of this kinase leads to phosphorylation of several transcription factors that induce the expression of gluconeogenic genes, namely PEPCK and G6Pase genes inducing gluconeogenesis, and inhibiting glycolysis by repressing glucokinase and glycolytic genes (such as piruvate kinase and fructose-2,6-bisphosphate; Jiang & Zhang, 2003; Agius, 2007; **Fig. 5**). Activation of PKA also triggers glycogen phosphorylase (stimulating glycogenolysis, via activation of phosphorylase kinase), and inactivates glycogen synthase (inhibiting glycogenesis) (Agius, 2007). The resulting glucose-1-phosphate besides being a potent inhibitor of glycogen synthase phosphatase and of glycogen synthesis is an important gluconeogenic intermediate. Glucose-1-phosphate is converted to glucose-6-phosphate and then to glucose through G6Pase (Jiang & Zhang, 2003; **Fig. 4**). These actions together, result in an increase of hepatic glucose delivery into the blood, re-establishing physiological glucose levels.

In the **postprandial state** (after a meal), when the levels of glucose rise because of intestinal glucose absorption, gluconeogenesis stops and the liver takes up more than one third of it to restore glycogen stores (Moore *et al*., 2003). During this period, **insulin** acts on the liver not by directly stimulating glucose uptake in this organ, but by blocking glycogenolysis and gluconeogenesis, and by stimulating glycogen synthesis (Aronoff *et al*., 2004; Klover & Mooney, 2004; **Fig. 4**). After a meal, high levels of **insulin** stimulates glycogen synthesis by activation of the linear signalling cascade IR/IRS/PI3K/Akt promoting the phosphorylation and inactivation of GSK3, which in turn dephosphorylates and activates GS and thus glycogen synthesis (Lee & Kim, 2007; **Fig. 5**). The inactivation of GSK3 by insulin also leads to the activation of the translation initiation factor eIF2B that result in protein synthesis (Lee & Kim, 2007; **Fig. 5**).

In addition, insulin suppresses gluconeogenesis in the liver by decreasing the expression and activity of several key enzymes of the gluconeogenic pathway. Through activation of its receptor in hepatocytes, insulin decreases the PEPCK and G6Pase gene expression via two major pathways: 1) the phosphatidylinositol 3-kinase (PI3K)/Akt pathway and 2) the mitogen-activated protein kinase (MAPK) cascade (Raf/MEK/ERK signalling cascade; less studied and elucidated than the first one) (Lizcano & Alessi, 2002; Barthel & Schmoll, 2003; **Fig. 5**).

It has been reported that insulin also inhibits PEPCK gene expression through the transcriptional factor sterol regulatory element-binding protein-1c (SREBP-1c) (Yamamoto *et al*., 2004; **Fig. 5**). According to Foufelle & Ferré (2002) SREBP-1c directly decreases PEPCK, but not G6Pase gene expression. Increases in SREBP-1c also result in increases in the expression of lipogenic genes (acetyl-CoA carboxylase and fatty acid synthase), stimulating synthesis of fatty acids from glucose and very low-density lipoprotein (VLDL)-triglyceride secretion (Magaña *et al*., 2000, Koo *et al*., 2001).

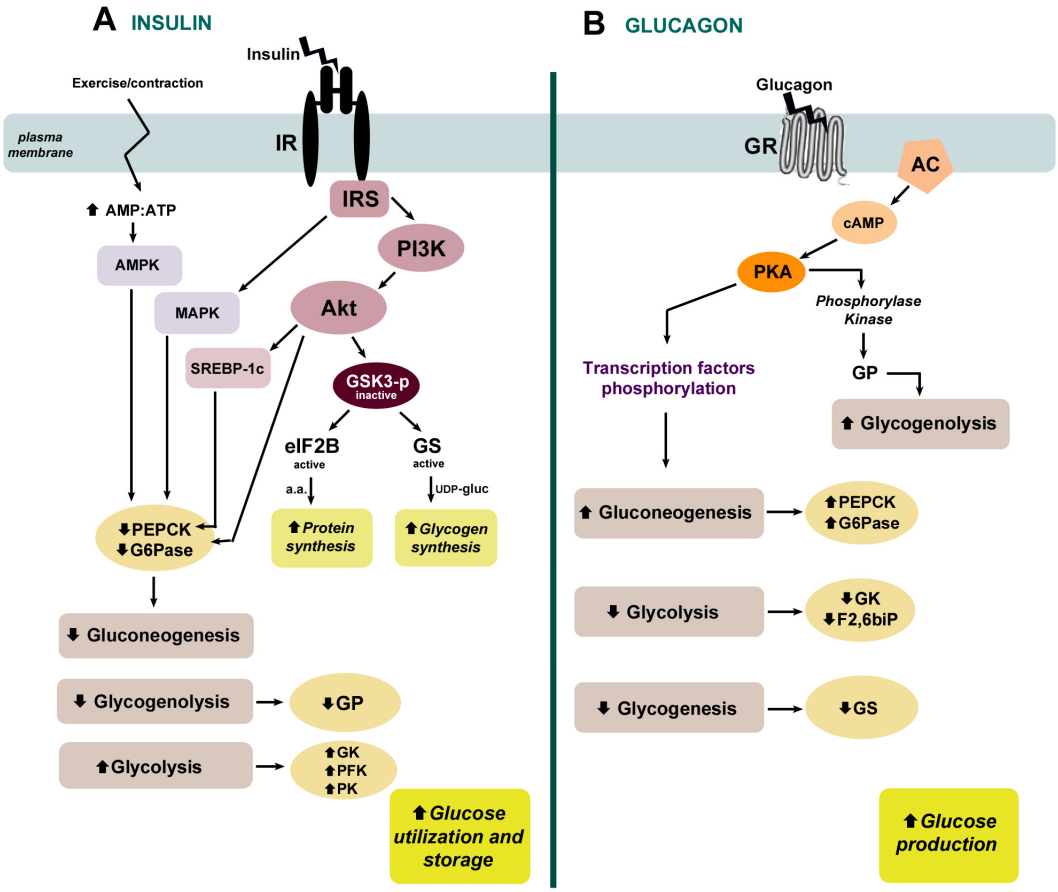

Fig. 5 – Schematic representation of insulin (A) and glucagon (B) signalling pathways in the liver. IR: insulin receptor; IRS: insulin receptor substrate; PI3K: phosphatidylinositol 3-kinase; GSK3-p: phosphorylated glycogen synthase kinase-3; GS: glycogen synthase; eIF2B: translation initiation factor; a.a.: amino acids; UDP-gluc: uridine diphosphate glucose; MAPK: mitogen-activated protein kinase; SREBP-1c: sterol regulatory element-binding protein-1c; PEPCK: phosphoenolpyruvate carboxykinase; G6Pase: glucose-6-phosphatase; AC: adenylate cyclase; PKA: protein kinase A; GP: glycogen phosphorylase; GK: glucokinase; F2,6biP: fructose-2,6-bisphosphate.

Moreover, another pathway is activated when the cells require energy (e.g. fasting state): the AMP-activated protein kinase (AMPK) pathway. Although AMPK is mainly associated with increase insulin-sensitive glucose uptake and fatty acid oxidation in skeletal muscle upon exercise stimulation, it is also involved with the inhibition of hepatic gluconeogenesis (Towler & Hardie, 2007; Barthel & Schmoll, 2003). Activation of AMPK in the liver leads to repression of both PEPCK and G6Pase gene expression, mimicking the effect of insulin (Towler & Hardie, 2007; **Fig. 5**).

The hepatic glucose uptake or release into the blood is mediated by the low-affinity high capacity facilitative glucose transporter GLUT2 present on the membrane of the hepatocyte. This transporter protein maintains intracellular glucose concentration in equilibrium with extracellular levels. Postprandially, when glucose enters the hepatocyte through GLUT2, it

is retained due to its conversion into glucose-6-phosphate by glucokinase (GK) (Mueckler, 1994; Brown, 2000). Thus, GLUT2 and GK remove significant amounts of glucose from the blood when it rises after a meal (Klover & Mooney, 2004; Ferre *et al*., 1996). Conversely, in the postabsorptive state, upon glucagon stimulation, the intracellular levels of free glucose increase in the hepatocyte leading to its efflux into the blood through GLUT2 (Olson & Pessin, 1996). Both GLUT2 and GK detect fluctuations of blood glucose concentrations, thus being called glucose sensors (Im *et al*., 2006). The expression of GLUT2 in the plasma membrane of hepatocytes is mainly regulated by blood glucose concentration (Rencurel *et al*., 1996). Rencurel and colleagues (1996) showed that glucose metabolism is necessary for the induction of the GLUT2 gene transcription in the liver. The stimulatory effect of glucose is counteracted by insulin, as was shown *in vivo* and in cultured rat hepatocytes studies (Burcelin *et al*., 1992; Postic *et al*., 1993; Im *et al*., 2005). Contrarily to GLUT2, GK expression in the liver is regulated by insulin and cAMP (although in pancreatic β-cells its activity and expression is regulated by glucose; Im *et al*., 2006). Additionally, many other factors, such as thyroid hormone (by regulation of GLUT2 mRNA and protein levels) and peroxisome proliferator-activated receptors gamma (PPARγ; through activation of GLUT2 and GK) have been reported to interfere with hepatic glucose uptake (Weinstein *et al*., 1994; Kim & Ahn, 2004). In the liver of diabetic animals GLUT2 expression is increased (Rencurel *et al*., 1996), whereas GK expression and activity is reduced (Ferre *et al*., 1996).

Although the liver with the switch to net uptake and suppression of hepatic glucose production contributes significantly to the postprandial decrease of blood glucose concentration, it may also manifest insulin resistance, similarly to what happens in skeletal muscle and adipose tissue (see sections 1.1.3.3. and 1.1.3.4.). Something that affects the efficacy of insulin action, results in intense effects on hepatic glucose homeostatic pathways and hence glucose homeostasis. Hepatic insulin resistance is essentially characterised by defects in the pathway of insulin signalling, which contribute for the onset of diabetes. Defects in insulin receptor phosphorylation, accompanied by the suppression of IRS transcription and a loss of IRS-associated PI3K activity (Kerouz *et al*., 1997; Valverde *et al*., 2003; Suzuki *et al*., 2004), results in impairment of the downstream cellular insulin action. Since there is a decrease of insulin response, the suppression of gluconeogenesis and glycogenolysis does not occur and what happens is an overexpression of gluconeogenic enzymes on one hand and reduced expression of glycolitic enzymes on the other. In addition, the expression of plasma cell membrane glycoprotein PC-1, which is an inhibitor of the insulin receptor, has also been shown to be elevated in the liver of both insulin-resistant humans and animals (Dong *et al*., 2005), contributing therefore for glucose intolerance and insulin resistance. Other factors can also contribute for the development of hepatic insulin resistance, although to a smaller extent. Increased expression and/or function of protein-tyrosine phosphatases, in addition to increased

production of proinflammatory cytokines particularly in adipose tissue (e.g. TNF-α and IL-6), may also be responsible for IRS dysfunction and liver insulin resistance (Klover & Mooney, 2004).

Therefore, both an impairment of glucose clearance in the postprandial state and a concomitant hepatic glucose production are pathophysiological features of T2DM.

1.1.3.3. The skeletal muscle

The skeletal muscle is another tissue that gives a considerable contribution for the control of glucose homeostasis, by increasing glucose uptake into its cells (myocytes). The skeletal muscle corresponds to 40-50% of the total body mass and is the major peripheral tissue responsible for glucose utilisation induced by insulin (up to 75% of insulin-dependent glucose disposal, a small fraction remaining to be taken up by adipose tissue) (Saltiel & Kahn, 2001).

The skeletal muscle stimulated by insulin takes glucose from the blood, and utilises it to produce energy and/or store it as glycogen (Huang & Czech, 2007). Although the pathways of glucose metabolism (glucose oxidation and glycogenesis) in skeletal muscle cells are influenced by insulin, the most important effect of this hormone in this tissue is the induction of glucose uptake by muscle cells. In fact, insulin stimulates the translocation of the glucose transporter GLUT4 from intracellular compartments to the plasma membrane and slightly decreases its internalisation (Sesti, 2007). In this section we will particularly attend to the mechanism related with this topic.

The glucose transport through GLUT4 is different from others of its family (e.g. GLUT2) because, among other aspects, it is acutely regulated in response to insulin, thus being usually called the insulin-sensitive glucose transporter (Brown, 2000). It is found in GLUT4-storage vesicles that are continuously cycled from intracellular pools to the plasma membrane. The major signal transduction pathway involved with insulin-stimulated GLUT4 translocation to the plasma membrane is the IRS1/PI3Kinase/Akt pathway (called PI3K-dependent pathway) (Chang *et al*., 2004; **Fig. 6A**). Insulin binding to its receptor (IR, with tyrosine kinase activity) in skeletal muscle cells (and also adipocytes), results in increased activity of the intrinsic tyrosine kinase, which leads to tyrosine phosphorylation of intracellular substrates, including insulin receptor substrate family (IRS). Phosphorylated IRS binds to the regulatory subunit of phosphatidylinositol 3-kinase (PI3K), and activating it produces polyphosphoinositide phosphatidylinositol (3,4,5)-trisphosphate (PIP3) that localises and interacts with phosphoinositide-dependent kinase 1 (PDK1). Activation of these kinases results in the activation of Akt/PKB and or atypical protein kinase C (PKCζ/λ) that in turn lead to the docking and fusion of GLUT4-storage vesicles with the plasma membrane, and consequent extracellular exposure of the GLUT4 protein (Saltiel & Kahn, 2001; Chang *et al*., 2004; James, 2005).

Despite the numerous evidences supporting the important role of PI3K pathway in GLUT4 translocation, several studies have shown that its activation is not sufficient for the increased glucose transport observed in response to insulin (Isakoff SJ *et al.*, 1995; Chang *et al.*, 2004), suggesting the requirement of other signalling pathways. In fact, a second signalling pathway has been described: the Cbl/TC10 pathway (linked to the exocystic complex and actin reorganization, also called PI3K-independent pathway) (Saltiel & Kahn, 2001; Chang *et al.*, 2004). It is localised in lipid rafts microdomains where some insulin receptors reside (**Fig. 6B**).

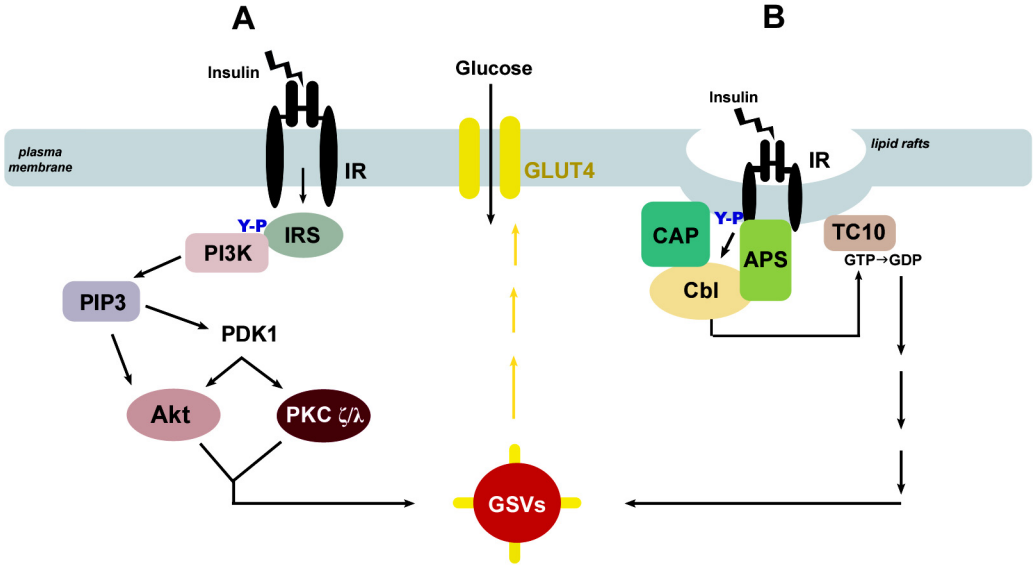

Fig. 6 – Simplified schematic representation of the two major signal transduction pathways of insulin-stimulated GLUT4 translocation: IRS1/PI3Kinase/Akt pathway (A) and Cbl/TC10 pathway (B). GSVs: GLUT4 storage vesicles; IR: insulin receptor; IRS: insulin receptor substrate 1; PI3K: phosphatidylinositol 3-kinase; PIP3: polyphosphoinositide phosphatidylinositol (3,4,5)-trisphosphate; PDK1: phosphoinositide-dependent kinase 1; PKCζ/λ: atypical protein kinase C; CAP: Cbl-associated protein; Y-P: tyrosine phosphorylation. [Adapted from Saltiel & Pessin, 2002]

Insulin binding to its receptors localised in these membrane subdomains results in IR activation and tyrosine phosphorylation of Cbl. This phosphorylation involves the adapter protein APS that is phosphorylated by the IR resulting in Cbl phosphorylation and recruitment together with CAP (Cbl-associated protein) to the lipid rafts. After that Cbl activates small G proteins TC10 that initiate a signalling pathway stimulating GLUT4 translocation to the plasma membrane. After TC10 activation the remaining signalling cascade is not well characterised (Saltiel & Kahn, 2001; Chang *et al.*, 2004; James, 2005). It is known that effectors of the PI3K pathway (such as PKCζ/λ) are recruited for the TC10 pathway and vice-versa, supporting the idea that both pathways are needed for a more efficient insulin-stimulated GLUT4 translocation and glucose uptake (Chang *et al.*, 2004).

Once inside the cell, glucose is phosphorylated by hexokinase (in the liver by glucokinase). Glucose-6-phosphate can then be oxidised in order to produce energy (ATP; via

activation of enzymes, such as pyruvate kinase) or stored as glycogen (via the activation of glycogen synthase) (Bouché *et al*., 2004; Sesti, 2007).

Skeletal muscle is the major site of insulin-stimulated glucose disposal and has also been suggested to be the primary tissue responsible for insulin resistance in the postabsorptive state (DeFronzo *et al*., 1985). In skeletal muscle, insulin resistance manifests itself primarily by decreased insulin stimulation of glycogen synthesis, although this is due to a defect in muscle glucose transport rather than to defects in hexokinase activity as was initially supposed (Cline *et al*., 1999).

Insulin resistance has been associated with changes in the relative abundance of insulin receptor isoform expression and the less sensitive IR/IGF-IR hybrid receptor. A significant increase in relative abundance of the $ExII^+$ IR isoform (the insulin receptor isoform with lower affinity to insulin) has been observed in skeletal muscle and adipose tissue of obese and T2DM subjects (Kellerer *et al*., 1993; Sesti *et al*., 1991). In these subjects, increased abundance of IR/IGF-IR hybrid receptors has also been observed, which reduces insulin sensitivity of target tissues and contributes to skeletal muscle and adipose tissue insulin resistance (since insulin binds to IR/IGF-IR hybrid receptors with lower affinity than to insulin homoreceptors) (Federici *et al*., 1996; Federici *et al*., 1998). Recently, defects in insulin signalling involving the IR/IRS-1/PI3K/PKCζ,λ/Akt/GLUT4 translocation cascade have been reported as of major relevance in the development of insulin resistance. Impaired insulin-stimulated phosphorylation of IR has been observed in skeletal muscle and adipocytes of obese and of T2DM patients (Nolan *et al*., 1994; Arner *et al*., 1987). Additionally, impaired insulin-stimulated phosphorylation of IRS and reduced PI3K activity, have been reported in skeletal muscle and adipocytes of T2DM patients (Björnholm *et al*., 1997; Krook *et al*., 2000). Skeletal muscle biopsies from glucose-tolerant and glucose-intolerant obese subjects and T2DM patients showed defects in insulin activation of PKCζ, but not Akt. In fact defects in Akt activation have been searched in skeletal muscle of obese people and T2DM patients, although controversial results were obtained.

Defects in insulin receptor and post-receptor signalling (IRS-1 phosphorylation, activation of PI3K and PKC, particularly the isoform ζ) with or without reduced Akt activation, have a central role in the development of insulin resistance, impairing a diverse range of metabolic and vascular actions. The reduced glucose uptake may result from impaired GLUT4 function or distribution in the cellular membrane. This corroborated what was mentioned before namely that the impaired glucose uptake, characteristic of insulin resistance, is mainly due to defects in the insulin-signalling pathways that regulate GLUT4-storage vesicles translocation to the plasma membrane (Brown, 2000; Sesti, 2007).

Accumulation of free fatty acids within myocytes (and hepatocytes) is also associated with the insulin resistance observed in T2DM patients. Free fatty acids compete with glucose for substrate oxidation in muscle cells. It is supposed that increased fatty acid oxidation leads to

insulin resistance due to increase in the ratios acetyl-CoA:CoA and NADH:NAD^+ in the mitochondria, which in turn inhibits several key glycolitic enzymes. This leads to accumulation of glucose-6-phosphate, which in turn inhibits hexokinase resulting in an increased of glucose in the cell and decreased glucose uptake (Sesti, 2007). Additionally, increased expression and/or function of inhibitors of insulin signalling (such as protein-tyrosine phosphatases and inhibitors of insulin receptor tyrosine kinase activity - e.g. membrane glycoprotein PC-1) has been observed in skeletal muscle and adipose tissue of T2DM (Ahmad *et al*., 1997; Ahmad *et al*., 1995; Goldfine *et al*., 1999), in addition to increased production of proinflammatory cytokines (e.g. TNF-α and IL-6) particularly in adipose tissue (Kern *et al*., 2001), which may contribute to the IRS dysfunction and impaired insulin action that characterises insulin resistance.

Since insulin resistance contributes to many cardiovascular and metabolic diseases, in addition to diabetes mellitus, a therapeutic attack through compounds that produce specific activation of IR, prolong IR and IRS-1 tyrosine kinase activity (by inhibiting phosphatases and serine kinases), increase PI3K activity, and/or remove signalling defects caused by cytokines would be beneficial to decrease insulin resistance and improve health status of a large number of patients (Bailey *et al*., 2007).

Furthermore, not only insulin stimulates muscle glucose uptake, physical exercise does too (Moore *et al*., 2003). It has been reported that exercise increases both insulin-independent muscle glucose uptake and insulin sensitivity, through two distinct pathways: (1) activating the muscle contraction pathway, and (2) enhancing insulin-stimulated pathway (Sinacore & Gulve, 1993; Moore *et al*., 2003). The stimulation of adenosine monophosphate kinase (AMPK) seems to be the major stimulus for the exercise-induced increase in glucose transport (Musi & Goodyear, 2003). With exercise (training), muscle contraction increases the AMP:ATP ratio, leading to the activation of AMPK. AMPK activation stimulates glucose uptake in muscle by increasing GLUT4 translocation of from intracellular pools to the plasma membrane (similar effect of insulin, although the underlying mechanisms and the fate of glucose are different) (Fujii *et al*., 2006; Towler & Hardie, 2007). Insulin signalling pathway stimulates glycogen synthesis, whereas AMPK induces glycolysis/oxidation (Towler & Hardie, 2007). There are also hormones (e.g. catecholamines), cytockines (e.g. adipokines) and other extracellular signals (e.g. free fatty acids) that can affect glucose uptake in skeletal muscle, by interfering with the AMPK system (Moore *et al*., 2003; Towler & Hardie, 2007). Exercise (through AMPK activation) increases the number of GLUT4 in the plasma membrane of myocytes resulting in an increase of insulin efficiency. The AMPK system, responsible for the health benefit of exercise is also the target of metformin (an antidiabetic drug; see section 1.3), suggesting that other AMPK activators could also be considered new therapeutic agents useful in the treatment of diseases such as obesity and T2DM (Towler & Hardie, 2007). GLUT4 transporter proteins have short half-lives, resulting in a fast loss of activity following cessation of training (Sinacore

& Gulve, 1993). Thus, exercise must be performed regularly in order to sustain beneficial effects on glucose disposal and therefore contribute to glucose homeostasis. In general, exercise is associated with lower plasma insulin concentrations and increased sensitivity of target organs, therefore being important in the prevention T2DM but also in the treatment. In fact, regular physical exercise is often prescribed to T2DM patients.

1.1.3.4. The adipose tissue

The adipose tissue is also involved in glucose homeostasis although its contribution is smaller (usually less than 10% of the whole body glucose uptake) (Ducluzeau *et al*., 2002). Adipocytes are not only storage cells but also produce and secrete a number of hormones (Rosen & Spiegelman, 2006).

Insulin, although most related in adipocytes to the conversion of excess fuel into triglycerides, also stimulates glucose uptake by adipocytes. Despite the fact that the amount of glucose uptake is small, adipose tissue plays an important role in the maintenance of blood glucose levels within the physiological range. It has been shown that mice with adipose-specific deletion of GLUT4 gene develop muscular and hepatic insulin resistance and consequent hyperglycaemia (Abel *et al*., 2001). As in the muscle the insulin-stimulated glucose uptake by adipocytes (observed in the postprandial state) is due to an increase of GLUT4 translocation to the plasma membrane. The two signalling pathways of GLUT4 translocation described above for skeletal muscle cells are also responsible for the insulin-stimulated glucose uptake in adipocytes: the IRS/PI3K/Akt and Cbl/TC10 pathways (Ducluzeau *et al*., 2002; see section 1.1.3.3). Both of these two insulin-dependent pathways are necessary for an efficient GLUT4 translocation and complete induction of glucose uptake. Once inside the cell, glucose is stored primarily as lipids (glucose used for the synthesis of fatty acids and glycerol-phosphate, components of triglycerides) via the activation of lipid synthetic enzymes (Sesti, 2007).

Insulin stimulates the activity of lipoprotein lipase (an enzyme that promotes the uptake of lipids from the blood) and gene expression of intracellular lipogenic enzymes (such as acetyl-CoA carboxylase and fatty acid synthase) (Kersten, 2001). This pancreatic hormone also inhibits lipolysis, by inhibiting the activity of adipocyte hormone-sensitive lipase, through cAMP-dependent (involving activation of phosphodiesterase) and cAMP-independent (involving activation of protein phosphatase-1) mechanisms (Anthonsen *et al*., 1998; Arner, 2005). These actions of insulin lead other tissues and organs to use glucose (instead of lipids) as source of energy, contributing for increased glucose utilisation. On the other hand, when low levels of lipids are in circulation (e.g. during fasting), catecholamines induce lipolysis by stimulating the activity of the hormone-sensitive lipase through a signalling pathway that involves cAMP and PKA activation (Arner, 2005). This results in an increase of fatty acids in

circulation available for energy production in muscle and conversion into ketones in the liver (Duncan *et al*., 2007). Recently, glucagon receptors were also found in the membrane of human adipocytes, which suggest that glucagon may also induce lipolysis (although to a smaller extent than catecholamines) (Duncan *et al*., 2007).

Insulin resistance is also often observed in adipose tissue (Rosen & Spiegelman, 2006). The numerous defects that where mentioned above, responsible for the insulin resistance detected in skeletal muscle (see section 1.1.3.3) are also reported for adipose tissue. Briefly, the decreased glucose uptake observed in adipocytes, characteristic of insulin resistance, is mainly due to defects in the insulin-signalling pathways that regulate GLUT4 translocation to the plasma membrane (Brown, 2000; Sesti, 2007). Reduced PKCζ activity, changes in insulin receptor isoform expression and IR/IGF-IR hybrid receptor abundance, increased function of protein-tyrosine phosphatases and cytokines (TNF-α and IL-6) are also involved in the development of insulin resistance in adipocytes (Sajan *et al*., 2004; Federici *et al.*, 1996; Ahmad *et al*., 1995; Kern *et al*., 2001).

Obese and diabetic animals have shown deficiency in adipocytes hormones, such as leptin and adiponectin and increased levels of resistin, TNF-α and IL-6 (Rosen & Spiegelman, 2006). Leptin besides its role in the inhibition of food intake and induction of energy expenditure has been shown to improve glucose tolerance and insulin sensitivity in muscle and liver. Adiponectin has also been shown to decrease insulin resistance in mice (Yamauchi *et al*., 2001). Resistin, however, is considered to possess hyperglycaemic activity, like the two cytokines –TNF-α and IL-6. They reduce glucose uptake by skeletal muscle and adipose tissue thus promoting insulin resistance (Rosen & Spiegelman, 2006). Similar to what was referred for skeletal muscle, therapeutic attack through compounds that interfere with tyrosine kinase activity of IR/IRS-1/PI3K, protein-tyrosine phosphatases, and so on, resulting in a decrease of insulin resistance are needed. However, other therapeutical approaches that also target adipokines (decreasing TNF-α and IL-6, and enhancing leptin and adiponectin synthesis) would be beneficial to decrease insulin resistance and improve health status of people with obesity and T2DM (Bailey *et al*., 2007; Rosen & Spiegelman, 2006).

From all the drugs that are currently used in T2DM treatment, thiazolidinediones (TZDs) specifically address the underlying insulin resistance of these patients (see section 1.3). TZDs enhance insulin action by activating the nuclear receptor peroxisome proliferator-activated receptor (PPAR) γ (Olefsky & Saltiel, 2000). The activation of this nuclear receptor results in the expression of several genes particularly involved in glucose and lipid metabolism, as well as in insulin signal transduction. In adipose tissue PPARγ activation increase the expression of lipoprotein lipase and some fatty acid transporters, resulting in fatty acid uptake into adipocytes (Etgen *et al*., 2004). Several other genes involved in glucose regulation and the synthesis of triglycerides in adipocytes (PEPCK and acylCoA synthetase) are also up-regulated

upon PPARγ activation. The activation of PPARγ also up-regulates CAP (Cbl-associated protein) that recruits Cbl to the insulin receptor, thus activating the second pathway of GLUT4 translocation improving the insulin stimulated glucose transport particularly in adipose tissue (to a smaller extent than in skeletal muscle), thus reducing insulin resistance (Etgen *et al*., 2004; Olefsky & Saltiel, 2000). PPARγ agonists (TZDs and non-TZDs agonists) showed to improve insulin sensitivity also by increasing adiponectin and suppressing resistin levels, a beneficial effect in insulin-resistant states. These agents also reduce several mediators of vascular inflammation, decreasing atherosclerosis and cardiovascular events (Etgen *et al*., 2004).

1.2. The small intestine – a contributor to glucose homeostasis

The small intestine is another organ involved in the control of glucose homeostasis. More than a digestive organ, the small intestine is the place where glucose is absorbed and, in some circumstances even produced. Therefore, the maintenance of plasma glucose within the physiological concentrations is also dependent of intestinal mechanisms.

Slowing carbohydrate absorption from the intestine (by inhibiting the activity of digestive enzymes and/or reducing the expression of sugar transporters) and even interfering with the hormone response associated with the absorption of these nutrients (incretin hormones), can provide health benefits particularly in T2DM patients. Therefore the small intestine is considered an emerging target organ, where new approaches should be explored for the treatment and prevention of several diseases such as diabetes mellitus.

1.2.1. Morphological and physiological aspects

The small intestine is part of the gastrointestinal tract and in addition to its contribution to the digestive process it is mainly involved in the absorption of the accumulated breakdown products (nutrients) as well as water, vitamins and electrolytes from the lumen of the organ (mucosal surface) into the blood and the lymph (serosal surface) (Caspary, 1992). The small intestine is subjected to several stimuli, such as dietary, hormonal factors, and pancreaticobiliary secretions that work as mediators of intestinal adaptation mechanisms (Jenkins & Thompson, 1994).

The small intestine has about 2.8 m of length in human (Rhoades & Pflanzer, 2003) and about 100 cm in rat (Miller, 1971), including the initial segment that begins from the pylorus – duodenum (small portion), followed by the jejunum and ileum (both of similar length). The ileum is the last part of small intestine that is merged with the large intestine (colon).

The small intestine is supplied with blood vessels, lymph vessels and nerves via the mesentery. The absorptive surface area of the epithelial-luminal interface is highly folded due to the Kerckring's folds (**Fig.7B**), the intestinal villi (singular villus; **Fig. 7C**) and the microvilli of the enterocytes (Burkitt *et al*., 1994; Despopoulos & Silbernagl, 2003). These morphological characteristics increase the absorptive surface, contributing for efficient nutrient absorption. Histologically, the jejunal portion of the small intestine (the one that was used and studied in this thesis) is composed by the following structures from outside inward: tunica serosa, layer of longitudinal muscle fibers, layer of circular muscle fibers, submucosa and tunica mucosa (Burkitt *et al*., 1994; **Fig. 7D**).

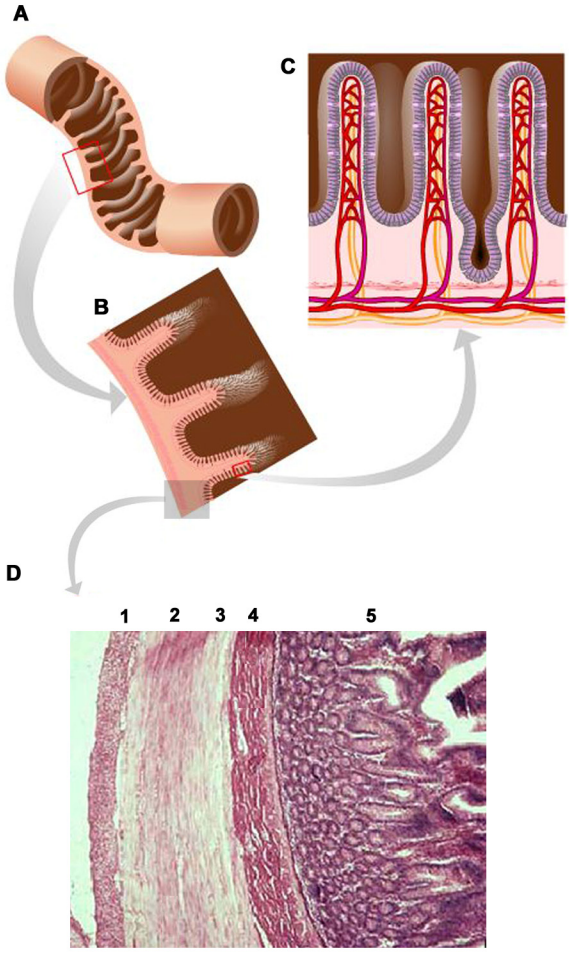

Fig. 7 – Schematic representation of the structure of the small intestine. A – General representation of the small intestine showing the Kerckring's folds (B); C – Intestinal villi; D – Jejunal section stained with haematoxylin and eosin showing the different intestinal structures. From outside to inside: 1 – *tunica serosa*; 2 – longitudinal muscle fibers; 3 – circular muscle fibers; 4 – submucosa; 5 – *tunica mucosa* (covered by villi with epithelial cells). [Images from www.uclan.ac.uk/facs/health/nursing/sonic/scenarios/scenario1GITlecture.htm;www.colorado.edu/intphys/iphy3415/histology/index.html]

The intestinal epithelium is organised into two morphologically distinct compartments – the crypts of Lieberkühn and the villi (Gordon, 1989) – and is in constant state of turnover, with cells proliferating/differentiating at the crypt (from a small number of stem cells that originate progenitors cells) and then migrating/maturating toward the villus tip (**Fig. 8**).

Towards the villus tip the proliferative progenitors will differentiate in enterocytes (up to 80% of all epithelial cells, with hydrolytic and absorptive capacity), enteroendocrine cells (small percentage of all epithelial cells, which produce several hormones, such as the incretin

hormones GLP-1 and GIP) and goblet cells (5% of all epithelial cells that secrete mucous that constitutes a protective barrier). Towards the bottom of the crypt, progenitor cells will originate Paneth cells (around 10 cells per crypt, associated with antimicrobial defence of the intestine) (Gordon, 1989; de Santa Barbara *et al*., 2003).

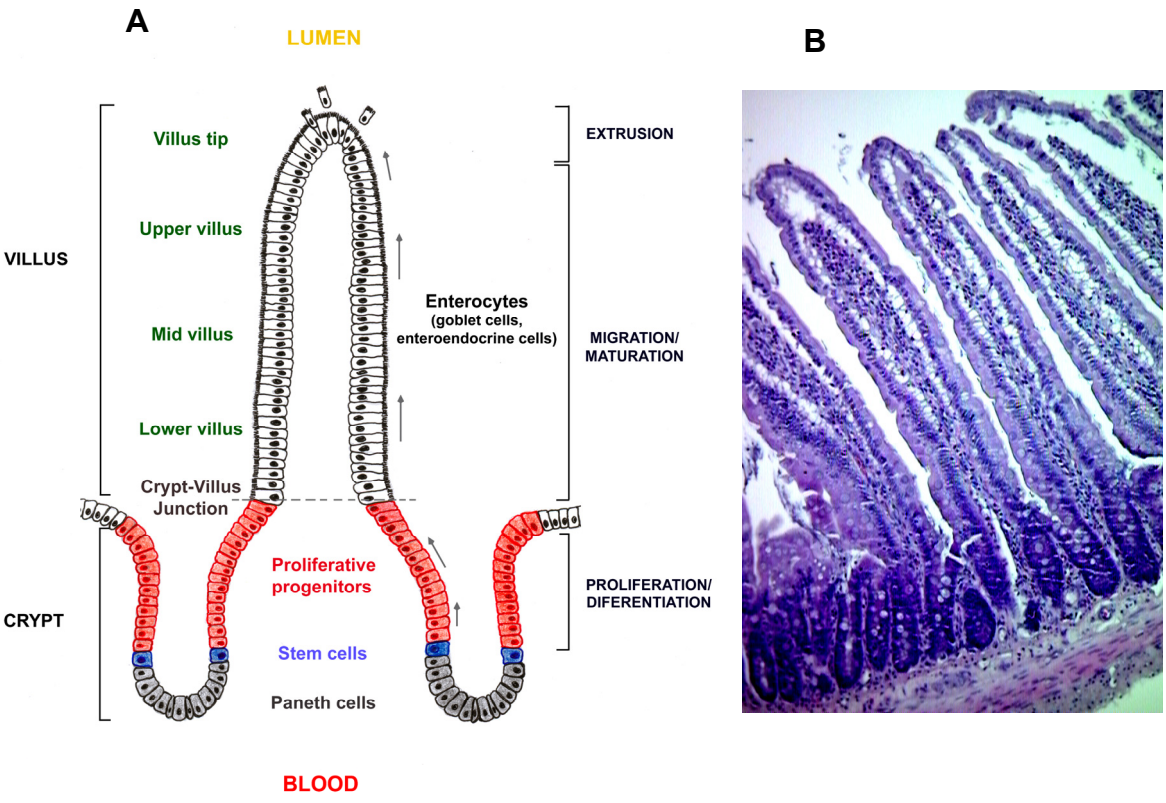

Fig. 8 – Schematic representation of a crypt-villus region in the small intestinal epithelium and the physiologic processes associated (A) [Adapted from Ferraris (2001)]. Representative image of rat small intestine section – intestinal villus and crypts, 100x amplified (B).

The cells that migrate up and along the sides of the villus, reach the villus tip 2-3 days in adult rodents or 3-6 days in humans after emerging from the crypt (Ferraris & Diamond, 1997; Lipkin, 1987; **Fig. 8**). Extrusion of damaged cells occurs near the villus tip 2-5 days after emerging from the crypt (Ferraris, 2001). Under normal circumstances there is a balance between the extrusion at the tip of the villus and proliferation in the crypt, which can be modified by nutritional and health status, and age (Raul & Schleiffer, 1996).

The cellular maturation (differentiation) state of enterocytes varies along the crypt-villus unit, and mature cells with full absorptive capacity are predominantly found in the middle and upper villus region.

It is particularly at the level of enterocytes that organic (carbohydrates, proteins, lipids and vitamins) and inorganic nutrients (water and ions) are absorbed. The process of intestinal nutrient absorption can be done with or without facilitative proteins.

Although in this thesis the process of carbohydrate absorption will be more emphasised, other important processes of nutrient absorption (proteins, lipids, vitamins and inorganic molecules) also take place at the level of intestinal epithelium. For example, the end products of protein digestion – amino acids and small peptides – are absorbed through a number of specific transporters (Na^+ independent or Na^+ and K^+ dependent carrier systems; Ganong, 1999). Protein absorption has many similarities with the mechanisms of absorption of carbohydrates. However, dipeptides and tripeptides can be absorbed through a carrier-mediated mechanism (dependent on H^+) contrarily to sugars (where only monosaccharides are absorbed; Ganong, 1999). Dietary lipids (triglycerides, cholesterol esters, phospholipids and fat soluble vitamins – A, D, E and K) are also absorbed through the intestinal epithelium. However, they are dispersed as vesicles and bile salt-mixed micelles for posterior absorption (by passive diffusion, although there is some evidence that carriers may be involved) across the lipid portion of the enterocyte's membrane into the cell (Ganong, 1999; Rhoades & Pflanzer, 2003), and after being rapidly esterified (in case of triglycerides) they are exocytosed in the form of chylomicrons to the central lacteal of the villus and then by intestinal lymph finally reach the systemic circulation (Rhoades & Pflanzer, 2003).

Carbohydrate absorption

The human diet contains a wide range of carbohydrates (CH), the vast majority of which are of plant origin. They constitute the main source of energy in human diets and are digested to glucose, fructose and galactose by salivary and pancreatic amylases, as well as by disaccharidases of the duodenal enterocyte brush-border membrane (lactase, sucrase, maltase and isomaltase) (Rhoades & Pflanzer, 2003; Drozdowski & Thomson, 2006*b*). After digestion monosaccharides are absorbed through the enterocytes (Wright *et al*., 2003; **Fig. 9**). **Enterocytes** are joined by tight junctions, which on the one hand restricts the movement of molecules from the lumen to the blood via the paracellular route, and on the other maintain the polarity of the enterocyte with an apical membrane (BBM) and a basolateral membrane (BLM, **Fig. 9**) (Woudstra & Thomson, 2002).

Due to their hydrophilic nature, glucose and the other monosaccharides cannot penetrate the lipid bilayer, and thus require specific transporter proteins, to facilitate their diffusion into cells. As was briefly mentioned above, the absorption of the free sugars – glucose, galactose and fructose – across enterocyte membranes occurs via Na^+-dependent (SGLT1) and independent (GLUT2 and GLUT5) membrane protein transporters. Once absorbed, the majority of galactose and fructose are converted to glucose in enterocytes (and the reminder in the liver) for further metabolism or storage (Wright *et al*., 2003).

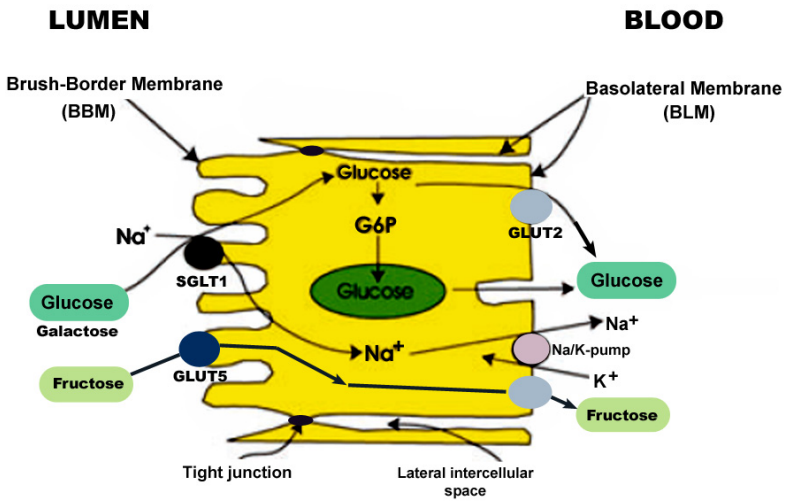

Fig. 9 – A schematic representation showing sugar transport across an enterocyte. The brush-border membrane (BBM) is the area of membrane exposed to the luminal contents. The basolateral membrane (BLM) is the remaining plasma membrane. Arrows indicate routes of monosaccharide absorption. [Adapted from Wright *et al*., 2007]

Briefly, SGLT1 is located to the brush-border or apical membrane (BBM) of the enterocyte and transports glucose and galactose, using the inward Na^+ gradient, from the intestinal lumen into the cytosol. Enterocyte basolateral Na^+/K^+-ATPase maintains the Na^+ gradient. It pumps Na^+ ions out and K^+ ions in across the basolateral membrane ($3Na^+/2K^+$-ATPase). The stoichiometry for SGLT1 transport is 1 glucose: 2 Na^+ ions: ~260 H_2O, which means that for each glucose molecule that is transported through the BBM of the enterocyte, 2 Na^+ ions and about 260 molecules of water enter the cell (Wright *et al*., 2004). The transport of glucose through SGLT1 is considered a secondary active transport, since the hydrolysis of ATP is indirectly coupled to glucose transport via the Na^+ electrochemical gradient (Wright *et al*., 2007). Some of the glucose transported by SGLT1 accumulates in the cytosol is used as fuel for the intestinal epithelial cell metabolism although the major fraction is transported out of the cell by the facilitative glucose transporter – GLUT2 localised in the basolateral membrane (BLM) of the enterocyte. GLUT2 is mainly localised to the basolateral membrane of the enterocyte and mediates the transport of glucose, galactose and fructose from the cytosol to the blood (Wright, 1998; Wright *et al*., 2003). Fructose is transported from the intestinal lumen into the blood via GLUT5 (a specific facilitative fructose transporter that is localised to the BBM) and GLUT2 (in the BLM of enterocytes). However, alternative mechanisms were recently proposed that change this dogma of intestinal sugar transport. The first one is that GLUT2 is not specifically located in the basolateral membrane. Its transient apical localisation in the enterocyte has also been reported, upon stimulation by dietary sugars or in a diabetic situation (Kellett & Brot-Laroche, 2005). Moreover, there is a glucose export mechanism to the blood independent of GLUT2, but

dependent on the previous phosphorylation of glucose by hexokinases, accumulated in endossomes, further dephosphorylation by G6Pase and finally exported by exocytosis into the blood (Stümpel *et al*., 2001; Wright *et al*., 2007).

The **SGLT1** glucose transporter is a 73 kDa protein that belongs to the sodium dependent glucose transporter family (which comprises 6 isoforms: SGLT1 - SGLT6; Wright *et al*., 2007). It contains 14 transmembrane α-helices with extracellular N and C terminus (**Fig. 10**), and has a single glycosylation site (between transmembrane 5 and 6) and two phosphorylation sites (one between transmembrane 6 and 7, and other between 8 and 9 transmembrane helices) (Wright, 1998).

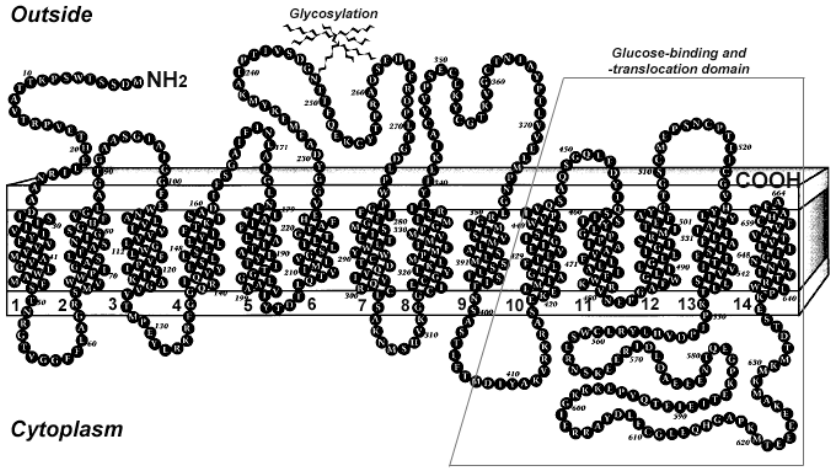

Fig. 10 – Secondary structure model for SGLT1 with 14 transmembrane helices. [Adapted from Turk *et al*., 1996]

The C-terminal region (namely helices 10-14) are required for sugar binding and transport (a high-affinity side on the outside and a low-affinity site on the inside face of the membrane) and the N-terminal region (namely helices 1-9) are required to couple Na^+ and also for the apical localisation of SGLT1 (Wright *et al*., 2004; Panayotova-Heiermann *et al*., 1997; Suzuki *et al*., 2001). SGLT1 is a high affinity low capacity Na^+-dependent glucose transporter that is mainly localised to the BBM of the enterocyte (small intestine), in the apical membrane of renal tubule cells (kidneys), cardiomyocytes (heart) and in the luminal membrane of intracerebral capillary endothelial cells (brain) (Bouché *et al*., 2004; Wright, 2001; Zhou *et al*., 2003; Elfeber *et al*., 2004).

Using the electrochemical gradient of Na^+, it transports glucose and galactose into the cell against their concentration gradients, although it can operate bidirectionally, depending on the direction of the Na^+-electrochemical gradients (Bouché *et al*., 2004; Wright *et al*., 2007). To allow the binding of glucose or galactose to SGLT1, two Na^+ ions first bind to the transporter in its extracellular side, producing a conformational change that permits glucose to bind. Upon

sugar binding, a second conformational change of the protein is produced and the two Na^+ and glucose are then facing the cytoplasmic side of the membrane. On that side glucose and sodium dissociate from the transporter (glucose first than Na^+), leaving it free again. This dissociation is promoted by the low affinity of cytosolic sites for glucose and Na^+ and by the low intracellular Na^+ concentration. The unloaded protein undergoes a last conformational change to again expose the binding sites to the external surface (Wright *et al*., 2003; Wright *et al*., 2007).

The transport of glucose through SGLT1 (with a K_m~0.1-0.6 mM) is phlorizin-sensitive (a well known high-affinity, non-transported, competitive inhibitor of this transporter with a K_i ~0.2 μM) and increases with increments in membrane potential (Wright *et al*., 2004; Wright *et al*., 2007). The Na^+ ions can be replaced by H^+ or Li^+ ions as a driver cation, although the affinity for glucose is much higher in the presence of Na^+. Some glucose analogs, such as the non-metabolised 3-O-methyl-D-glucoside, are also transported by SGLT1, while fructose, although similar to glucose, is not transported by it (Wright *et al*., 2003). SGLT1 is very important in glucose and galactose absorption, since mutations in its gene account for defects in the absorption of these sugars, leading to the development of glucose-galactose malabsorption (Turk *et al*., 1991; Wright *et al*., 2002). Glucose-galactose malabsorption is an inherited disease that results from mutations in the SGLT1 gene, causing severe diarrhoea that requires complete removal of glucose, galactose and lactose from the diet (Wright *et al*., 2002).

In addition, SGLT1 is considered a multifunctional protein since it acts as a Na^+ uniporter, a water co-transporter (upon glucose cotransport) and even a water and urea channel (Wright *et al*., 2003).

At the small intestinal level, the density of SGLT1 is highest in the jejunum, intermediate in ileum and lowest in duodenum (Yoshida *et al*., 1995). Hwang and collaborators (1991) showed that SGLT1 protein is restricted to the BBM of mature enterocytes, and that mRNA abundance increase from the lower villus to the tip. The high rate of sugar transport in enterocytes of the villus tip is due to the transcription of the SGLT1 gene in these mature enterocytes, subsequent translation of the mRNA and direct insertion of functional SGLT1 into the BBM of these cells. Yoshida and collaborators (1995) have shown that all enterocytes in the villus were positive for SGLT1 (not only those of the villus tip), and that there is an increase in its amount from the lower villus to the villus tip.

Regulation of SGLT1 is essential for the provision of glucose to the body and, thus is important for the maintenance of glucose homeostasis. Numerous factors that influence sugar transport through the regulation of SGLT1 have been described. Dietary sugars, activation of protein kinases (such as PKA and PKC), heat shock protein 70 (Hsp70), the intracellular regulatory protein – RS1, and transcription factors (HNF-1, Sp1 and Foxl1), are among others, the most emphasised (Ferraris 2001; Wright *et al*., 1997; Ikari *et al*., 2002; Veyhl *et al*., 2003; Katz *et al*., 2004; Martín *et al*., 2000).

Similarly to other intestinal nutrient transporters, SGLT1 is adaptively regulated by the type and amount of nutrients that enter the intestinal lumen. Thus, in most animal species, dietary sugars regulate the activity, as well as the levels of SGLT1 in the BBM of enterocytes (Dyer *et al*., 1997; Ferraris 2001). It has been shown in rats and also in humans that the presence of sugars (carbohydrate rich diet) increases the activity as well as the expression of SGLT1 in enterocyte BBM (Ferraris 2001; Dyer *et al*., 1997). In addition, intestinal expression of SGLT1 also exhibits circadian periodicity (Rhoads *et al*., 1998).

Protein kinases (PKA and PKC) also have a role in the regulation of SGLT1 activity. Hirsch *et al*. (1996) and Wright *et al*. (1997) have shown that when rabbit SGLT1 is expressed in *Xenopus laevis* oocytes, PKA and PKC activities modulate the number of SGLT1 in the plasma membrane and this occur by controlling the trafficking (exo- and endocytosis mechanisms) of this transporter protein. PKA activation increases the rate of transport in oocytes expressing rabbit SGLT1, whereas PKC activation decreases it (Wright *et al*., 1997). Although PKC inhibit rabbit and rat SGLT1, it stimulates the rate of transport when human SGLT1 is expressed. Thus, it seems that the regulation of vesicle trafficking by PKA and PKC is dependent on the structure of the transporter expressed (Wright *et al*., 1997). Recently, it was shown that the regulatory protein RS1 is able to down-regulate the expression of SGLT1 (in *Xenopus laevis* oocytes), and that this is dependent on dynamin and PKC. In fact it was shown that RS1 participates in transcriptional and posttranslational regulation of SGLT1. RS1 inhibits the transcription of SGLT1 in confluent LLC-PK1 cells derived from porcine kidney (Korn *et al*., 2001). This regulatory protein also inhibits SGLT1 trafficking to the plasma membrane through a posttranslational mechanism that modulates dynamin-dependent trafficking of intracellular vesicles to the plasma membrane. RS1 also modulates the PKC-dependent short-term regulation of SGLT1 (by decreasing the rate of transport trough human SGLT1 upon activation of PKC) (Veyhl *et al*., 2003). Recently it was demonstrated that RS1 regulated the exocytotic pathway of SGLT1 by inhibiting the dynamin-dependent release of SGLT1-containing vesicles from the trans-Golgi network (Kroiss *et al*., 2006). Several transcriptional factors, such as Foxl1, HNF-1 and Sp1, were also reported to be involved in the regulation of SGLT1 gene expression. Other proteins are involved in the regulation of activity and expression of SGLT1 in the BBM of epithelial cells. The Hsp70 has been shown to form a complex with SGLT1, being involved in the translocation of this sugar transporter to the BBM. According to Ikari and collaborators (2002), this interaction between Hsp70 and SGLT1 increases the expression and the activity of SGLT1 on the apical membrane of renal tubule cells, and consequently high levels of Hsp in the BBM up-regulates glucose uptake. The fluidity of plasma membrane also affects the activity of SGLT1. It was shown that fluidisation of the plasma membrane (by increasing the phospholipids-to-cholesterol ratio) or removing cholesterol from the membrane, strongly decreased glucose transport (Drozdowski & Thomson, 2006*b*;

Woudstra & Thomson, 2002). This leads to the conclusion that the activity of SGLT1 is optimal in an environment of low fluidity of the plasma membrane.

The dogma that all three monosacharides (glucose, galactose and fructose) are transported across the enterocyte basolateral membrane by a passive process (according their concentration gradients) through GLUT2 is the most commonly accepted.

The glucose transporter **GLUT2** belongs to the facilitative GLUT family of transporters. Similarly to the other 12 family members of the mammalian facilitated hexose transporters, GLUT2 possess 12 transmembrane helices with intracellular N and C terminus and a larger intracellular loop (connecting the 6th and 7th helices; Mueckler, 1994; Bouché *et al*., 2004) (**Fig. 11**). The transport of glucose trough this carrier is saturable, stereoselective and bidirectional. GLUT2 is found predominantly in the small intestine, liver, kidney and pancreatic β-cells (Hogan *et al*., 1991; Bouché *et al*., 2004). It corresponds to a low-affinity glucose transporter protein (K_m > 50 mM) that has a high transport capacity. The K_m for galactose and fructose is about 66 mM (Walmsley *et al*., 1998; Wright *et al*., 2003). This sugar transporter is sensitive to inhibitors such as phloretin and cytochalasin B (Thorens, 1993; Wright *et al*., 2003). At the intestinal level, the rate of glucose transport through GLUT2 is proportional to the glucose levels in the intestinal lumen. There is no doubt that the main pathway responsible for sugar exit from enterocytes involves the basolateral GLUT2. However, recently it has been proposed another pathway that involves trafficking of vesicles containing glucose, which requires glucose phosphorylation within the cell (into glucose-6-phosphate), accumulation in endossomes, and posterior dephosphorylation and release into the blood by exocytosis (Wright *et al*., 2007).

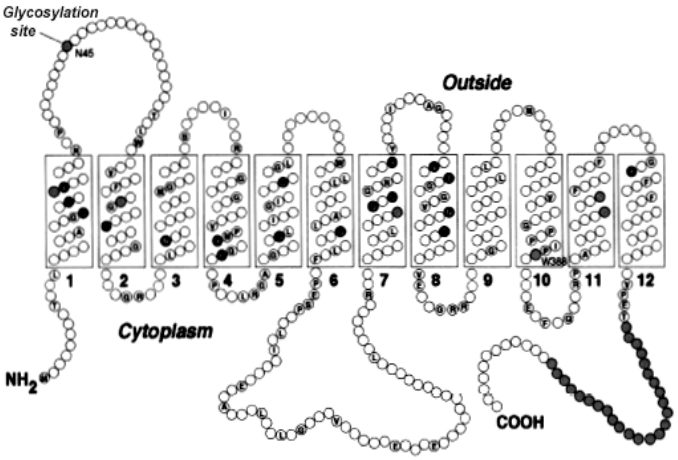

Fig. 11 – Secondary structure model for GLUT2 with 12 transmembrane helices. [Adapted from Bell *et al*., 1993]

Recently, however, it has been shown that GLUT2 can also be recruited to the BBM of enterocyte (within minutes – short term regulation) in situations such as an increase luminal glucose concentration (after a carbohydrate rich meal), diabetes and when a psychological stress are present (Kellet & Brot-Laroche, 2005; Boudry *et al*., 2007). In fact, Kellet & Brot-Laroche (2005) showed that there is a mechanism of GLUT2 insertion into the apical membrane that is promoted by the glucose transport through SGLT1, during assimilation of a carbohydrate enriched meal.

It is well established that luminal nutrients interfere structurally and functionally with small intestine and particularly with its absorptive cells. In the postabsorptive sate, glucose in the intestinal lumen is absent and therefore the expression of intestinal glucose transporters is at the basal level, as a result of the translocation of vesicles containing glucose transporters (by endocytosis) back to the intracellular compartments (Hirsch *et al.*, 1996). In fact, most of the SGLT1 resides in intracellular reserve pools (microtubule-associated vesicular structures) and only a smaller fraction appears in the BBM, as it was demonstrated in the enterocyte model cell line Caco-2 (Kipp *et al*., 2003). The significant amounts of SGLT1 in these intracellular pools can be translocated to the apical membrane and are therefore involved in the regulation of SGLT1 expression in the apical membrane (Kipp *et al*., 2003), in response to several stimuli (e.g. dietary sugars and diabetes). Protein kinases (A and C) have been shown to alter sugar transport activity by regulating the trafficking of vesicles containing SGLT1 transporter between intracellular pools and the plasma membrane (Hirsch *et al.*, 1996).

In some situations, such as during assimilation of a carbohydrate rich meal, the amount of D-glucose and D-galactose in the lumen is high leading to an increase in the number of functional SGLT1 and GLUT2 in the apical membrane of enterocytes (Ferraris & Diamond, 1989; Dyer *et al*., 1997; Kellet & Brot-Laroche, 2005) and consequent increase in glucose uptake, leading to changes in blood glucose levels. Luminal glucose regulates the activity and expression of SGLT1 by mechanisms that are independent of glucose metabolism (Solberg & Diamond, 1987; Dyer *et al*., 1997). It was considered that glucose binds to the intestinal epithelial sensor and generates an intracellular signal in enterocytes that via G-protein-coupled receptor linked to a cAMP/PKA signalling cascade leads to an increase in the SGLT1 mRNA and protein expression (Dyer *et al*., 2003; see section 1.2.2.1).

The **enteroendocrine cells** are, among other specialised cell types of the intestinal mucosa, important in the regulation of energy intake and the maintenance of glucose homeostasis through the actions of their secretions on the insulin and glucagon secretion by the endocrine pancreas (Drucker, 2007). The enteroendocrine cells are responsible for the synthesis of numerous hormones such as GLP-1, GLP-2, secretin, GIP and gastrin. Among this variety of gut hormones, two peptides (GLP-1 and GIP) are secreted by enteroendocrine L-cells and K-cells, respectively, during glucose absorption (after an oral glucose load or a meal) and potentiate the glucose induced insulin secretion from the pancreatic β-cells (Efendic & Portwood, 2004; Drucker, 2007). This phenomenon in which enteral glucose administration provokes greater insulin secretion than intravenous administration is called incretin effect (Efendic & Portwood, 2004). The incretin effect in healthy subjects depends on the amount of glucose given, and usually accounts for up to 60% of the insulin secretory response following an oral glucose lead (Nauck *et al*., 1986*b*). GIP and GLP-1 are involved in the maintenance of blood glucose levels within a narrow range, belonging therefore to the group of glucoregulatory

hormones. GIP and GLP-1 produce pancreatic and extrapancreatic effects, in order to contribute for glucose homeostasis.

GLP-1 is the incretin hormone to which more physiological actions have been attributed in the control of energy intake and assimilation. GLP-1 and GIP are called incretin hormones because they enhance β-cell response to glucose. Therefore, at pancreatic level, GLP-1 (and also GIP) acts as a potent insulin secretatgogue, stimulating glucose-dependent insulin secretion (Mojsov *et al*., 1987; see section 1.1.3.1). In addition, they also increase insulin biosynthesis, promoting insulin gene transcription (through the activation of the transcription factor PDX-1) and mRNA stability (through polypyrimidine tract binding protein (PTB)) (Stoffers *et al*., 2000; Wang *et al*., 1995). These actions are the result of activation of pathways that involve cAMP and PKA, as well as pathways that increase the intracellular concentration of Ca^{2+} (Drucker, 2007). GLP-1 and GIP also exert trofic effects, by inducing β-cell neogenesis and proliferation, and inhibiting apoptosis (through a mechanism that involve PI3K, PKB/Akt and/or PKCζ) (Doyle & Egan, 2007; Brubaker & Drucker, 2004).

GIP and GLP-1 exert common effects at the level of the pancreas, although GLP-1 is able to suppress glucagon secretion by α-cells of Langerhans islets contrarily to GIP (Drucker, 2007; Aronoff *et al*., 2004).

In addition to its pancreatic effects, GLP-1 and GIP also have a series of peripheral actions, all of them in order to promote enhanced glucose tolerance.

At the level of adipose tissue, only GIP produces insulin-like lipogenic actions and potentiate lipid storage (Drucker, 2007). By its turn, GLP-1 exerts effects on the central nervous system and cardiovascular system. GLP-1 enhances memory and neural survival and improves cardiovascular function after ischemia (Drucker, 2007). GLP-1 receptor stimulation was considered a key mechanism required not only for glucose-dependent insulin secretion but also for the function of the hepatoportal vein glucose sensor (Burcelin *et al*., 2000). It is also known that GLP-1 is involved in the inhibition of gastric emptying, gastric acid secretion (like GIP) thus promoting satiety and weight loss (Drucker, 2007). Although numerous studies have been developed in order to clarify the mechanisms of action of the incretin hormones (particularly GIP and GLP-1), the mechanisms linking nutrient absorption and GLP-1 secretion remains uncertain.

Another important aspect concerning incretin hormones is its short half-life. The half-life of biological active GIP is about 7 and 5 minutes in healthy and T2DM individuals, respectively, while for GLP-1 it is less than 2 minutes in healthy subjects (Deacon *et al*., 2000). These short half-lives are particularly due to degradation by a serine protease dipeptidyl peptidase IV (DPP-IV) enzyme and clearance by the kidney (Kieffer *et al*., 1995; Drucker, 2007). DPP-IV cleaves GIP and GLP-1 abolishing their capacity to stimulate insulin secretion,

and has even been suggested that their metabolites (particularly GLP-1-(9,36)) may act as GLP-1R antagonist (Vahl *et al*., 2003).

In T2DM this incretin effect is about half at that in healthy subjects, contributing for postprandial hyperglycaemia. The incretin contribution for the total insulin response is of about 73% in healthy individuals and only 36% in diabetic patients (Nauck *et al*., 1986*a*; Efendic & Portwood, 2004). This decrease in the incretin effect observed in T2DM is mainly attributed to a loss of GIP-regulated insulin secretion (probably due to decrease in the β-cell sensitivity to GIP, reduction of GIP insulinotropic effect, reduction in the expression of GIP receptors in β-cells and/or defects in post-receptor signalling pathways) as well as to a decreased secretion of GLP-1 (Efendic & Portwood, 2004). Recently, it was showed that taste signalling elements, namely the α-gustducin, and both type 1 G-protein-coupled taste receptors (T1R) – T1R2 and T1R3 – are co-expressed in mouse and human enteroendocrine cells (Margolskee *et al*., 2007; Jang *et al*., 2007). This shows that these cells are also involved in intestinal glucose sensing, and that taste-sensing mechanisms exist in the intestinal epithelium (Dyer *et al*., 2007; see section 1.2.2.1).

Recently, the small intestine was considered the third gluconeogenic organ, participating with about 13% of the total endogenous glucose production in diabetes (Mithieux G, 2001). Low levels of plasma insulin (during fasting and diabetic state) induce the expression of key enzymes of gluconeogenesis (glucose-6-phosphatase and PEPCK), leading to net glucose production from the main glucose precursor in the intestine – glutamine (Croset *et al*., 2001). These authors showed that the expression of pyruvate carboxylase is repressed and thus the gluconeogenic precursor's alanine and lactate are not used like in liver, but are "substituted" with glutamine. Alanine and lactate are released by the small intestine in the portal vein and are then actively taken up by the liver. In this way, the small intestine not only contributes directly for the endogenous glucose production with its net glucose production by using glutamine, but contributes also indirectly for the hepatic gluconeogenesis (Croset *et al*., 2001). However, small intestinal gluconeogenesis can be suppressed by insulin infusion. Thus, the small intestine is considered a new insulin-sensitive tissue (Croset *et al*., 2001).

In summary, plasma glucose regulation is a complex phenomenon where many pancreatic and gut hormones produce effects on numerous target tissues such as the liver, pancreas, muscle and adipose tissue. However, the importance of the small intestine as an active organ for glucose homeostasis is rising. Mechanisms concerning the enteroendocrine hormone signalling pathways (including interaction between hormone and its receptor), transport of glucose across the intestinal epithelium (including regulation of expression and/or activity of glucose transporters), among others, are relevant in glucose homeostasis and deserve more attention.

1.2.2. Adaptive epithelial responses of the small intestine – regulation of glucose transport

Changes in the concentration of luminal nutrients, hormone and ATP levels and even transcription factors are some examples of signals that induce intestinal adaptations. Morphological and physiological adaptations include changes in crypt depth and villus length, in enterocyte proliferation and nutrient uptake (glucose, amino acids and electrolytes), changes in cell migration rate and in reprogramming nutrient transporters expression (Ferraris & Diamond, 1997; Drozdowski & Thomson, 2006*a*). These adaptations usually occur under dietary regulation, in the presence of diseases (such as diabetes, small bowel disease), fasting and malnutrition and with aging, among others (Drozdowski & Thomson, 2006*a*). Some adaptations provide benefits, minimizing damages caused by, for example, the loss of a portion of small intestine (loss of absorptive surface area), as happens in the small intestinal resection. However, other adaptations such as those described for diabetes may have deleterious effects with enhanced nutrient uptake, which exacerbate hyperglycaemia (Woudstra & Thomson, 2002). The adaptations in intestinal sugar absorption may result from nonspecific or specific mechanisms.

According to Ferraris & Diamond (1997) **nonspecific mechanisms** of intestinal adaptations include:

1) changes in the surface area (which in turn include changes in cell size – hypertrophy – or in the total number of cells – hyperplasia – leading to alterations in mucosal area and thickness, intestinal villus weight and even total length);
2) changes in electrochemical gradient for Na^+ (which are responsible for changes in Na^+ dependent nutrient transport across the BBM, such as glucose uptake trough SGLT1);
3) changes in plasma membrane lipid composition (which may change the passive permeability of the membrane and the activity of transporters like SGLT1);
4) changes in the ratio of transporting to nontransporting cells (through differences in rates of cell division, maturation/differentiation, migration and exfoliation).

Specific mechanisms of intestinal adaptations include:

1) changes in the turnover number of transporters (i.e. number of substrate molecules transported per unit time, which affect the V_{max} of the transporter; e.g. changes in V_{max} induced by a high carbohydrate (HC) diet);
2) changes in the affinity constants (K_m) of a transporter for its substrate also affects the rate of transport (e.g. changes in K_m during enterocyte maturation process);

3) changes in site density of transporters per enterocyte (e.g. changes in the number of SGLT1 induced by a HC diet).

These changes can be modulated by hormones (glucocorticosteroids, growth hormone, glucagon-like peptide 2 (GLP-2), insulin-like growth factor 1 (IGF-1), among others) and diet.

Since the major studies of this thesis focus the effects of sage water extracts on the adaptive intestinal responses particularly to diabetes and diet, the following sub-section will introduce the regulation of intestinal sugar transport and the major intestinal adaptations to diet and diabetes (a pathological stress situation).

1.2.2.1. Adaptations to diet

Enterocytes are every day exposed to several nutrients that vary according to the type of meal. The intestine has the ability to adapt to variations in dietary concentration and composition (Ferraris, 2001; Sanderson & Naik, 2000). Changes in dietary constituents correspond to environmental signal that result in the expression of a variety of genes that in addition to other short term effects determine intestinal adaptation (Jump & Clarke, 1999). Luminal nutrients may even stimulate intestinal growth, at least in part, due to an increase in the expression of both glucagon-like peptide 2 and insulin-like growth factor 1 (Nelson *et al*.,2008).

It is well established that a carbohydrate rich diet induces increases in intestinal sugar transporters, promoting higher rates of sugar absorption (Cheeseman & Maenz, 1989; Ferraris 2001).

Two time scales seem to be involved with the dietary regulation of glucose transport, a short-term response that takes minutes and a long one taking days. A short-term regulation can be considered when, within approximately 1 hour, enterocyte glucose transporters respond to a change in luminal glucose concentration (Sharp *et al*., 1996). Long-term regulation corresponds to increases in glucose transport, one day after introduction of the carbohydrate rich diet (Ferraris, 2001). The reverse dietary switch causes a decline in glucose transport within 2-3 days (Ferraris & Diamond, 1992; Karasov *et al*., 1983). Consumption of carbohydrate rich diets increases intestinal sugar transport, particularly due to an increase in the amounts of SGLT1 in the BBM of enterocytes. Animals fed a HC diet showed high levels of SGLT1 in BBM reflected in changes in phlorizin binding site density and GLUT2 in BLM associated with an increase in glucose absorption (Ferraris & Diamond, 1992; Cheeseman & Maenz, 1989). Ferraris & Diamond (1993) showed that changes in site density of glucose transporters induced by dietary carbohydrate initially appeared in crypt cells (and some lower villus cells) before spreading over the course of several days to the tip of the villus. Also according to these authors (1992) after

crypt cells perceive the signal, nutrient transporters are programmed irreversibly (and not reprogrammed in mature enterocytes) and the time lag of changes in glucose uptake induced by diet was mainly due to cell migration times. But how and where is the signal perceived?

Since the gustatory G-protein α-gustducin was detected in BBM of rats enterocytes, it was suggested that mechanisms of glucose sensing in intestinal epithelium could be similar to those identified in taste cells of the lingual epithelium and are involved in the enhanced expression of SGLT1 shown in response to luminal sugars or sweeteners (Höfer *et al*., 1999; Margolskee *et al*., 2007). T1R2+T1R3 taste receptor seem to be the luminal sugar sensor (Dyer *et al*., 2007). Up-regulation of SGLT1 upon a high carbohydrate diet was not obtained in T1R3- and α-gustducin-knockout mice, which indicates that these taste transduction elements are involved in the inducible pathway that regulates SGLT1 expression when glucose is present in the lumen (Margolskee *et al*., 2007). However, according to these authors there is a constitutive level of SGLT1 expression that maintains its basal levels and is independent of luminal sugar sensing by T1R3 and/or α-gustducin. Because SGLT1 is expressed in enterocytes whereas T1Rs and α-gustducin are expressed in enteroendocrine L- and K-cells (the same that produce and secrete GLP-1 and GIP, respectively) a chemical signalling pathway that links these two types of intestinal epithelial cells must exist although not well characterised.

The increase in SGLT1 expression in the BBM after induction by carbohydrate-rich diet does not seem to be accompanied by parallel increases in mRNA (Lescale-Matys *et al*., 1993). This is a controversial matter, however, since several authors reported that increases in glucose transporters expression are correlated with increases in their mRNA (Miyamoto *et al*., 1993; Wood *et al*., 2000). Intestinal luminal infusion of glucose (but not intravenous) induces a significant increase in SGLT1 activity and protein levels in the BBM, but not in mRNA abundance, which suggests that regulation of SGLT1 expression by the diet seems to be the result of translational or post-translational mechanisms, as well as new enterocyte reprogramming (Lescale-Matys *et al*., 1993; Ferraris & Diamond, 1997).

Although the majority of studies have been done with laboratory animals, SGLT1 expression is also regulated by diet in the intestine of humans (Dyer *et al*., 1997). Human BBM vesicles exposed to luminal nutrients showed an increase in glucose transport, together with increases in the amount of SGLT1 protein (Dyer *et al*., 1997).

Additionally, changes in other dietary constituents, such as sodium and fibre, also modulate SGLT1 activity and expression. It is clear that SGLT1 activity is regulated by the levels of its substrates (glucose and Na^+) in the diet. Reduction of Na^+ in the lumen, due to a consumption of a low-salt diet, leads to a reduction of SGLT1 activity and consequently of intestinal glucose transport (Ferraris, 2001; De La Horra *et al*., 2001). Moreover, chronic consumption of dietary fiber leads to non-specific changes in the small intestine. It is known that the presence of fiber in the diet affects intestinal motility, intestinal mass and length,

enterocyte migration rate, life span and turnover, and even microbial metabolism (Chiou *et al*., 1994; Ferraris 2001). This regulation induced by fiber may confound the specific effects on intestinal glucose transport induced by changes in diet composition. Many studies reported that high fibre content in the diet decreases intestinal glucose transport. However, conflicting results have been obtained, where fiber may increase or not affect intestinal glucose transport (Ferraris, 2001).

Intestinal fructose absorption also increases in the presence of a high fructose or sucrose diet. According to Burant & Saxena (1994), this increase in fructose transport is a result of increases in enterocyte levels of GLUT5 protein and mRNA. Diet-induced changes in BBM transport are considered higher for fructose (2.5-3 fold) than for glucose (1.5-2 fold) (Ferraris & Diamond, 1997). Also the time scale is different. Four to eight hours seem to be necessary to increase fructose transport in response to dietary fructose, whereas the diet-induced increase of glucose transport is only achieved after one day. This difference resides in the fact that GLUT5 activity can be reprogrammed in mature enterocytes lining the villus, whereas SGLT1 activity is not fully modulated by diet in villus cells where vesicle translocation only occurs, but in crypt cells with the reprogramming of new enterocytes (Ferraris, 2001).

The amount and the type of dietary lipids also alter the functioning of the intestine. The lipid composition of the plasma membrane is affected by several dietary lipids (for example dietary fatty acids influence the BBM phospholipid fatty acid composition) but not by dietary cholesterol content. In fact BBM cholesterol composition is tightly regulated by the activity of an enterocyte microsomal membrane desaturase (Keelan *et al*., 1994). Modifying the lipid composition of the plasma membrane by dietary lipids usually leads to changes in the activity of membrane-bound proteins, among others SGLT1 and GLUT5 (Brasitus *et al*., 1989; Meddings *et al*., 1990). Dietary lipids that rise BBM fluidity increase the uptake of lipids while decreasing the uptake of glucose (Meddings, 1989; Meddings & Theisen, 1989). This seems to be associated with increases in the saturation of BBM phospholipids fatty acid (low membrane fluidity; low phospholipids-to-cholesterol ratio) that in turn promotes SGLT1 activity (Drozdowski & Thomson, 2006*b*). It has been suggested that dietary lipids may also change gene expression and consequently membrane composition and/or nutrient transport through the activation of the following transcriptional factors: PPARs, SREBP1c, HNF-4 and NFkB (Jump & Clarke, 1999). Dietary lipids bind to these transcriptional factors and interfere with gene transcription and consequently with protein synthesis of nutrient transporters (Sanderson 1998; Thiesen *et al*., 2003). However, lipids may also participate in signal transduction by activating second messengers, such as cAMP, Ca^{2+} and diacylglycerol, thus interfering with signalling pathways, such as those mediated by protein kinase C and mitogen-activated protein kinase (Huwiler *et al*., 2000).

Moreover, long-term **food deprivation** rapidly induces adaptive intestinal responses. A short period of fasting (2-3 days) induces alterations in intestinal mucosa, particularly villus hypoplasia (reduced DNA synthesis and crypt cell proliferation) and thus mucosal atrophy (reduced mucosal mass; Raul & Schleiffer, 1996). Also a reduction in BBM hydrolase activities (e.g. sucrase and aminopeptidase) is achieved with 24 hours of fasting, except for lactase (Raul *et al*., 1982). Recently, it was also shown that also starvation produces progressive small intestine atrophy, (characterised by decreased intestine weight, length and total epithelial cell number), and decreased villus density (Chappell *et al*., 2003). The mechanisms by which these changes occur involve decreased proliferation and migration of intestinal epithelial cells, accompanied by increased apoptosis in these cells (Chappell *et al*., 2003).

Ferraris & Diamond (1997) reported that starvation decreases intestinal nutrient transport per cm of intestine and suggested that this was due to a decrease in intestinal mass. However, these authors also report that the intestinal sugar transport per mg of intestine increases with food deprivation. This fact could be due to increases in the ratio of mature (transporting) to immature (nontransporting) cells (Ferraris & Diamond, 1997).

Fasting can also lead to increases in electrochemical gradient for Na^+ and to changes in the fluidity of the BBM, contributing to enhance transiently glucose transport across the intestinal epithelial cell membranes (Gupta & Waheed, 1992; Ferraris & Carey, 2000). An overnight fast can lead to a significant increase in the V_{max} in jejunum and ileum, which is accompanied by an increase in the electrical gradient for Na^+ entry. Changes in hexose uptake induced by starvation are similar to those obtained upon glucagon treatment (in fed rats), which suggested that raised levels of glucagon may be one factor responsible for these changes induced by starvation (Debnam & Thompson, 1984). However, food deprivation can lead to different responses of the intestinal mucosa. In sheep, food deprivation leads to decreased 3-O-methyl-α-D-glucose transport due to a global process to save energy (lowering the activity of Na^+/K^+-ATPase) or due to specific down-regulation of SGLT1 (Gabel & Aschenbach, 2002). In contrast, studies in mice suggested that chronic (but not acute) energy restriction increases the capacity of the intestine to absorb glucose (Ferraris *et al*., 2001). These alterations induced by a starvation period can be reversed rapidly by refeeding (Williamson, 1978). To maintain the normal small intestine architecture, more than 50% of the *ad libitum* diet is required as suggested by studies done in mice (Chappell *et al*., 2003). In summary, the adaptive changes of intestinal mucosa to food deprivation seem to depend on the duration of deprivation, the biological system, among other physiological aspects.

1.2.2.2. Diabetes modifications

Intestinal changes observed in experimental animal models of diabetes mellitus (such as those induced by the drugs streptozotocin (STZ) or alloxan) are considered important models to study effects on glucose and lipid absorption, and have proven to be good approaches in the study of the control of hyperglycaemia and hyperlipidaemia.

Mucosal hyperplasia (characterised by increased villus length), as well as hypertrophy of the villi of the proximal small intestine, have been shown in rats with chronic diabetes (Debnam *et al*., 1995; Fedorak *et al*., 1991). In addition, physiological changes occur in the small intestine of diabetic animals and humans (Debnam *et al*., 1990; Fedorak *et al*., 1987; Burant *et al*., 1994; Dyer *et al*., 2002). The increase in surface area (due to hypertrophy and hyperplasia) was not considered by several authors as completely responsible for the observed enhancement of active absorption of glucose in the small intestine of experimentally induced diabetic rats (Fedorak *et al*., 1987; Burant *et al*., 1994). A specific mechanism of intestinal adaptations seemed to underlie the enhanced glucose transport observed.

Studies of phlorizin binding showed that this increase is accompanied by an increased expression of SGLT1 transporters in the crypt region of the villus (Fedorak *et al*., 1987). Additionally, Burant and collaborators (1994) showed that the increase in glucose transport through the enterocyte of STZ-diabetic animals is due not only to increases in SGLT1 expression in BBM, but also GLUT2 in the BLM of the jejunum and ileum (contrarily to the decrease in GLUT4 reported for muscle and adipose tissue of diabetics). According to these authors the diabetes-induced increase in the expression of glucose transporters is due to a premature expression of these transporters (SGLT1 and GLUT2) in the enterocyte, along the crypt-villus axis, which causes a cumulative increase in transporter protein during enterocyte maturation. The uptake of fructose is also increased in insulinopenic diabetes.

The protein content of SGLT1, GLUT2 and GLUT5 increased from 1.5 to 6 times in jejunum and ileum enterocytes from STZ-diabetic rats (Burant *et al*., 1994). This increase seemed to be accompanied by changes in the expression of sugar transporter genes along the crypt-villus axis, since a 4 to 8 times increase in the amount of mRNA was observed (greater for SGLT1 and GLUT2 than for GLUT5). According to these authors, the expression of these genes also begins to appear in more immature cells of the lower villus and crypt. These increases in the levels of transporters correspond to an “adaptive” response by the organism in order to increase nutrient absorption and what the body perceives is a state of starvation. However, this increase in the absorptive capacity of the small intestine along with hyperphagia associated with diabetes contributes to exacerbate hyperglycaemia. It has also been proposed that GLUT2 is “trafficked” to the BBM of rat jejunum and the levels of GLUT2 in the apical

membrane are increased in the intestine of STZ-induced diabetic rat (Helliwell *et al*., 2000; Kellet & Helliwell, 2000).

The increase of glucose transport associated with diabetes attributed to the presence of more carriers per cell, may also result from the fact that diabetes can lead to increased number and longer life span of functionally mature cells along the villus, and even to an enhanced electrochemical gradient across the BBM (through increases in Na^+/K^+-ATPase activity) (Debnam *et al*., 1995; Ferraris & Diamond, 1997).

Also in humans, an increase in the capacity of the intestine to absorb monosacharides is present in T2DM patients, relative to health controls. The abundance of SGLT1 and GLUT5 increased significantly (4.3 and 4.1-times, respectively) in the BBM of T2DM patients compared with healthy controls. This increase in the protein expression was accompanied by a 3-times increase in the mRNA of the sugar transporters (SGLT1, GLUT5 and GLUT2) of the intestine of diabetic patients (Dyer *et al*., 2002). An increase of 1.5- to 2-fold in the abundance and activity of disaccharidases (sucrase and lactase), accompanied by a 1.5-fold increase in the abundance of microvillar structural proteins – villin and β-actin, was also observed in the intestine of T2DM patients (Dyer *et al*., 2002). Therefore, the increased capacity of the human diabetic intestine to absorb monosaccharides seems to be due to structural changes of the small intestine (as demonstrated by the abundance of the structural proteins villin and β-actin) combined with a specific increase in the expression of the intestinal sugar transporters – SGLT1, GLUT2 and GLUT5.

A HC diet increased the expression of SGLT1 in the small intestine of mice, with predominant increase in crypt enterocytes (see previous section; Ferraris *et al*., 1992), similarly to what is obtained with experimentally induced diabetes in rats. But is there any effect of a HC diet on the intestinal sugar absorption in a diabetic condition? HC diet seems to increase the already enhanced rates of glucose absorption in models of diabetes. Diet-induced increases in sugar uptake rate combined with an enlarged absorptive mucosa resulted in much higher rates of total intestinal sugar absorption compared with non-diabetic mice fed a HC diet (Ferraris *et al*., 1993). According to Ferraris *et al*. (1993), chronic diabetes enhanced sugar transport in mice (by non-specific increases in intestinal mass, although we already know that this evidence does not completely explain the enhanced absorption capacity) and dietary carbohydrate further stimulated sugar transport in these mice by specific increases in glucose transport per mg of tissue. This can be explained by the presence of more carriers per unit surface area (cm^2), due to a rapid translocation of pre-existing carriers to the BBM, or to less recycling of the carriers from the membrane.

Recently, Casirola & Ferraris (2006) showed that α-glucosidases inhibitors, such as the oral andiabetic agent acarbose, prevent the diet-induced increase in intestinal glucose uptake in diabetic mice. Treatment with acarbose (antidiabetic drug currently used to reduce postprandial

hyperglycaemia) does not affect mRNA and protein expression of SGLT1 and GLUT2 (Paiva *et al*., 2002) although it regulates intestinal sugar transport by affecting luminal sugar concentrations. Casirola & Ferraris (2006) using diabetic mice showed that acarbose (by inhibiting intestinal α-glucosidases) decreases the amount of glucose available in the lumen, thus inducing adaptive decreases in glucose transport. This effect reduces postprandial glycaemic excursions, particularly beneficial for the treatment of T2DM and obesity.

Also the intestinal absorption of lipids is increased in diabetic rats, and can be normalised by administration of insulin, Langerhans islet transplantation or with a polyunsaturated fatty acid diet (Thomson & Rajotte, 1984; Thomson & Rajotte, 1985; Thomson *et al*., 1987). The altered lipid transport observed in diabetic animals can be associated with changes in lipid composition of the intestinal BBM (Keelan *et al*., 1990; Keelan *et al*., 1985). Changes in the Na^+ electrochemical gradient across BBM of enterocytes are also observed in a diabetic situation. Lower Na^+ permeability of the BBM was observed in diabetic rats, which in turn enhances the electrical and chemical driving force for active Na^+-dependent glucose uptake (by reducing glucose-independent movement of Na^+ across this membrane) (Debnam & Ebrahim, 1989).

1.3. Oral drug therapy in type 2 diabetes mellitus

Since T2DM is strongly related to sedentary lifestyle and wrong dietary habits, it represents the type of diabetes to which treatment and/or prevention through dietary strategies makes more sense. Long term T2DM treatment is difficult to achieve and in light of the dramatic epidemic proportions of this type of diabetes, there is great interest in identifying and implementing interventions to prevent or delay its onset (Curtis & Wilson, 2005).

The ideal treatment of T2DM should reverse insulin resistance and β-cell dysfunction and prevent or reverse long-term complications. The first step in the treatment of T2DM is lifestyle changes such as changes in diet (opting for low fat diets with high content of fiber and low glycaemic index) and increasing daily physical activity (**Table 2**). Although lifestyle modifications (e.g., diet, physical exercise, weight loss and stop smoking) are the basis of diabetes therapy, most patients will also require pharmacotherapy to achieve the better glucose concentration, and in some cases they need exogenous insulin administration (Triplitt, 2007). In fact, in most cases of T2DM patients, diet and exercise are not sufficient to achieve adequate glycaemic control, and when the establishment of the control aggravates it is required to introduce an oral hypoglycaemic agent (Feinglos *et al*., 2005).

The main pharmacological drugs that have been used in the treatment of T2DM are: **α-glucosidase inhibitors**, to delay intestinal carbohydrate absorption (e.g. acarbose); **biguanides**

to target hepatic insulin resistance (e.g. metformin); **insulin secretagogues or sulphonylureas**, to increase pancreatic insulin secretion and also increase glucose-induced insulin secretion (e.g. glibenclamide; gliclazide); **insulin sensitisers or thiazolidinediones**, to target adipocyte and muscle insulin resistance (e.g. troglitazone; rosiglitazone); and **intestinal lipase inhibitors**, to inhibit fat absorption and promote weight loss in obese patients (e.g. orlistat) (**Table 2**; Cheng & Fantus, 2005; Cheng, 2005).

Table 2 – Oral pharmacological approaches used in the treatment of T2DM.

Pharmacological approach					
Class	**Drug**	**Action**	**Molecular target**	**Mechanism of action**	**Site(s) of action**
Biguanides	Metformin	Reduce insulin resistance Reduce hepatic glucose output	AMPK	Increases liver and muscle insulin sensitivity Inhibits hepatic gluconeogenesis	Liver, muscle
Sulphonylureas	Glibenclamide Glicazide Glimepiride	Increase insulin secretion (insulin secretagogues)	Sulphonylurea receptor	Bind to sulphonylurea receptor on β-cell, leading to closure of ATP-sensitive K^+-channels	Pancreatic islet β-cells
Non-sulphonylureas (Meglitinide analogs)	Repaglinide Nateglinide	Acute increase of insulin secretion (insulin secretagogues)	Sulphonylurea receptor	Bind to sulphonylurea receptor on β-cell, leading to closure of ATP-sensitive K^+-channels	Pancreatic islet β-cells
Thiazolidinediones	Pioglitazone Rosiglitazone	Reduce insulin resistance (insulin sensitisers)	PPARγ	PPARγ agonist (increase liver, adipose and muscle insulin sensitivity)	Liver, fat, muscle
α-Glucosidase inhibitors	Acarbose	Delay absorption of carbohydrates	α-Glucosidase	Inhibits α-Glucosidase	Intestine
Agents that reduce fat absorption	Orlistat	Reduce weight	Lipase	Inhibits gastric and pancreatic lipase	Intestine
GLP-1 analogs (*)	Exenatide (Exendin-4)	Stimulates glucose-dependent insulin secretion Inhibit glucagon secretion Delay gastric emptying (incretin mimetic)	GLP-1 receptor	GLP-1 receptor agonist	Pancreatic islet β-cells
DPP-IV inhibitors (*)	Vildagliptin	Enhance incretin actions	DPP-IV enzymes	Inhibits DPP-IV	Capillaries close to the intestinal cells

(*) Emerging therapies awaiting results of clinical studies. AMPK: adenosine monophosphate-activated protein kinase, PPARγ: peroxisome proliferators-activated receptor-gamma; GLP-1: glucagon-like peptide-1; DPP-IV: dipeptidyl peptidase IV.

Biguanides, particularly metformin, has been used to treat diabetes since the middle ages. The major action of this oral agent is to decrease hepatic glucose production, by decreasing gluconeogenesis and thus glucose output. Metformin also increases liver and skeletal muscle insulin sensitivity (Cheng & Fantus, 2005). The key mediator of beneficial effects of metformin seems to be the adenosine monophosphate-activated protein kinase (AMPK). Zhou and colleagues (2001) showed that metformin activates hepatic and muscle AMPK that results in phosphorylation and inhibition of acetyl-CoA carboxylase (catalyzes the rate-limiting step of lipogenesis) and also decreases expression of SREBP-1c, thereby decreasing gene expression of lipogenic enzymes. In addition to blocking fatty acid synthesis, metformin also promotes fatty acid oxidation. It has been shown that metformin is also beneficial in weight loss and in the improvement of lipid profile. Reductions of plasma levels of free fatty acids, triglycerides and very-low-density lipoproteins were observed in patients with high baseline levels (Abbasi *et al*., 1997; Landin *et al*., 1991). Thus, metformin has significant cardiovascular benefits. The major side effects that affects one-third of patients are nausea, anorexia or diarrhoea (Williams & Pickup, 2004).

Sulphonylureas stimulate insulin secretion and are thus called insulin secretagogues. They bind to the sulphonylurea receptor on the β-cell plasma membrane, resulting in the closure of the ATP-sensitive K^+ channels, membrane depolarisation and opening the voltage-dependent Ca^{2+} channels, calcium influx and exocytosis of insulin contained in vesicles (Cheng & Fantus, 2005). The major side effects are hypoglycaemia (mainly with glibenclamide and additional risk factors, such as, excess alcohol intake and impaired renal function) and weight gain (Williams & Pickup, 2004; Cheng & Fantus, 2005).

Non-sulphonylureas (insulin secretagogues) have its mechanism of action similar to those of sulphonylureas, namely closure of K_{ATP} channels, leading to Ca^{2+}-dependent insulin secretion. However, the binding site to the sulphonylurea receptor and the kinetics are different (Cheng & Fantus, 2005). The response is faster and the half-life is shorter, which result in an acute increase in insulin secretion (Cheng & Fantus, 2005). They are used to control postprandial hyperglycaemia and they can also produce hypoglycaemia (Williams & Pickup, 2004).

Thiazolidinediones (TZDs) are another class of oral agents to treat T2DM. They are insulin sensitisers that enter the cell and bind to the peroxisome proliferators-activated receptor-gamma (PPARγ) that is a nuclear receptor expressed mainly in adipocytes (but also in muscle and liver). Once these nuclear receptors become ligand-activated transcription factors, they are involved in the regulation of expression of numerous genes of carbohydrate and lipid metabolism (Cheng & Fantus, 2005). TZDs improve insulin sensitivity, by enhancing the expression of insulin-sensitive genes (e.g. GLUT4, lipoprotein lipase, fatty acid transporter protein, fatty acyl CoA synthase), increasing glucose uptake, adipocyte lipogenesis, and

decreasing plasma fatty acid levels. TZDs can additionally reduce insulin resistance, because they decrease production of cytokines, such as, TNF-α and resistin (Cheng & Fantus, 2005). Thus TZDs treatment leads to a reduction in circulating triglycerides levels, modest increases in HDL levels and decreased blood pressure. The major side effects of TZDs are weight gain, edema, anemia and even congestive heart failure (Williams & Pickup, 2004; Cheng & Fantus, 2005).

Alpha-glucosidase inhibitors, such as acarbose, are used in the treatment of T2DM to decrease intestinal carbohydrate absorption. They act primarily by inhibiting the activity of digestive disaccharidase enzymes (α-amylase and α-glucosidases) in the intestine, thus reducing particularly postprandial glucose levels. Clinical trials with acarbose also showed lowering effects on HbA_{1c} and also a reduction in plasma triglyceride levels (Cheng & Fantus, 2005). The main side effects are gastrointestinal, such as flatulence, diarrhoea and muscle spasms that can be diminished with time (Williams & Pickup, 2004).

Agents that reduce fat absorption, such as orlistat, act locally in the gastrointestinal tract. They inhibit the activity of gastric and pancreatic lipases, involved in the digestion of triglycerides (dietary fat) into absorbable free fatty acids and monoglycerides. This leads to a decrease in energy absorption and weight loss Cheng & Fantus, 2005). Gastrointestinal side effects can occur frequently, such as flatulence, steatorrhoea, increase frequency of defection and fecal incontinence (Williams & Pickup, 2004; Cheng & Fantus, 2005).

Recently, new therapeutical approaches for T2DM that mimic the action of the gut hormone GLP-1 have been developed. As referred above GLP-1 is an incretin hormone that stimulates glucose induced insulin secretion in pancreatic β-cells (Mojsov *et al*., 1987). In addition to its effects on insulin secretion, GLP-1 exerts other physiological actions relevant to the control of postprandial hyperglycaemia observed in T2DM, including stimulation of insulin biosynthesis, inhibition of glucagon secretion, inhibition of gastric emptying and acid secretion, reduction of food intake and trophic effects on the endocrine pancreas (Gallwitz, 2006; see section 1.2.1). The function of this incretin hormone depends on elevated levels of glucose in the plasma therefore it does not produce hypoglycaemia, which makes GLP-1 favourable for application as therapeutic agents. However, GLP-1 has to be administered parenterally and has a short half-life (due to the rapid inactivation by the enzymes DPP-IV), which makes it unsuitable for daily use. Because of this, two strategies have been developed. The first one is the development of **GLP-1 analogs** (e.g. liraglutide and exenatide) rendering natural GLP-1 resistant to the degradation by DPP-IV and with a GLP-1-like action (GLP-1 receptor agonists). Another approach was the development of **DPP-IV inhibitors** (drugs that increase endogenous GLP-1 by inhibiting DPP-IV enzymes) (Riddle & Drucker, 2006). These DPP-IV inhibitors (e.g. vildagliptin) not only extended the half-life of this incretin hormone, stimulating pancreatic insulin secretion, but also reduce hepatic glucose production by suppressing pancreatic

glucagon secretion in diabetic patients. In contrast with the some therapies mentioned above that can prevent or delay T2DM, GLP-1 based therapies, namely by administration of exogenous synthetic GLP-1 receptor agonists, long-acting GLP-1 analogs or through the inhibition of DPP-IV, seems also to have benefits in diabetes treatment (Cheng, 2005; Gallwitz, 2006).

The particular drug treatment used in a patient with T2DM should be determined by the physician having into account the balance between β-cell impairment and insulin resistance in that case. When oral agents are ineffective, insulin is required to achieve better control. In fact, sometimes monotherapy reveals insufficient glycaemic control, thus combination therapy has been tested in order to achieve a better glycaemic control and also reduction risk of cardiovascular disease. None of the drugs alone corrects all of the pathogenic abnormalities found in T2DM, and some randomised placebo-controlled trials tested the combination of two therapeutical agents (e.g. biguanide plus a secretagogue/sulphonylurea (Glucovance®) or a thiazolidinedione (Avandament®); insulin plus sulphonylurea, among others) (Krentz & Bailey, 2005; Herbaut, 2005). However, the cost/benefit ratio of combined therapy remains to be evaluated in large randomised trials.

According with Jermendy (2005) the evidence from large clinical trials established that T2DM is a preventable disease, and drug therapy should not be considered as a routine for preventing T2DM.

1.4. Other therapeutical targets and approaches

Parallel to the emerging approaches for the control of glycaemia based in GLP-1 analogs and DPP-IV inhibitors, new hepatic targets have been identified and compounds that counteract the glycaemic response to glucagon have been identified.

These new targets include the glucagon receptor (inhibiting glucagon binding or affecting the coupling between glucagon receptor and the adenylate cyclase) and key enzymes involved in the two processes responsible for the hepatic glucose production – gluconeogenesis and glycogenolysis. Other targets have also been reported such as protein tyrosin phosphatases (PTPs; that reduce insulin action, leading to insulin resistance; Koren & Fantus, 2007) and oxidative stress (LDL oxidation, AGEs formation, among others) (Wiernsperger, 2003).

In order to reduce glucagon action on the liver, glucagon receptor antagonists (GRAs) have been identified, suggesting a useful strategy for the treatment of T2DM (Jiang & Zhang, 2003). These GRAs (nonpeptide small molecules) act by inhibiting the binding of glucagon to its receptor or the potential of glucagon to stimulate cAMP production, thus decreasing hepatic glucose output (Jiang & Zhang, 2003; Agius, 2007). However, it is important to find

compounds that can be used as alternative to GRAs, since it was shown that they cause accumulation of glucagon in plasma and/or pancreatic islets (Agius, 2007). In this way, compounds that affect the coupling between glucagon receptor and adenylate cyclase; that inhibit the activity of key enzymes of gluconeogenesis or glycogenolysis seem more promising.

These alternative approaches to GRAs are compounds that function as inhibitors of gluconeogenic enzymes (e.g. peroxovanadium compounds that are inhibitors of glucose-6-phosphatase and AMP-mimetic compounds or fructose-2,6-biphosphate that are inhibitors of fructose-1,6-biphosphatase); inhibitors of glycogenolytic enzymes (e.g. active-site (glucose analogue) inhibitors, AMP-site and indole-carboxamine site inhibitors are the major pharmacological glycogen phosphorylase inhibitors that mimic the action of several physiological ligands) and activators of glycolitic enzymes (e.g. glucokinase activators (GKAs) that dissociate this enzyme from its inhibitor, the glucokinase regulatory protein, or increase its affinity for glucose) (Agius, 2007).

Generically, these compounds cause a lowering of blood glucose levels (sometimes dose-dependently), by stimulating hepatic glucose phosphorylation, glycolysis and glycogenesis through gene therapy (regulating the expression of key enzymes involved in these processes). However, the administration of these compounds lead to a variety of side effects including hypoglycaemia, excessive glycogen accumulation (e.g. due to accumulation of glucose-6-phosphate), induction of lipogenic genes and increase in plasma and hepatic triglycerides that may compromise their application (Agius, 2007).

Almost all of these compounds have been identified by pharmaceutical industries, in *in vitro* and *in vivo* studies with animals (particularly rodents). Studies with humans are fewer and proof of efficacy of such compounds free of side effects in T2DM patients is still awaited. The main purpose of the work presented in this thesis was to find in the known medicinal plants new therapeutical targets and approaches that produced a more effective control of glycaemia without the undesirable side effects.

1.5. Nutrition and type 2 diabetes mellitus

Dietary approaches that reduce risk of several diseases, such as cardiovascular diseases, cancer and age-related diseases, are also recommended as particularly beneficial to reduce the epidemics of T2DM, obesity and associated complications. Interventions based on changes in the diet have been shown to reduce 30 to 50% the incidence of diabetes and associated cardiovascular complications in high-risk groups (Pan *et al*., 1997).

The major approach of dietary therapy for T2DM patients is to reduce absorption rates, which allow plasma clearance of glucose and make the sense of satiety, last longer. The main

factors that contribute for that approach are: increase frequency and decrease meal size; increase viscous soluble fibre in diet (e.g. pectin and β-glucan); select low-glycaemic index foods (e.g. dried legumes, barley and pasta) and the use of inhibitors of carbohydrate digestion and thus absorption (e.g. acarbose) (Jenkins *et al*., 2004). The advantage of prolonging carbohydrate absorption seems to be related to the more efficient metabolism of glucose over time (Jenkins *et al*., 2004).

High intake of carbohydrates with a high-glycaemic index (a relative measure of the increment in plasma glucose per gram of ingested carbohydrate) increase insulin resistance compared with intake of low-glycaemic index foods (Willett *et al.*, 2002). Low fat diets with high content of fiber and low-glycaemic index have been associated with lower risk to develop T2DM.

Saturated fat should be reduced and replaced with monounsaturated fat, such as olive oil or polyunsaturated fats. Dietary cholesterol is especially detrimental in diabetic people therefore the consumption of some foods (e.g. eggs) should be minimal. Diets higher in monounsaturated fatty acids, fibers and low levels of carbohydrates have been reported to reduce cholesterol and postprandial blood glucose levels (Hung *et al*., 2003). Fish rich in ω3 fatty acids and with lower levels of triglycerides, are recommended. Thus, 2 to 3 servings of fish per week are beneficial, and have been reported that reduces cardiovascular complications in diabetes (Kris-Etherton *et al*., 2003).

Foods that usually improve glycaemic control and reduce the risk of cardiovascular diseases are grains (e.g. brown rice and oats) and foods with high content of fibers (e.g. cereals, fruits and vegetables) (Williams & Pickup, 2004).

The "diabetic foods" that contain sorbitol or fructose as sweeteners (instead of sucrose) are not recommended because they provide calories to the body. They usually raise blood glucose, triglycerides and cholesterol levels (Bantle, 2006). Thus, for people with T2DM, trying to lose weight, these sweeteners are not recommended. The increase in consumption of this type of sweeteners is associated with the growing prevalence of obesity and cardiovascular complications (Bantle, 2006; Lorenzy, 2007). Therefore, sucrose should not be removed completely from the diabetic diet, although its consumption must be moderate (Williams & Pickup, 2004).

Dietary plans for diabetic patients should take into account energy intake to energy expenditure ratios and quality of fat and carbohydrate, rather than quantity alone. Therefore these diets seem to be beneficial in decreasing insulin resistance, improving glycaemic control and blood lipid profile (Hung *et al.*, 2003). These types of diets are also the most effective in weight reduction and therefore relevant to the control of obesity.

However, even though right dietary choices constitutes one of the most important behavioural aspects of diabetes treatment and prevention, patients fail to adhere to

recommendations (for dietary management and physical exercise), which contributes for the frustration of preventive plans.

1.6. Diabetes Prevention

Subjects with impaired glucose tolerance (IGT), impaired fasting glycaemia (IFG) and/or obesity are undoubtedly candidates for application of T2DM prevention, because they are at high risk. Therefore they should opt for weight reduction strategies, such as physical exercise, in order to prevent this disease. Regular physical exercise is an important component of the healthy lifestyle for everyone. Numerous healthy benefits can be achieved from physical exercise, including improvement of long-term glycaemic control, as well as lipid profiles and of insulin sensitivity (Steppel & Horton, 2004; see also section 1.1.3.3). According to the Da Quing study performed in IGT subjects, diet and exercise reduced the risk to develop T2DM in 31% and 46% respectively, whereas diet plus exercise reduce in 42% (Pan *et al*., 1997, **Table 3**).

Other recent clinical trials have provided proof that progression to T2DM is not inevitable, even in people at high risk, and that interventions designed to reverse or ameliorate the pathophysiology of glucose intolerance can delay its onset. These clinical studies (randomised placebo-controlled trials) have demonstrated that the risk of T2DM could be decreased by non-pharmacological (lifestyle modification, weight reduction strategies) or by pharmacological interventions (such as with metformin, acarbose or orlistat) in subjects with IGT and obesity as well as with troglitazone in women with previous gestational diabetes (Jermendy, 2005).

Table 3 summarises these major recent studies on diabetes prevention and specifies, among other information, the population involved in the study as well as the type of intervention in each one.

There are numerous pharmacotherapeutic agents available for the treatment of T2DM, although besides their incomplete effectiveness, they are either too expensive and/or have undesirable side effects. Five to ten percent of the population with diabetes experience secondary failure, that can be due to decreasing β-cell function, poor adherence to treatment, weight gain, reduction of exercise, changes in diet, or illness (Triplitt, 2007).

Table 3 – Recent Major Diabetes Prevention Studies (adapted from Curtis & Wilson, 2005).

Study (Year)	Population	N	Design	Intervention	RR↓ vs Placebo
Da Quing (1997)	> 25 y IGT	577	Randomized controlled trial	Diet and Exercise	42%
DPS (2001)	40-65 y BMI=25 kg/m^2 IGT	522	Randomized controlled trial	Diet and Exercise	58%
DPP (lifestyle, 2002)	= 25 y BMI=24 kg/m^2 IGT	3234	Randomized controlled trial	Diet and Exercise	58%
DPP (metformin, 2002)	= 25 y BMI=24 kg/m^2 IGT	3234	Randomized controlled trial	Metformin	31%
TRIPOD (2002)	Hispanic nondiabetic woman with history of gestational diabetes	235	Randomized controlled trial	Troglitazone	56%
STOP-NIDDM (2002)	40-70 y BMI=25-40 kg/m^2 IGT FPG 100-140 mg/dl	1429	Randomized controlled trial	Acarbose	25-36%
XENDOS (2004)	30-60 y BMI=30 kg/m^2	3305	Randomized controlled trial	Orlistat	33.7%

N – number of intervenients; y – year; RR – Reduced Risk; BMI – Body Mass Index; IGT – Impaired Glucose Tolerance; FPG – Fasting Plasma Glucose.

With aging, sulfonylureas and insulin therapy significantly increases the risk of severe hypoglycaemia. Biguanides are effective in the treatment of older patients with diabetes, although these medications can be contraindicated because of congestive heart failure, renal insufficiency, liver disease, or may not be tolerated because of gastrointestinal adverse effects (Josse *et al*., 2003; Meneilly & Tessier, 1995). As a result, there has been increasing interest in the use of alternative therapeutic agents for the treatment of diabetes especially in the elderly population. However, prevention is still a good possibility to alleviate the huge increase in T2DM prevalence.

In Chinese Medicine, Native American Medicine and Tibetan Medicine it is common to appeal to dietary measures and traditional plant therapies to the management of diabetes (Vuksan & Sievenpiper, 2005; Kaleem *et al*., 2006). Jia *et al*., (2003) reported that the use of these naturally derived agents together with conventional drug treatments, such as a chemical agent or insulin, allows the use of lower doses of the drug and/or decreased frequency of administration which decreases the side effects commonly observed. The World Health Organisation (WHO) has also recommended the evaluation of the plants' effectiveness in conditions where we lack safe modern drugs (Day, 1998).

Natural products are the source of oral antidiabetic drugs that have been used in the treatment, prevention or to delay the progression of diabetes. The best example is metformin.

The biguanide metformin was first derived from *Galega officinalis* and is now the only example of an approved antidiabetic drug that was developed from an herbal source with a long history of use for diabetes (Bailey, 1992; Vuksan & Sievenpiper, 2005). It has been reported that metformin prevents the progression of impaired glucose tolerance to overt T2DM. According to the Diabetes Prevention Program trial metformin reduced by 31% the risk to develop T2DM in subjects with IGT (Knowler *et al*., 2002). Thus, this compound in addition to its use in treatment of T2DM (see section 1.3) can also be beneficial in prevention.

Acarbose, a well-known α-glucosidase inhibitor, was first isolated in 1975 from *Actynomycetals* species, and is today produced industrially using developed strains of *Actinoplanes sp.* SE 50/110 (Perion *et al*., 2003; Brunkhorst & Schneider, 2005). The results of the STOP-NIDDM trial showed that acarbose by delaying carbohydrate digestion and thereby reducing postprandial hyperglycaemia, reduced the risk for developing diabetes in individuals with IGT. This indicates that the most prescribed α-glucosidase inhibitor can also be considered useful in the prevention of T2DM, and also shows the potential of other products that decrease postprandial glucose absorption.

Both metformin and acarbose supports the idea that medicinal approaches that include natural sources may be promising in T2DM prevention and also treatment.

1.7. Medicinal plants as sources of new bioactive compounds

In recent decades we have seen a resurgent in the interest in traditional plant treatments for diabetes. We have witnessed to a growing public interest and awareness of complementary and natural types of medicine.

Food restriction and traditional plant treatments were the cornerstone of antidiabetic therapies before the introduction of insulin therapy in 1922 (Day, 1998). At the moment we do not know any plant that substitutes insulin therapy essential for T1DM and none is known as a natural source of this hormone. This is probably the main reason why occidental societies continue preferring insulin administration to manage diabetes. However, as said by Day (1998) "although insulin is a life-saver is not a cure-all". For the majority of T2DM patients, treatment with insulin is not the ideal strategy since these patients are sufficiently resistant to this hormone that even high levels of insulin in circulation are insufficient to control hyperglycaemia (Day, 1998).

Despite the numerous preventive strategies and pharmacological interventions, the management of T2DM remains grossly unsatisfactory, and the disorder is still growing and gaining epidemic proportions. The cost-effectiveness of diabetes treatment is high and becoming a real concern, thus the necessity for more effective and cheaper management

modalities is today widely acknowledged. Complementary and alternative medicine approaches that include herbs may be promising.

There is a growing database of clinical trials that have been investigating the effects of several herbs in diabetes. Herbs such as ginseng (*Panax* spp.), ivy gourd (*Coccinia indica*), garlic (*Allium sativum* and *Allium cepa*), holy basil (*Ocimun sanctum*), fenugreek (*Trigonella foenum graecum*), milk thistle (*Silibum marianum*), gurmar (*Gymnena sylvestre*), bitter melon (*Momordica charantia*), *Aloe vera* and *Ginkgo biloba* are examples of herbs with clinical information in diabetes (Vuksan & Sievenpiper, 2005).

This ethnopharmacological approach that involves ethnobotany and screening of traditional systems of medicine for candidate therapies may be more effective than the random *in vitro* "high-throughput" screening for new drug therapies preferred by pharmaceutical companies, especially for complex metabolic disorders such as diabetes. The mechanisms of action include the delay of glucose absorption in the gut (e.g. *Aloe vera*, ginseng, prickly pear cactus, fenugreek), the increase of glucose disposal (e.g. ginseng, fenugreek, fig leaf, ivy gourd, bitter melon), and the glucose stimulated insulin secretion (e.g. ginseng, garlic, holy basil, bitter melon, gurmar) (Vuksan & Sievenpiper, 2005).

The antioxidant activity of medicinal plants that may contribute to their antidiabetic effects, have been attributed to a number of constituents, where phenolic compounds, alkaloids, flavonoids and glycosides are the most important. The chronic hyperglycaemia that characterises diabetes is associated with oxidative stress in many tissues. High levels of glucose produce more reactive species, which lead to oxidative damage in the cells (glucotoxicity) (Robertson & Harmon, 2006). Antioxidants are emerging as therapeutic agents since they scavenge free radicals, preventing damages in the cell (Ratnam *et al*., 2006). Studies with diabetic animals showed that pharmacologic protection against oxidative stress (through the use of antioxidants) reduces diabetes progression (Tanaka *et al*., 1999; Harmon *et al*., 1999; Kaneto *et al*., 1999). Once antioxidants are part of the diet (foods and herbs) their biopharmaceutical properties should be exploited.

1.7.1 The genus *Salvia*

Several species of *Salvia* (family Lamiaceae) have been used as medicinal plants since ancient times. The genus name *Salvia* comes from the Latin *salvāre* meaning "to save" or "to heal" and comprises about 900 known species (Dweck, 2000).

The ancient use of *Salvia* species (namely *Salvia officinalis*) as medicinal plants is shown by several old reports. As cited by Dweck (2000), in an Anglo-Saxon manuscript we can

read “why should man die when he has sage?”. Sage species have been extensively used as pharmaceutical herbs, and because of the wide range of medicinal effects sage enjoys the reputation of being a universal remedy. Most of the plant material used is still collected from natural stands, especially in some countries of the Eastern Mediterranean (such as former Yugoslavia, Albania, Greece and Turkey) (Karamanos, 2000).

Aerial parts of these plants usually contain as bioactive compounds: flavonoids (such as luteolin-7-glucoside, apigenin), phenolic acids (such as rosmarinic acid, caffeic acid, and ferulic acid) and triterpenoids (such as ursolic acid) as well as essential oils (such as monoterpenoids) (Lima *et al*., 2005; el-Sayed *et al*., 2001).

Several species of the genus *Salvia* are empirically used to treat diabetes mellitus. *S. fruticosa*, *S. officinalis*, *S. lavandulifolia* and *S. plebeia* have been studied in normal and alloxan- or STZ-diabetic animals (Zarzuelo *et al*., 1990; Alarcon-Aguilar *et al*., 2002). According with these authors, the best results were obtained with *S. lavandulifolia*. *Salvia miltiorrhiza* is another sage specie that is commonly used in the traditional Chinese medicine that although direct antidiabetic properties have not been attributed, can be considered beneficial for diabetic patients. In fact it has been shown to be effective in reducing the risk to develop diabetic associated cardiovascular complications. In China, pharmaceutical preparations of *S. miltiorrhiza* are widely used particularly for the treatment of cardiovascular and cerebrovascular diseases (e.g. angina pectoris, hyperlipidaemia, and acute ischemic stroke) (Zhou *et al.*, 2005). It was shown that a pharmaceutical preparation of *S. miltiorrhiza*, also due to its antioxidant activity, inhibits vascular endothelial expression of adhesion molecules and smooth-muscle proliferation *in vitro*, as well as, decreases cholesterol levels in mice (Ling *et al*., 2008). From all of its compounds salvianolic acid B also showed to be a promising compound to be used in the treatment of neurodegenerative diseases associated with oxidative stress, by protecting human neuroblastoma SH-SY5Y cells from induced-apoptosis (Tian *et al*., 2008).

S. fruticosa and *S. officinalis* were those particularly studied in this thesis. Alarcon-Aguilar *et al*. (2002) showed the hypoglycaemic activity of *S. officinalis* water ethanol extracts and also referred an antidiabetic potential for *S. fruticosa* and *S. lavandulifolia*. Perfumi *et al.* (1991) showed hypoglycaemic effects of *S. fruticosa* water extract in oral glucose tolerance tests in both healthy and alloxan-diabetic rabbits, but not after an intraperitoneal glucose tolerance test, suggesting that *S. fruticosa* water extract might inhibit the mechanisms of intestinal glucose absorption. Until the beginning of the work presented in this thesis, no other studies had been reported to confirm the antidiabetic potential of these sage species.

1.7.1.1 *Salvia fruticosa* Mill.

Salvia fruticosa Miller (**Fig. 12**) is also named *Salvia triloba* L. (due to the morphological characteristic of the leave with three lobes) or even Greek sage (because its abundance in Greece). It is an endemic sage species of the Eastern Mediterranean basin. Its total native range extends from Sicily and Southern Italy, through the southern part of the Balkan Peninsula to West Syria (Karousou *et al*., 2000). Furthermore, it is found as a naturalised plant in parts of the Western Mediterranean region namely in Greece, Malta, Spain and Portugal (Karousou *et al*., 2000).

Fig. 12 – *Salvia fruticosa* Mill. (*Lamiaceae*) plant.

Many activities have been attributed to *S. fruticosa*, including its pharmacological effects on the central nervous system (e.g. sedative, hypnotic, skeletal muscle relaxant, analgesic, memory enhancing, neuroprotective, antiparkinsonian, Imanshahidi & Hosseinzadeh, 2006). Additionally, some of *S. fruticosa* extracts have revealed anti-inflammatory and antiulcerogenic (Kaileh *et al*., 2007; El-Sayed *et al*., 2006) and antioxidant activities (Exarchou *et al*., 2002). Water extracts of leaves of *S. fruticosa* have been used in popular medicine of the eastern Mediterranean regions as a hypoglycaemic agent (Karousou *et al*., 2000). In what concerns to the antidiabetic activities of *S. fruticosa*, there are few studies that have looked at this potential.

These important bioactivities attributed to *S. fruticosa* are a result of active compounds (that act alone or in synergism) present in the respective extracts. Among the huge variety of compounds that constitute them, some deserve more attention. Compounds such as flavonoids (e.g. luteolin-7-glucoside), phenolic acids (e.g. rosmarinic acid, caffeic acid, ferulic acid), triterpenoids (e.g. ursolic acid, oleanolic acid) and volatile compounds (essential oils, e.g. 1,8-cineole, camphor) are some of sage constituents that are certainly involved in its bioactivities.
For all of this, *S. fruticosa* is a medicinal plant that deserves more attention, not only because the properties that have been attributed, but also others that it can have and are still not found and/or confirmed.

1.7.1.2 *Salvia officinalis* L.

Salvia officinalis L. (**Fig. 13**) is also known as common sage or garden sage. According to the Pharmacopeia of Portugal, this plant is native of Mediterranean countries, although it is today cultivated all over the world. *Salvia officinalis* is morphologically similar to *Salvia fruticosa*, both small, evergreen perennial plants with short woody stems that branch extensively, with blue and in some cases violet flowers. The most visible difference is present in the leaves. While in *S. fruticosa* we can see three lobules, in *S. officinalis* we only identify one. The species name *officinalis* means medicinal (Dweck, 2000; Miura *et al*., 2002).

Fig. 13 – *Salvia officinalis* L. (*Lamiaceae*) plant.

S. officinalis has been used in the preparation of cosmetic products and has been used for centuries in folk medicine for the treatment of a variety of disorders and as a culinary spice.

Sage extracts, particularly essential oil (EO), are also widely used in the food, drug, beverage, cosmetic and fragrance industries. Sage has become an important source of antioxidants used in food industry, which had additional wider implications for the dietary intake of natural antioxidants (Deans & Simpson, 2000). Recent studies with some phenolic compounds (such as rosmarinic acid, caffeic acid and luteolin-7-glucoside) present in sage plants revealed protective effects against oxidative damages induced in HepG2 cells (human hepatoma cell line). They decreased the GSH depletion induced by tert-butyl hydroperoxyde, which is of great importance to preserve cell viability (protecting against oxidative damage) (Lima *et al*., 2006). In addition, water and methanolic extracts of *S. officinalis* proved to be effective in protecting liver cells (HepG2 cell line) from the oxidative damage induced by tert-butyl hydroperoxyde, suggesting their use in the prevention and/or treatment of liver diseases (Lima *et al*., 2007). Furthermore, a water extract (tea) of *S. officinalis* showed effects on lowering fasting plasma glucose levels in healthy rats and a metformin-like effect on hepatocyte primary cultures of these animals by increasing hepatocyte glucose uptake and inhibiting gluconeogenesis. Although these effects were not observed in STZ-induced diabetic rats, they suggest the use of sage in the prevention of T2DM, as a food supplement (Lima *et al*., 2006).

The increasing scientific evidence of the health benefits of these sage species make them important medicinal plants with numerous applications, namely in pharmaceutical and cosmetic industries.

The work here presented comprises *in vivo* and *in vitro* studies where the antidiabetic efficacy and the safety of sage water extracts (prepared as tea) were assessed.

In order to study the antidiabetic potential of *S. fruticosa* tea at the level of intestine (on carbohydrate absorption capacity), an experiment with healthy and diabetic rats was performed and will be presented in the chapter 2. In particular, the effects of *S. fruticosa* tea on the inhibition of the characteristic diabetes-induced increase in the expression of the glucose transporter SGLT1 will be discussed.

Afterwards, another *in vivo* experiment with healthy rats was performed, in which a different model of induction of SGLT1 was applied (through diet manipulation). With this experiment we intended to confirm the effect of *S. fruticosa* tea on the regulation of SGLT1 (chapter 3). In order to identify active principles of this sage tea, a solution of rosmarinic acid (the most abundant phenolic compound of the sage species studied in this thesis) was administered to another group of animals and the results show this to be the active principle in the mixture and will also be discussed in this chapter.

Then, with the aim to verify if the effects of sage tea on intestinal expression of glucose transporters were mediated through an action on carbohydrate digestion, a quantification of *in vitro* inhibition of α-amylase activity was made and the results are presented in chapter 4. Also in this chapter an *in vivo* study with rats submitted to treatment with two sage compounds (luteolin-7-glucoside and ursolic acid) will be presented. Their effects on plasma glucose levels, liver glycogen content and lipid profile will be discussed.

In chapter 5 two studies concerning the evaluation of antidiabetic activities of a *S. officinalis* tea will be described. The first study was done with healthy rats treated *in vivo* with *S. fruticosa* tea. The response of primary cultures of hepatocytes from these rats to the pancreatic hormones insulin and glucagon was then studied *in vitro*. In order to verify if *S. officinalis* tea drinking produces in humans the same beneficial effects observed in animals, the last part of this chapter reports on a pilot trial carried out in an attempt to find effects particularly useful in the prevention and/or treatment of T2DM.

Finally, in chapter 6 the major results of all the experiments will be globally discussed. Moreover, future perspectives will also be presented.

1.8. References

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Chapter

2

Is *Salvia fruticosa* tea effective in a diabetic condition?

2.1. Chapter overview

Traditional medicine has attributed numerous medicinal properties to *Salvia fruticos*a Mill (Greek sage), including antidiabetic. However, the studies that have been performed in order to prove its antidiabetic potential are few and effectiveness and mechanisms of action unknown.

In a preliminary study performed in our laboratory, SFT treatment did not show effects on glucose clearance mechanisms after an intraperitoneal glucose tolerance test in rats (Azevedo *et al*., 2006)[1], and it also did not show effects on the activity of the digestive enzyme α-amylase *in vitro* (see chapter 4; Azevedo *et al*., submitted)[2]. However, because the intraperitoneal glucose tolerance test bypasses the intestine, effects at this level would not be revealed. Therefore, with the present study the effects of SFT treatment at the level of intestinal glucose transporter expression (Na^+/glucose cotransporter – SGLT1 and facilitative glucose transporter 2 – GLUT2) and on the incretin hormone glucagon-like peptide-1 (GLP-1) were studied in healthy and streptozotocin (STZ)-induced diabetic rats.

Our results showed that SFT treatment for 14 days stabilised fasting blood glucose levels in STZ-diabetic animals, and did not affect glucose levels of healthy animals. Although no effects were obtained on liver glycogen, at the level of intestinal epithelium SFT treatment significantly diminished the diabetes associated increase in SGLT1 and in a similar manner also heat shock protein 70 (Hsp70) expression in the brush-border membrane (BBM) of enterocytes.

In what concerns the expression of GLUT2, Na^+/K^+-ATPase and Hsp70 in jejunal mucosa homogenates, although diabetes significantly induced their expression, no effects of SFT were observed. No effects of sage tea on GLP-1 immunoreactive cells were also observed, although the reduction of plasma insulin characteristic of diabetic animals was smaller in SFT treated diabetic rats.

In conclusion, this study shows that the small intestine can be considered a target organ for therapeutical interventions in diabetes. Particularly the effects on fasting blood glucose and SGLT1 expression in enterocyte's BBM suggest SFT to be beneficial in the reduction of postprandial hyperglycaemia, helpful in the control of T2DM progression.

Salvia fruticosa tea showed to be a promising medicinal plant to be used in the control of the progression of diabetes mellitus and since it is easily accessible, its inclusion in dietary strategies for the control of T2DM progression should be considered.

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[1] Azevedo MF, Lima CF, Fernandes-Ferreira M, Almeida MJ & Pereira-Wilson C (2006) *Salvia fruticosa* tea reduces the expression of SGLT1 in rat intestinal epithelium. *Acta Physiologica* **186**, 234.

[2] Azevedo MF, Camsari Ç, Valentao PCR, Andrade PB, Seabra RM, Braga PSC, *et al*. (2008) Sage plants as potential sources of antidiabetic compounds. Submitted to *J Agric Food Chem*.

2.2. Manuscript

This chapter comprises the following manuscript:

Azevedo MF, Lima CF, Wilson JM, Fernandes-Ferreira M & Pereira-Wilson C. Control of diabetic hyperglycaemia by a sage herbal tea involves regulation of Na^+/glucose cotransporter expression in the brush-border membrane of enterocytes. Submitted to *Diabetologia*.

Control of diabetic hyperglycaemia by a sage herbal tea involves regulation of Na^+/glucose cotransporter expression in the brush-border membrane of enterocytes

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Abstract

Salvia fruticosa (Greek sage) is a medicinal plant to which antidiabetic properties have been attributed. Our previous results suggested that the effects of *S. fruticosa* tea (SFT) drinking on glucose regulation might be at intestinal level although not through α-amylase inhibition. The aim of the present study was to characterise the effects of SFT treatment on the intestinal epithelium, namely on Na+/glucose cotransporter 1 (SGLT1) and facilitative glucose transporter GLUT2 expression, as well as on enteroendocrine L cells. Effects on plasma insulin levels were also assessed. Sage tea was given in replacement of water for 14 days to groups of healthy and streptozotocin (STZ)-induced diabetic rats. Blood glucose, plasma insulin levels and liver glycogen content were measured. Expression of SGLT1 in enterocyte brush-border membrane (BBM), and facilitative glucose transporter GLUT2, Na^+/K^+-ATPase pump and heat shock protein70 (Hsp70) in intestinal mucosa whole cell homogenates was determined by Western blotting. Effects on GLP-1 were evaluated by immunohistochemistry. In diabetic rats, a significant increase in blood glucose and decrease in plasma insulin was observed. SFT treatment stabilised fasting blood glucose levels in diabetic animals. No effects were obtained on liver glycogen as expected. A significant increase on SGLT1, GLUT2, Na^+/K^+-ATPase and Hsp70 expression was observed in the intestinal epithelium of diabetic animals. SFT treatment significantly diminished the diabetes associated increase in SGLT1 and Hsp70 in BBM. No effect of sage was observed on the expression of GLUT2, Na^+/K^+-ATPase and Hsp70 in jejunal mucosa homogenates. Diabetic rats treated with SFT showed smaller decrease in plasma insulin concentrations, than STZ-diabetic control. No effects were observed on GLP-1 immunoreactive

cells. The effects on fasting blood glucose and SGLT1 expression in enterocyte's BBM suggest SFT to be beneficial in the reduction of postprandial hyperglycaemia, particularly helpful in the control of T2DM progression.

Keywords: Salvia fruticosa Mill; Streptozotocin-induced diabetes; SGLT1 expression; Intestinal glucose transport; Antidiabetic.

1. Introduction

Diabetes mellitus is a chronic endocrine disorder characterised by persistent hyperglycaemia that results from defects in insulin secretion and/or insulin stimulation of glucose utilisation by peripheral tissues. Type 2 diabetes mellitus (T2DM) which corresponds to 90 to 95% of all cases of diabetes is more prevalent in populations with western-type diets lifestyles affecting 10 to 20% of adults in many developed countries (Bell & Polonsky, 2001). This type of diabetes is the result of both insulin resistance and insufficient insulin production (Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2003).

Upon digestion of a carbohydrate rich meal, glucose is absorbed mainly in the jejunum of the small intestine. The Na (+)-glucose cotransporter (SGLT1) and the facilitative glucose transporter 2 (GLUT2) are the main intestinal sugar transporters responsible for transporting glucose from the intestinal lumen into the blood. SGLT1 is located to the apical membrane or brush-border (BBM) of the enterocyte and transports glucose and galactose from the intestinal lumen into the cytosol, using the inward Na^+ gradient maintained by the basolateral Na^+/K^+-ATPase. GLUT2 is mainly expressed in the basolateral membrane (BLM) of the enterocyte and transports glucose, galactose and fructose from the enterocyte cytosol to the blood (Wright, 1998). Induction of diabetes in experimental animals is also associated with an increase in SGLT1 (about 6-fold) expression in jejunal enterocytes, resulting in increased monosaccharide absorption (Burant *et al*., 1994). In humans, T2DM is also accompanied by an increase in SGLT1 expression (of 4.3-fold) in the apical membrane of enterocytes (Dyer *et al*., 2002). This increase in the capacity for glucose absorption is also due to an increase in villus length (hyperplasia), and not to changes in intestinal absorptive properties (Debnam *et al*., 1995; Dyer *et al*., 2002). Protein levels of the facilitative sugar transporter GLUT2 have also been shown to be 3-fold increased in the intestine of both diabetic rats and patients (Burant *et al*., 1994; Dyer *et al*., 2002). This increase in the capacity for glucose absorption in diabetic individuals tends to aggravate the undesirable postprandial hyperglycaemia, thus strategies that delay digestion and absorption of intestinal glucose are beneficial in a diabetes scenario. This therapeutical goal is

currently addressed through the oral antidiabetic drug acarbose (α-amylase and α-glucosidase inhibitor) (Chiasson *et al*., 2002).

Contributing to the diabetic postprandial hyperglycaemia is a reduction in the incretin effect (insulin response as a result of glucose orally ingested is higher than if a similar rise in plasma glucose is produced by intravenous infusion). The incretin effect, through the release of the gastrointestinal hormones glucagon-like peptide 1 (GLP-1) and gastric inhibitory polypeptide (GIP) upon glucose absorption is responsible for up to 60% of the postprandial insulin secretion. T2DM patients show a loss of GIP-regulated insulin secretion and reduced GLP-1 secretion but not action, which make GLP-1 a therapeutic target for this type of diabetes (Gallwitz, 2006). Because GLP-1 is rapidly degraded by dipeptidyl peptidase (DPP)-IV it has a very short half life (less than 2 min) and recent attempts at developing GLP-1 analogs and DPP-IV inhibitors have been made with the purpose to restore the incretin effect in diabetic patients (Deacon *et al*., 2000; Combettes & Kargar, 2007).

In spite of the diversity of therapeutic targets and number of different drugs available, the currently used oral antidiabetic agents for the treatment of T2DM are not completely effective particularly in preventing diabetic complications. This, and the fact that the disease is attaining epidemic proportions justify the search for new drugs and therapeutical targets that help treat and preferably also prevent the progression of this disease (Spiller & Sawyer, 2006; Harrigan *et al*., 2001). Traditional medicine has empirically identified plants, such as *Salvia fruticosa* Mill. (Greek sage), with antidiabetic properties that provide good source material for the search of novel active compounds.

In the present study the antidiabetic potential of *S. fruticosa* water extract (hereafter referred to as tea) was investigated. In previous studies *S. fruticosa* tea (SFT) treatment did not show effects on glucose clearance mechanisms after an intraperitoneal (ip) glucose tolerance test performed in rats (Azevedo *et al*., 2006). Moreover, it also did not show effects on the activity of the digestive enzyme alpha-amylase *in vitro* (Azevedo *et al*., submitted *b*). Taken together, these previous results indicate that sage tea may act on the gastrointestinal tract although not through inhibition of digestive enzymes. Therefore, effects at the level of intestinal glucose transporter expression, in particular SGLT1 and GLUT2 and on the incretin hormone GLP-1 were assessed in healthy and STZ-diabetic rats. The SGLT1 activity has been shown to be dependent on the co-expression of the heat shock protein 70 (Hsp70) in BBM (Ikary *et al*., 2002). Therefore, the expression of Hsp70 in intestinal whole cell homogenates and in the BBM subfraction of enterocytes was also determined.

2. Materials and methods

2.1. Plant material and preparation of S. fruticosa water extract

Salvia fruticosa plants were cultivated in an experimental farm located in Merelim, Braga, Portugal, and were collected in June 2004. The aerial parts of plants were air dried and kept a -20ºC. Since sage is traditionally used as a tea, a water extract of *S. fruticosa* was routinely prepared as previously described by Azevedo *et al.* (submitted *b*). The preparation produced a 2.8±0.1 mg of extract dry weight per ml of infusion, containing rosmarinic acid (577.29 μg/ml), 6-hydroxyluteolin-7-glucoside (104.78 μg/ml) and the heteroside of an unidentified flavone (99.13 μg/ml) as the most representative phenolic compounds, and 1,8-cineole (61.74 μg/ml), camphor (25.22 μg/ml) and α-terpineol (5.77 μg/ml) as the major volatile compounds (Azevedo *et al*., submitted *b*).

2.2. Animals

Male Wistar rats (6 weeks) were purchased from Charles River Laboratories (Barcelona, Spain) and acclimated to the Bioterium animal facilities of the Life and Health Science Research Institute (ICVS), University of Minho, for at least one week before the start of the experiments. During the experimental period, animals were maintained under controlled temperature (20 ± 2ºC), and humidity (55 ± 10%) with a 12 h light: 12 h dark cycle and given food and tap water *ad libitum*. The animals used in the experiment were kept and handled in accordance with European regulations (European Union Directive 86/609/EEC) and the NIH guidelines on animal care and experimentation (National Institutes of Health, 1985).

2.3. Induction of experimental diabetes

Diabetes was induced by a single intraperitoneal injection of freshly prepared streptozotocin (STZ, Sigma-Aldrich, St. Louis, MO, USA) solution (45 mg/Kg in 0.1 M-acetate buffer, pH 4.5 given in a volume of 1ml/Kg body weight; Siddique *et al*., 1987) to overnight-fasted rats. Control animals received a sham injection with buffer only. Diabetes was identified by polydipsia, polyuria and by measuring non-fasting plasma glucose levels 48 h after injection of STZ. Before the experiment, animals were kept under standard conditions for one week to stabilise their diabetic state (Fig. 1). At the end of this period, rats that developed moderate diabetes – fasting blood glucose levels of 250-350 mg/dl – were taken for the experiment (Hamdan & Afifi, 2004).

2.4. Experimental design

In this experiment, twenty four rats were used and divided into four groups (two healthy and two STZ-diabetic) of six rats each. After that, animal groups were subjected to the following treatments for 14 days (**Fig. 1**): group 1 – healthy rats drinking water; group 2 – healthy rats drinking SFT; group 3 – STZ-induced diabetic rats drinking water; group 4 – STZ-induced diabetic rats drinking SFT. Water/tea (SFT) and food were given *ad libitum* and the beverage was renewed daily. The replacement of water by SFT did not change food and beverage consumption, or animal body weight increase.

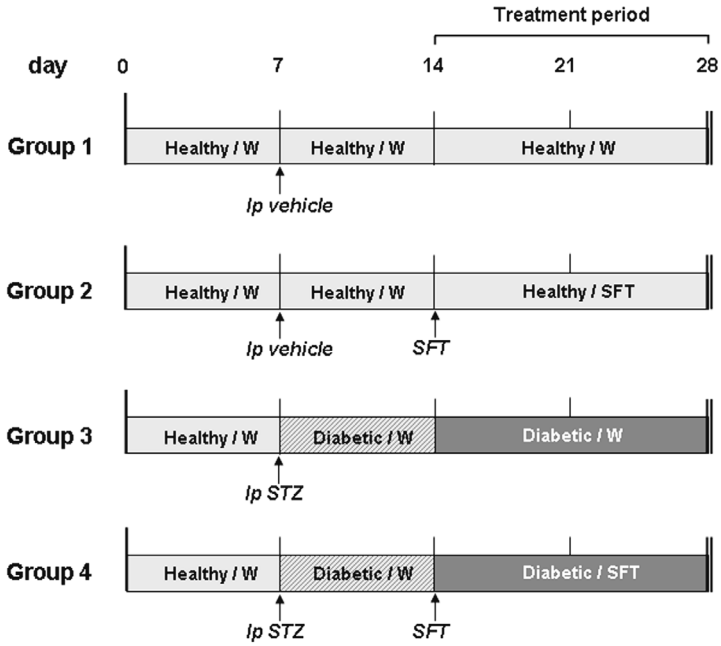

Fig. 1. Schematic representation of the experimental design. Twenty-four rats were divided in to 4 groups of 6 animals each. STZ= streptozotocin; ip= intraperitoneal injection; Healthy= non-diabetic animals: Diabetic=STZ induced-diabetic animals. SFT treatment was from day 14th until 28th of experiment. Double vertical bars on the right side of each group indicate terminal sampling.
□ healthy condition, ▨ developing diabetes and ■ tea/water treatment established diabetic condition.

At the end of the treatment, 16 h-fasted animals were killed by decapitation and the intestinal mucosa (40 cm of jejunum) scraped off on ice with a glass microscope slide, after washing with PBS pH 7.4 (with 40 mM-PMSF in ethanol added fresh).
The intestinal mucosa was immediately frozen in liquid nitrogen and stored at -80ºC until use. Prior to scraping a small piece of intact jejunum (from the middle of the above 40 cm region) was collected as well as pancreatic tissue for fixation in 4%-paraformaldeyde in PBS pH 7.4, for 24 h at 4ºC. The tissues were then stored in ethanol 70% (v/v) until being processed for paraffin embedding. Blood samples were also collected to measure activity of liver transaminases.

2.5. Preparation of Brush-Border Membrane Vesicles

Brush-Border Membrane Vesicles (BBMV) were prepared from frozen jejunal mucosal scrapings using a combination of cation precipitation and differential centrifugation as described previously (Shirazy-Beechey *et al*., 1990) with few modifications. Briefly, the jejunal mucosa scrapings were thawed on ice and then placed in 50 ml of ice-cold Buffer 1 (100 mM-mannitol, 2 mM-HEPES, pH 7.1 adjusted with 2-M Tris). The tissue was homogenised with a Waring Blender (Waring Commercial, Torrington, CT, USA) (2 × 30 s) and then filtered through 100 µm nylon mesh, and the volume was made up to 100 ml with ice-cold Buffer 1. A solution of 2.5 M-$MgCl_2$ was added to the tissue homogenate to give a final concentration of 10 mM. After stirring on ice for 20 min the solution was centrifuged at 3,000 × *g* (Eppendorf 5804 R, Hamburg, Germany) at 4ºC for 10 min to remove debris. The supernatant was further centrifuged at 30,000 × *g* (Beckman Avanti J-25I, Beckman, Germany) at 4ºC for 30 min and the pellet homogenised in a Buffer 2 (100 mM-mannitol, 2 mM-HEPES, pH 7.5 with 2 M-Tris, and 0.1 mM-$MgSO_4$ added after pH adjustment with Tris) with a glass homogeniser. The homogenate was then centrifuged at 30,000 × *g* at 4ºC for 45 min and the final purified BBMV was suspended in a Buffer 3 (300 mM-mannitol, 20 mM-HEPES, pH 7.5 with 2 M-Tris, and 0.1 mM-$MgSO_4$ added after pH adjustment). BBMV were then frozen in liquid nitrogen and stored at -80ºC until use. The enrichment of the brush-border marker (by measuring the activity of alkaline phosphatase; Pekarthy *et al*., 1972) was about 10 times the mucosa whole cell homogenate. Protein content was measured with the Bradford Reagent (Sigma-Aldrich, St. Louis, MO, USA) using bovine serum albumin as a standard.

2.6. Western blot analysis of SGLT1, GLUT2, Na^+/K^+-ATPase and Hsp70 proteins

The abundance of SGLT1 protein was measured by quantitative Western blotting as described previously (Hirsh & Cheeseman, 1998; Wilson *et al*., 2004), with slight modifications.

The BBMV preparations from control and treatment animals were solubilised in Laemmli's buffer (Laemmli, 1970), vortexed, and heated for 15 min at 70ºC. Then, 25µg of protein were loaded in each well and separated by SDS-PAGE (8% T resolving gel with 4% T stacking gel), using the mini-PROTEAN 3 electrophoresis cell (BioRad Laboratories, Inc., Hercules, CA, USA). Proteins were transferred onto Hybond-P polyvinylidene difluoride membrane (GE Healthcare, UK) by electrotransfer for 1h, at room temperature (Mini Trans-Blot Cell, BioRad Laboratories, Inc., Hercules, CA, USA). Membranes were blocked in 5% (w/v) non-fat dry milk in TPBS (0.05% (v/v) Tween 20 in PBS) pH 7.4, for 1 h, at room temperature and then incubated with rabbit antibody to rat SGLT1 (raised in rabbits against a peptide comprising

amino acids 582-600: EEDPKDTIEIDAEAPQKEK of rat SGLT1) (Elfeber *et al*., 2004) diluted 1:500 in antibody dilution buffer (1% BSA/TPBS), overnight at 4ºC. After rinsing with TPBS, membranes were incubated with donkey anti-rabbit IgG horseradish peroxidase conjugated secondary antibody (GE Healthcare, UK) diluted 1:30,000 in 5% non-fat dry milk, TPBS for 1h, at room temperature. Membranes were rinsed again, then developed using the enhanced chemoluminescence detection solution (ECL, from GE Healthcare, UK) and exposed to film. A single band was detected by this method with an apparent molecular mass of 75 kDa.

The same procedure was used to quantify the abundance of GLUT2 (Mw 52 kDa), Na^+/K^+-ATPase (Mw 110 kDa) and Hsp70 (Mw about 70 kDa) in whole cell homogenates of jejunal mucosa, using a rabbit polyclonal antibody (Chemicon International, Temecula, CA, USA) diluted 1:2,500, a α5 mouse monoclonal antibody (Takeyasu *et al*., 1988) diluted 1:500, and a mouse monoclonal antibody (Sigma-Aldrich, St. Louis, MO, USA) diluted 1:4,000 in 1% BSA/TPBS, overnight at 4ºC, respectively. The Na^+/K^+-ATPase antibody was obtained as culture supernatant from the Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, under contract N01-HD-7-3263 from The National Institute of Child Health and Human Development. The expression of Hsp70 was also measured in BBM samples using the same procedure and antibody. The intensity of the immunoreactive bands detected in the BBM and intestinal mucosa whole cell homogenates were measured from digitised images, calibrated with a Kodak gray scale using Sigma Scan Pro (v5) software (SPSS Chicago, IL, USA). Results are presented as percentage of band area intensity of HC-HC group.

2.7. Immunohistochemistry

The immunolocalisation of SGLT1, GLUT2 and GLP-1 in jejunum of rat small intestine was carried out following the immunohistochemistry method described by Wilson *et al.* (2004) with few modifications. Jejunal tissue was processed and embedded in paraffin, and 5 μm sections were mounted on 3-aminopropyltriethoxysilane (Sigma-Aldrich, St. Louis, MO, USA) coated glass slides, dewaxed in Clear-Rite (Richard-Allan, Kalamazoo, MI, U.S.A.), and rehydrated and subjected to citraconic anhydride 0.05% (v/v) pre-treatment in a 98ºC bath, for 30 min, when necessary (Naminatsu *et al*., 2005). Slides were then air-dried, and the tissue was circled with hydrophobic barrier (Super PAP PEN, Sigma-Aldrich). After rehydration in TPBS, the slides were incubated for 20 min in blocking reagent (5% normal goat serum/1% bovine serum albumin in TPBS pH 7.4) followed by the respective primary antibody diluted in 1% BSA in TPBS, overnight at room temperature in humidity chambers. Sections were then rinsed in TPBS and incubated with a secondary goat anti-rabbit or mouse biotin conjugated antibody, followed by a streptavidin conjugated Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA) for 1 h at 37ºC with rinsing in-between. The slides were rinsed again in TPBS and then the

sections were coversliped using an aqueous mounting media containing antifade compounds (10% (v/v)-Mowiol, 40% (v/v)-glycerol, 0.1% (w/v)-DABCO, 0.1 M-Tris, pH 8.5).

Negative controls were processed on the same slide in an identical manner (substitution of primary antibody with normal rabbit serum with an equivalent dilution).

The antibodies against SGLT1 (Elfeber *et al*., 2004), GLUT2 (Chemicon International, Temecula, CA, USA), and GLP-1 (Abcam, Cambridge, UK) were used diluted 1:100, 1:200 and 1:1,000, respectively.

The number of GLP-1 expressing L-cells/cm of villus length was assessed using the software LAS AF (version 1.4.1) from Leica Microsystem (Wetzlar, Germany). The average intensity of GLP-1 signal in immunoreactive L-cells, the average of β-cell insulin signal and percentage of active β-cell in Langerhans islets were measured using the Sigma Scan Pro (v5) software (SPSS Chicago, IL, USA).

2.8. Biochemical analysis

2.8.1. Plasma transaminases activities

The alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were measured spectrophotometrically in rat plasma following the NADH oxidation method (at 30ºC) at 340 nm on a plate reader (Spectra Max 340pc, Molecular Devices, Sunnyvale, CA, USA), as previously described by Lima *et al.* (2005).

2.8.2. Glucose measurement

Blood glucose levels were monitored with the Accutrend® GCT device (Roche diagnostics GmbH, Mannheim, Germany) using Accutrend® test strips for glucose (Roche diagnostics GmbH, Mannheim, Germany), during the experimental period.

2.8.3. Insulin measurement

The content of insulin in rat plasma was measured using an ELISA-based commercial kit – Rat Insulin EIA Kit (SPI-BIO, Montigny-le-Bretonneux, France) – following the manufacturer specifications.

2.8.4. Liver glycogen content

The liver glycogen content was quantified with amyloglucosidase as described previously (Keppler & Decker, 1974) with minor modifications reported in Azevedo MF *et al*. (submitted *b*).

2.9. Statistical Analysis

Data are expressed as means ± standard errors of the means (SEM). For statistical analysis a two-way ANOVA was employed followed by the Newman-Keuls multiple comparison test, to compare physiological conditions (healthy vs. diabetic) and the effect of *in vivo* beverage (water *vs S. fruticosa* tea (SFT)) (SigmaStat, version 2.03; SPSS Inc., San Rafael, CA, USA). *P* values ≤ 0.05 were considered statistically significant.

3. Results

3.1. Effects of SFT treatment on transaminase activities

In order to identify possible liver toxicity of SFT treatment, the activities of plasma transaminases (ALT and AST) were measured. As shown in **Table 1**, SFT drinking for 14 days did not significantly increase ALT or AST activity in the plasma of both healthy and diabetic animals.

Table 1 – Effect of 14 days SFT treatment on rat plasma transaminase activities (alanine aminotransferase – ALT and aspartate aminotransferase – AST).

		Plasma Transaminases	
	***In vivo* beverage**	**ALT (U/l)**	**AST (U/l)**
Healthy	Water	22.6±2.0	31.1±2.6
	Tea	23.4±1.3	32.2±5.2
Diabetic	Water	25.5±1.7	20.3±2.9
	Tea	24.2±1.2	22.7±2.2

Values are means ± SEM (*n*=6)
Mean values were not significantly different when compared with the respective control group (P>0.05).

3.2. Effects of SFT treatment on fasting blood glucose, plasma insulin and liver glycogen content

During the experimental period, rat blood glucose levels were monitored regularly to ascertain possible effects of SFT treatment. One week after the *ip* injection of STZ (at day 14) fasting blood glucose increased from about 110 mg/dl in healthy rats to 305 mg/dl in STZ-induced diabetic animals (**Fig. 2A**). During the 14 days of treatment glycaemic levels increased in STZ-diabetic water drinking controls and remained stable in SFT drinking STZ-diabetic

animals (**Fig. 2A**). After fourteen days (at day 28) treatment with SFT diabetic animals showed significantly lower levels of glucose in the blood (**Fig. 2A**).

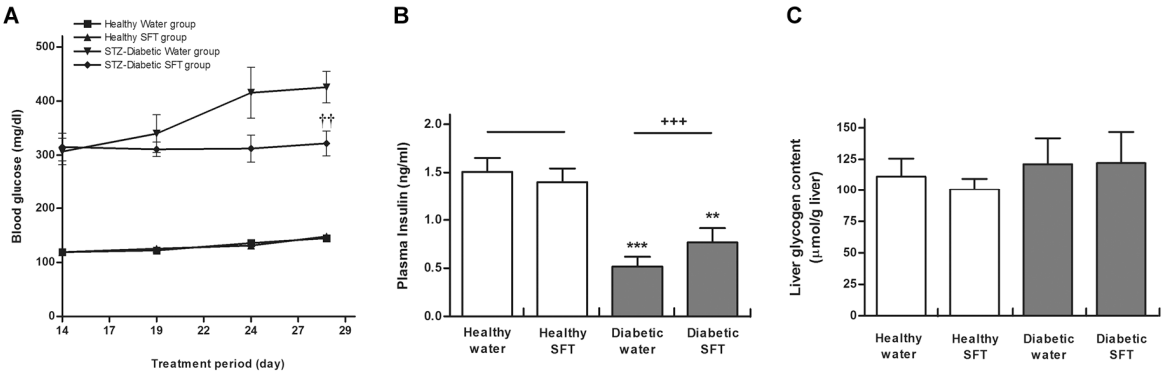

Fig. 2. Effect of 14 days treatment with *Salvia fruticosa* tea (SFT) on fasting blood glucose (A) and plasma insulin concentrations (B) and liver glycogen content (C) of healthy (white bars) and STZ-induced diabetic (grey bars) animals. Values are means ± SEM, *n*=5-6. Diabetic rats showed high levels of blood glucose (during the entire treatment period: from day 14th until 28th) and lower plasma insulin concentrations when compared with healthy animals (P<0.001). $^{\dagger\dagger}P$<0.01 when compared with diabetic water group. $^{***}P\leq 0.001$ and $^{**}P\leq 0.01$ when compared with the respective healthy control.

Plasma insulin concentration measured in fasted animals significantly decreased in STZ-induced diabetic rats (**Fig. 2B**). Diabetic water drinking rats showed a reduction of plasma insulin concentration caused by STZ administration of about 65%, while diabetic SFT treatment animals showed a smaller reduction (about 45%).

The liver glycogen content was also determined and as shown in **Fig. 2C** no significant differences were obtained. Neither STZ-induction of diabetes, nor treatment with SFT affected the amount of glycogen in rat liver.

3.3. Effects of SFT treatment on SGLT1, GLUT2 and Na^+/K^+-ATPase expression

After STZ-induction of diabetes and treatment with SFT, the expression of intestinal glucose transporters (SGLT1 and GLUT2) and Na^+/K^+-ATPase was quantified. As shown in Fig. 3, a significant increase (85%) in SGLT1 expression on BBM was obtained in STZ-induced diabetic rats when compared with healthy rats. When water was replaced by SFT for 14 days in diabetic animals, the increase in SGLT1 was only about 30% (**Fig. 3**). In healthy animals sage tea drinking did not change SGLT1 expression.

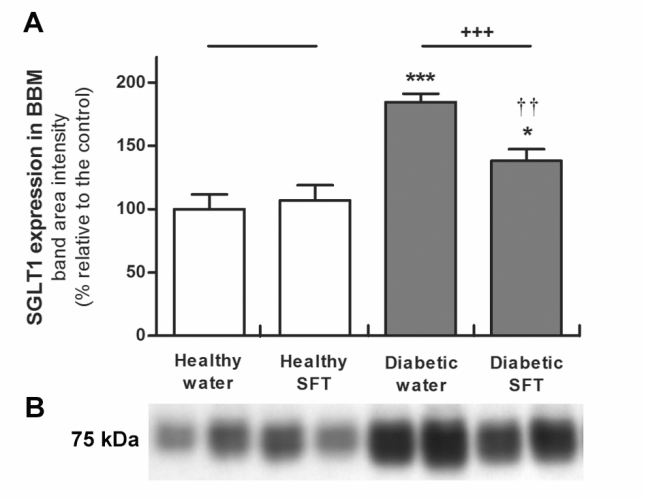

Fig. 3. Western blot analysis of SGLT1 expression in jejunal brush-border membrane (BBM) of healthy (white bars) and STZ-induced diabetic rats (grey bars) treated with water or sage tea (SFT) (A). (B) – Representative immunoblot from two animals from each treatment group. Values are means ± SEM, *n*=5-6. Two-way ANOVA, indicates that the difference between diabetic (W+SFT) and healthy (W+SFT) rats was significant ($P<0.001$). The post-hoc test Student Newman Keuls indicates significant differences, $^{***}P<0.001$ and $^{*}P<0.05$ when compared with the respective healthy control group, and $^{††}P<0.01$ when compared with the diabetic water group.

Effects of SFT treatment on the basolateral facilitative glucose transporter GLUT2 and Na^+/K^+-ATPase expression were measured in enterocyte whole cell homogenates by Western blotting. The expression of both GLUT2 and Na^+/K^+-ATPase in jejunal mucosa was significantly increased (by 29 and 41%, respectively) in STZ-induced diabetic rats when compared with healthy controls (**Fig. 4A-B**). Na^+/K^+-ATPase expression in diabetic water drinking animals increased significantly (59%) when compared with the respective healthy control, while in SFT treated animals this increase was of a smaller magnitude (25%, **Fig. 4B**).

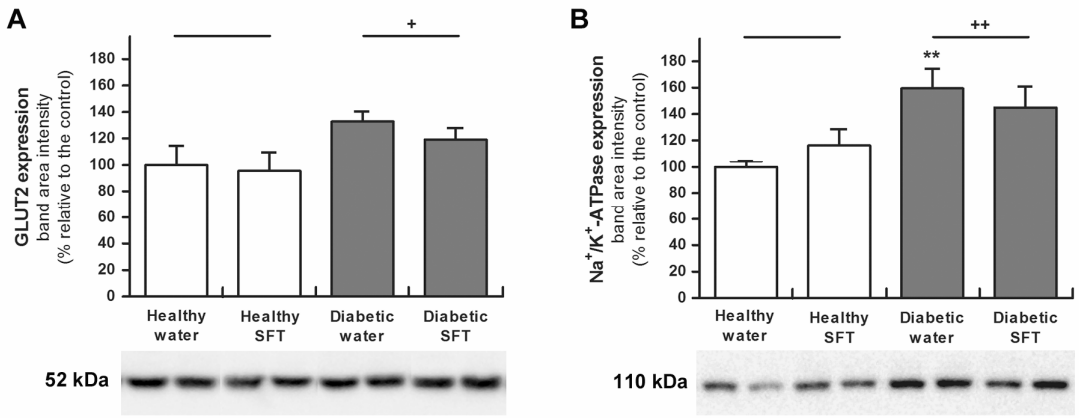

Fig. 4. Western blot analysis of GLUT2 (A) and Na^+K^+-ATPase (B) expression in jejunal whole cell homogenates of healthy and STZ-induced diabetic rats and the respective representative immunoblots from two animals from each treatment group. Values are means ± SEM, *n*=5-6. Two-way ANOVA,

indicates that the difference between diabetic (W+SFT) and healthy (W+SFT) was significant ($P<0.05$ and $P<0.01$, respectively). The post-hoc test Student-Newman-Keuls indicates significant differences, ** $P<0.01$ when compared with the healthy water group.

Both SGLT1 and GLUT2 were immunolocalised in paraffin-embedding tissue, and representative immunofluorescence images are presented in **Fig. 5**. As expected, SGLT1 was detected on the apical membrane (BBM) and GLUT2 on the basolateral membrane (BLM) of the enterocytes in both healthy and STZ-diabetic animals. Overall, the most obvious histological change induced in STZ-diabetic animals was the increase in villi length (data not shown), which in agreement with the literature (Debnam *et al*., 1995).

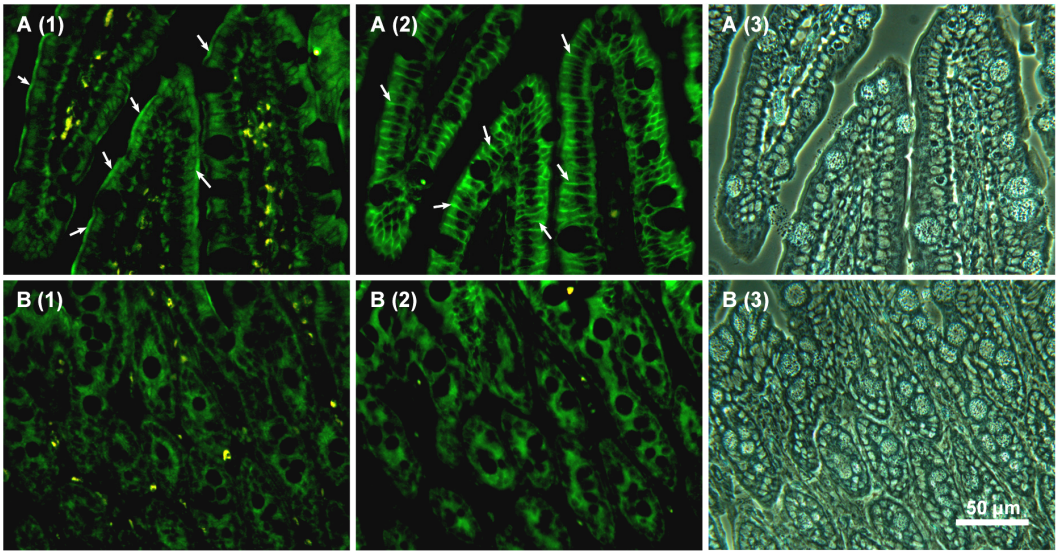

Fig. 5. Representative images [(A) – tip villus region; (B) – crypt region] showing immunolocalisation of SGLT1 (1) and GLUT2 (2) to the brush-border membrane and basolateral membrane of enterocytes, respectively. Tissue also visualised by phase contrast for orientation. Arrows indicate SGLT1 and GLUT2 immunofluorescence signal, respectively.

3.4. Effects of SFT treatment on Hsp70 expression

Expression of Hsp70 in intestinal mucosa whole cell homogenates increased significantly (about 30%) in STZ-diabetic rats when compared with healthy controls, as shown in **Fig. 6A**. On the other hand, while the expression of this protein on the subcellular fraction of enterocyte BBM was not affected between healthy and diabetic condition (**Fig. 6B**), differ significantly between water and tea treated groups (P = 0.043, **Fig. 6B**). No effect of SFT treatment was observed in whole cell homogenates, although in BBM subfraction a significant

difference between water and SFT treated diabetic animals was almost obtained ($P = 0.069$; **Fig. 6B**).

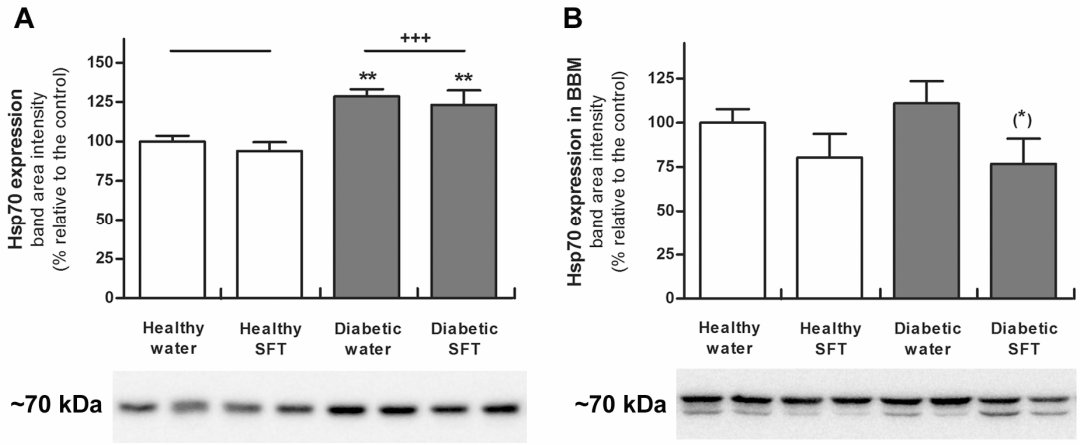

Fig. 6. Western blot analysis of Hsp70 expression in jejunal whole cell homogenates (A) and brush-border membrane (B) of healthy and STZ-induced diabetic rats. The respective representative immunoblots from two animals from each treatment group is also shown. Values are means ± SEM, n=5-6. Two-way ANOVA indicates that diabetic (W+SFT) and healthy (W+SFT) were not significantly different ($P>0.05$), but W and SFT treatment (healthy and diabetic) were significantly different ($P = 0.043$). The Student-Newman-Keuls post-hoc test indicates an almost significant difference, $^{(*)}P = 0.069$ when compared with diabetic water group. The two bands obtained in (B) were analysed together.

3.5. Effects of SFT treatment on the incretin hormone GLP-1

The incretin hormone GLP-1 stimulates glucose-dependent insulin secretion and the inhibition of glucagon secretion which grants it clinical relevance in the treatment of diabetes (Baggio & Drucker, 2004). Therefore, effects of SFT treatment on GLP-1 expressing L-cells of intestinal epithelium were assessed by immunofluorescence (**Fig. 7**). Our results show that the number of GLP-1 expressing L cells/cm villus significantly decreased in STZ-diabetic rats when compared with healthy controls. On the other hand, the GLP-1 signal intensity (per cell) was significantly higher in STZ-diabetic rats compared with healthy controls. SFT treatment did not affect either the number of GLP-1 expressing cells (**Fig. 7A**) or the GLP-1 signal intensity per cell (**Fig. 7B**).

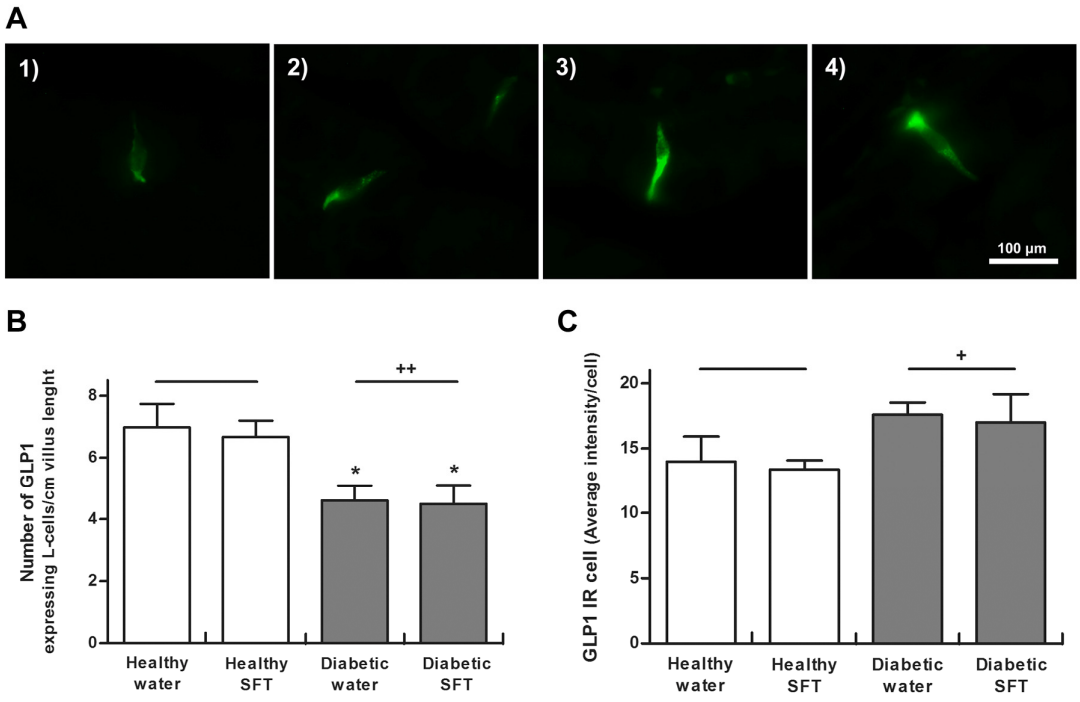

Fig. 7. Effects of *S. fruticosa* tea (SFT) treatment on STZ-induced reduction of GLP-1 producing cells (L-cells) in the jejunal epithelium. A – Representative immunofluorescence micrograph of GLP-1 localisation to intestinal epithelial enteroendocrine L-cell: in (1) – healthy water group; (2) – healthy SFT group; (3) – diabetic water group; (4) – diabetic SFT group; B – The number of GLP-1 expressing enteroendocrine L-cells (expressed per cm of villus length); C – Intensity of GLP-1 signal on the same cells. Values are means ± SEM, n=5-6. Two-way ANOVA, indicates that the difference between diabetic (W+SFT) and healthy (W+SFT) was significant (P<0.01 and P<0.05, respectively). The post-hoc test Student-Newman-Keuls indicates that $^{*}P \leq 0.05$ when compared with the respective healthy control.

4. Discussion and conclusions

The present study shows that 14 days of SFT treatment was effective in the control of the progressive increase of fasting blood glucose levels observed in diabetic animals, although without effect on plasma insulin levels and liver glycogen content. This effect was accompanied by a significant reduction of the increase in SGLT1 expression in BBM of enterocytes associated with diabetes. Control over carbohydrate digestion and absorption is beneficial in the management of type 2 diabetes since it helps contain postprandial hyperglycaemia excursions thereby improving glycaemic control and reducing the risk of diabetic complications. The effects of SFT treatment limiting the increase in SGLT1 expression associated with diabetes, provide a novel target (modulation of the expression of intestinal SGLT1) and a novel therapeutic strategy in the treatment and possibly also in the prevention of diabetes.

In our study, we found an 84% increase in SGLT1 protein levels in the apical membrane of enterocytes from diabetic rats (due to STZ-induction of diabetes) when compared with healthy controls. Fourteen days of SFT treatment (initiated one week after induction of diabetes) limited this increase of SGLT1 expression to about 30%. However, because SFT was only effective in diabetic animals, means that it is only active when the mechanisms of induction of intestinal glucose transporters are present. Recently, Casirola & Ferraris (2006) showed that the α-glucosidase inhibitors (e.g. acarbose) are also able to prevent the diet-induced increases of intestinal glucose transport in diabetic mice, although the mechanism through which they are effective seems to be different from the one of *S. fruticosa* tea. It was demonstrated that the α-glucosidase inhibitors regulate intestinal glucose transport by decreasing luminal glucose concentrations (through inhibition of digestive enzymes) and not by affecting mRNA or protein expression of the sugar transporters (SGLT1 and GLUT2) (Casirola & Ferraris, 2006; Paiva *et al*., 2002). Contrarily, sage tea prevents the diet-induced increases of intestinal glucose transport in diabetic rat, not by inhibiting carbohydrate digestion and thereby decreasing the amount of glucose in the lumen, but by decreasing the expression of SGLT1 in the BBM of enterocytes. Thus, the present study highlights the importance of sage tea as possible alternative compounds to α-glucosidase inhibitors such as acarbose, for the control of intestinal glucose absorption.

Also in the intestinal epithelium of diabetic patients several changes are observed that lead to exacerbation of hyperglycaemia in diabetes (Ferraris & Diamond, 1997). However, although many studies have reported that diabetes enhances intestinal glucose absorption also in humans, the mechanisms that underlie this effect are poorly understood and there are no therapeutic attempts to limit it. Increases in villus length, on the density of apical SGLT1 and on the basolateral and diabetes-induced apical facilitative glucose transporter GLUT2 seem to be involved (Debnam *et al*., 1995; Burant *et al*., 1994; Kellett *et al*., 2008). The increased expression of SGLT1 has been shown to be associated with an increase in transport capacity (Dyer *et al*., 2002). Moreover, an increase in the enterocyte basolateral Na^+/K^+-ATPase expression has been reported in association with diabetes (Wild *et al*., 1999). In agreement with this, our results in rats also show a significant increase in Na^+/K^+-ATPase as well as GLUT2 expression (29% and 41%, respectively) in intestinal epithelium whole cell homogenates of STZ-diabetic rats compared with healthy controls. No effect of SFT treatment on the levels of both these proteins in jejunal enterocytes was observed.

It has been repeatedly shown that the expression of the Hsp70 is decreased in the liver of diabetic rats (Yamagishi *et al*., 2001), in skeletal muscle of T2DM patients (Kurucz *et al*., 2002) as well as in heart and vascular system (Hooper, 2001; Chung *et al*., 2008). According to Hooper (1999), decreased levels of heat shock proteins in T2DM may be a primary factor that leads to the development of diabetes and consequent organ damage, since they are important

cellular defences against oxidative stress, which is involved in the pathophysiology of this type of diabetes. These lower levels of Hsp70 correlate with lower glucose uptake rates characteristic of insulin resistance. Moreover, it has been recently reported that Hsp70 forms a complex with SGLT1 involving this heat shock protein in SGLT1 translocation to the apical membrane. According to Ikari and colleagues (2002, 2003), this interaction between Hsp70 and SGLT1 increases not only the expression but also activity of SGLT1 on the apical membrane of renal tubule cells and consequently high levels of Hsp in BBM up-regulates glucose uptake. The present study show an increase in Hsp70 expression in jejunal mucosa whole cell homogenates (about 30%) but not in BBM of tea treated diabetic rats. This increase in whole cell Hsp70 is in contrast with what is known for other tissues.

Sage tea drinking did not affect the Hsp70 expression in jejunal mucosa whole cell homogenates, however in enterocyte's BBM of healthy and diabetic rats SFT significantly reduced the expression of this protein (P=0.043 by the two way-ANOVA), which seems to indicate a possible effect of this extract on glucose transport activity. In agreement with this, these results correlate with those obtained for SGLT1 and seem to be consistent with the reported interactions between these two proteins. Our results suggest that in addition to expression, SFT treatment may be decreasing SGLT1 activity through the inactivation of the signalling pathway that leads to the formation of Hsp70-SGLT1 complexes. In this context, contrarily to what generically Hooper (1999) and Chung *et al*. (2008) considered (that increasing Hsp70 levels protects the organism from diabetes), a decrease in Hsp70 expression at the enterocyte BBM may be beneficial since it contributes to the control of plasma glucose levels through decreasing SGLT1 expression and activity, and therefore glucose absorption.

There is evidence for the role of insulin in the control of SGLT1 expression through mechanisms that may involve vesicle translocation. In STZ-diabetic rat, the normal levels of SGLT1 expression were restored after insulin treatment, although no changes in mRNA were observed (Fujii *et al*., 1991; Kurokawa *et al*., 1995). In fact, several proteins of the insulin signalling cascade are related with vesicle translocation of glucose transporters in myocytes and adipocytes, such as the protein kinase C in the insulin-dependent GLUT4 translocation. In other study performed in our laboratory, *in vivo* treatment with an aqueous solution of rosmarinic acid (the most abundant phenolic compound of this sage tea) resulted in a significant decrease in PKC expression in enterocytes (Azevedo *et al*., submitted *a*). Therefore, insulin results in a decrease in membrane expression of SGLT1 in enterocytes whereas in other cell types insulin stimulation results in increase membrane expression of glucose transporters.

Effects on the incretin hormone GLP-1 would be important components of any intestinal effects of SFT. GLP-1 potentiates the glucose-induced insulin secretion by the β-cells where it also has trofic effects (Perfetti *et al*., 2000; Doyle & Egan, 2007). Several studies show a reduced postprandial plasma concentration of GLP-1 in T2DM patients compared with control

subjects (Vilsboll *et al*., 2001; Lugari *et al*., 2002), mainly due to a decrease in GLP-1 secretion by enteroendocrine L-cells (Vilsboll *et al*., 2003). This decrease in GLP-1 secretion observed in diabetics may also have a negative impact on β-cells of the pancreas. Recently, there has been a growing interest on the possible use of GLP-1 based therapies in the treatment of T2DM, namely the administration of exogenous synthetic GLP-1 receptor agonists, long-acting GLP-1 analogs or DPP-IV inhibitors (Cheng, 2005; Gallwitz, 2006). We quantified SFT treatment on GLP-1 immunoreactive cells of the intestine (enteroendocrine L-cells). Our results show that the number of GLP-1 expressing cells was smaller per cm of villus in diabetic animals, compared with healthy controls, and SFT did not increase their numbers. There was significantly higher GLP-1 signal intensity in intestinal epithelial L-cells of STZ-diabetic rats compared with healthy controls, and SFT was without effect on this parameter.

In addition, SFT treatment did not produce apoptotic effects on the intestinal epithelial cells, as was obtained by the TUNEL assay technique (data not shown). However, effects on cellular proliferation seem to be involved since a decrease, although slight, on the expression of the proliferating cell nuclear antigen (PCNA) protein in jejunal mucosa homogenates of SFT treated diabetic rats was observed, when compared with water control (data not shown). The results of PCNA expression show a similar pattern to those obtained for SGLT1 expression, which lead to the suggestion that SFT may be reducing SGLT1 levels in BBM of enterocytes by a mechanism that may also involve cellular proliferation.

We should also take into account the results concerning liver glycogen content. SFT treatment did not alter the content of glycogen in the liver of both healthy and diabetic rats. It seemed also to have no effect on plasma insulin levels. SFT was also without effect on the regeneration of β-cell mass detectable by immunohistochemistry (data not shown). These results together with those of GLP-1 lead us to suggest that SFT does not interfere with mechanisms of liver glycogen deposition and β-cell proliferation.

SFT treatment also did not produce liver toxicity, as shown by the activities of plasma transaminases (ALT and AST), which may indicates that the drinking of this tea can be considered safe. Additionally, sage plants are good sources of antioxidant compounds (Lima *et al*., 2005) and *S. fruticosa*, in particular, is very rich in rosmarinic acid. As reported in the literature, antioxidants are important compounds used to reduce oxidative stress, namely by decreasing glucotoxicity in many cells, such as pancreatic β-cells (which *per se* have lower antioxidant defences) (Robertson & Harmon, 2006). Studies performed with rat islets of Langerhans and in islet cell cultures (HIT-T15) exposed to chronic hyperglycaemia (an oxidative environment) showed a significant decrease in insulin mRNA levels and insulin secretion (Robertson & Harmon, 2006). Treatment with the antioxidant N-acetylcysteine protected β cell lines and rat islets against deterioration of insulin gene expression induced by exposure to high levels of glucose (Tanaka *et al*., 1999). Several individual phenolic compounds

present in water extract of this plant and of a relate species (*S. officinalis*) have shown to improve cellular antioxidant status by protecting reduced glutathione (GSH) levels in the cell (Lima *et al*., 2005; Lima *et al*., 2006). The high antioxidant content of sage may help explain the slight increase in insulin intensity of pancreatic β-cells (by protecting the cells against oxidative damage), but may be also on the basis of the control of fasting hyperglycaemia observed in SFT treated diabetic rats. Thus, the antioxidant potential of sage compounds may be assisting the antidiabetic effects of SFT.

In conclusion, with the present study we addressed the importance of the small intestine and its role in glucose homeostasis. *Salvia fruticosa* tea drinking is effective in the amelioration of diabetic glycaemic control. This effect seems to be, at least in part, due to a reduction of SGLT1 expression in BBM of enterocytes with consequent reduction of postprandial glucose hyperglycaemia. The mechanism behind this effect of sage treatment is different from that of acarbose, which decreases carbohydrate digestion. Sage tea seems to be interfering with transcriptional or posttranscriptional mechanisms of SGLT1 BBM expression, although there may also be effects at the level of crypt cell proliferation and migration rates. In this study *Salvia fruticosa* tea showed to be promising in the control of diabetes progression and because of its low cost and high acceptability its inclusion in dietary strategies for the control of diabetes should be considered. Furthermore, this study showed that the intestinal epithelium seems to be a relevant target for new therapeutical interventions in diabetes.

Acknowledgements

We thank Dr. Hermann Koepsell from the University of Würzburg, Germany for kindly supplying the SGLT1 antibody, and Dr. Jane Dyer from the University of Liverpool, England, for providing us with protocol for brush-border membrane preparation.
M. F. A. and C. F. L. were supported by the Foundation for Science and Technology, Portugal, grants SFRH/BD/12527/2003 and SFRH/BPD/26316/2006, respectively. This work was supported by the Foundation for Science and Technology, Portugal, research grant POCI/AGR/62040/2004.

Abbreviations

BBM: brush-border membrane; BBMV: brush border membrane vesicles; BLM: basolateral membrane; GLUT2: facilitative glucose transporter 2; PVDF: polyvinylidene difluoride; SFT : *Salvia fruticosa* tea; SGLT1: Na+-glucose cotransporter 1; STZ: streptozotocin; T2DM: type 2 diabetes mellitus.

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2.3. Supplementary data

1) Effects of *S. fruticosa* tea (SFT) treatment on pancreatic islet mass

Besides the exacerbated intestinal glucose absorption observed in T2DM, a deficit in the β-cell mass, an increased β-cell apoptosis, and impaired insulin secretion are also present in this type of diabetes (Butler *et al*., 2004). The islet anatomy in T2DM usually shows an approximately 50 – 65% deficit in β-cell mass (Matveyenko & Butler, 2006). Therefore, compounds that induce the recovery of β-cell mass can be beneficial for these patients, since could contribute for the control of hyperglycaemia.

In line with this, pancreatic samples from healthy and STZ-induced diabetic rats of the experiment described in the manuscript were collected and used to determine the effects of *S. fruticosa* tea (SFT) treatment on islet regeneration (through β cell insulin expression). The effects were evaluated by immunohistochemistry, as described in the manuscript for GLP-1, with minor modifications. Sections were subjected to 1 % (v/v)-SDS pre-treatment at room temperature for 5 min (Brown *et al*., 1996), rinsed and then incubated with primary rabbit polyclonal antibody to insulin (PROGEN Biotechnik GmbH, Heidelberg, Germany) diluted 1:100, in 1 % BSA in TTBS overnight at room temperature in humidity chambers. Slides were finally incubated with a secondary goat anti-rabbit or mouse biotin conjugated antibody, followed by a streptavidin conjugated Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA) incubation for 1 h at 37ºC with rinsing in-between.

The number of GLP-1 expressing L-cells/cm of villus length was assessed using the software LAS AF (version 1.4.1) from Leica Microsystem (Wetzlar, Germany). The average intensity of GLP-1 signal in immunoreactive L-cells, the average of β-cell insulin signal and percentage of active β-cell in islets of Langerhans were quantified using the Sigma Scan Pro (v5) software (SPSS Chicago, IL, USA).

The results show in STZ-diabetic animals a significant decrease in the percentage of active β-cells in islets of Langerhans and a decrease in β-cell insulin signal intensity compared to water drinking controls (**Fig. 1**).

A slight increase in the intensity of insulin signal from β-cells in sage tea drinking diabetic animals compared with diabetic control was observed, although SFT treatment seems not to have increased islet regeneration (since there was no detectable increase in the number of insulin producing β-cells).

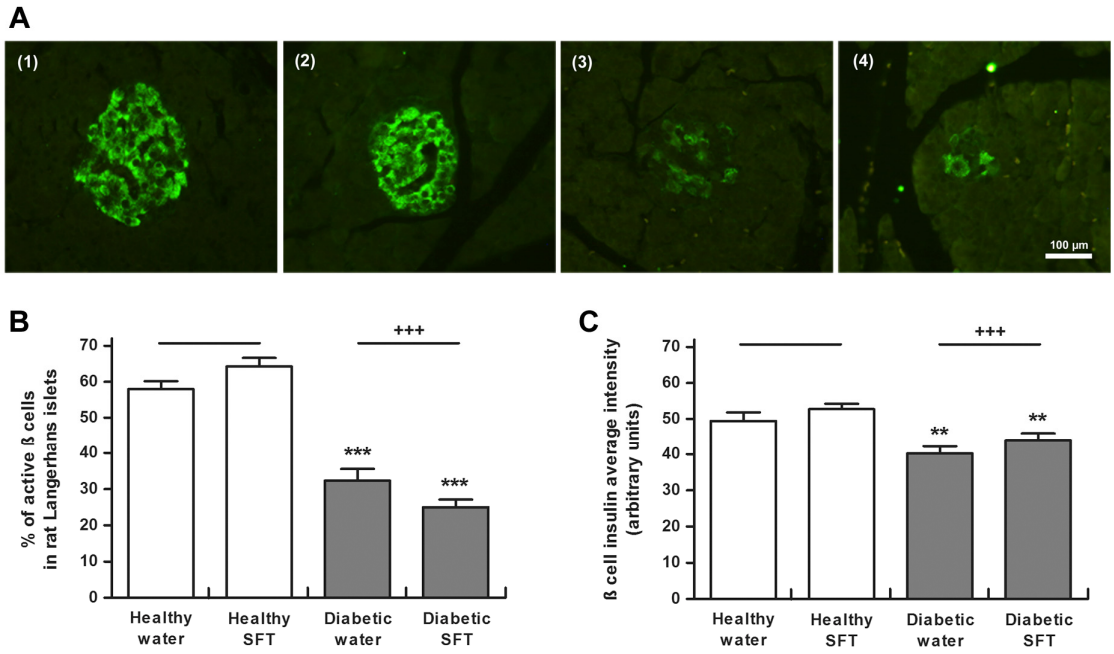

Fig. 1 Effects of *S. fruticosa* tea (SFT) treatment on STZ-induced decrease of insulin-producing β-cells. A – Immunofluorescence images of insulin signal in pancreatic islets β-cells: (1) healthy water group; (2) healthy tea group; (3) diabetic water group; (4) diabetic tea group). B – Percentage of active β-cells in rat islets of Langerhans. C – Intensity of insulin signal in immunoreactive β-cells. The % of active β-cells was determined by measuring the total islet area (A_T) observed in phase contrast field and the immunofluorescence insulin signal area (A_{IF}), using the following formula: % active β-cells = $A_T \times 100 / A_{IF}$. Values are means ± SEM, n=6. Two-way ANOVA, indicates that the difference between diabetic (W+SFT) and healthy (W+SFT) was significant ($^{+++}P<0.001$). The post-hoc test Student-Newman-Keuls indicates that $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$ when compared with the respective healthy control.

2) Evaluation of effects of *S. fruticosa* tea (SFT) treatment on proliferation of intestinal epithelium

Another feature of diabetes mellitus is an increase in intestinal epithelium cell proliferation (Adachi *et al*., 2003). The constant and rapid renewal of intestinal epithelial cells is essential for maximal nutrient absorption, adaptation to changes in diet, and repair from mucosal injury (Dahly *et al*., 2002). As occurs in other cell types (e.g. vascular smooth muscle cells), an increase in the proliferation rate of enterocytes is also observed in diabetes (Gizard & Bruemmer, 2008; Adachi *et al*., 2003).

Using samples of jejunal mucosa whole cell homogenates of the animals of the same experiment, described in the manuscript enclosed in this chapter, effects of SFT treatment on

epithelial cell proliferation were also assessed by measuring the enterocyte expression of the proliferating cell nuclear antigen (PCNA). The expression of PCNA was quantified by Western blotting, as described in the manuscript for the expression of SGLT1 and other proteins, using a mouse monoclonal antibody against rat PCNA (Abcam, Cambridge, UK).

Our results show that SFT treatment seems to interfere with intestinal epithelial cell proliferation only in STZ-induced diabetic rats, although not significantly (**Fig. 2**).

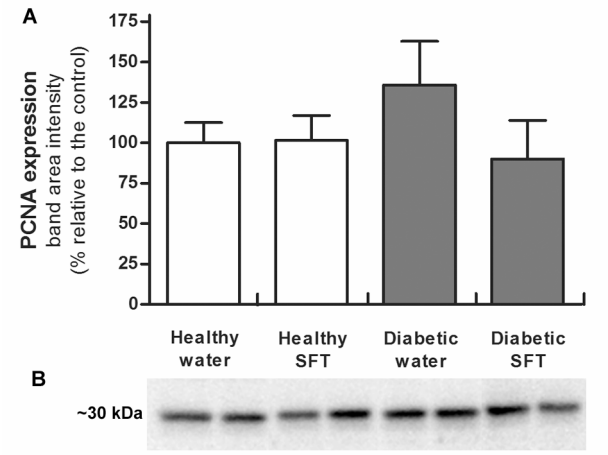

Fig. 2 Western blot analysis of PCNA expression in jejunal whole cell homogenates of healthy and STZ-induced diabetic rats (A). Representative immunoblots from two animals from each treatment group (B). Values are means ± SEM, *n*=5-6. Two-way ANOVA indicates that diabetic (W+SFT) and healthy (W+SFT) were not significantly different ($P>0.05$).

3) Evaluation of effects of *S. fruticosa* tea (SFT) treatment on apoptosis of intestinal epithelium cells

Hyperglycaemia leads to many and different cellular responses, which in certain circumstances can result in cell death. High plasma glucose levels induces oxidative and nitrosative stress in many cell types causing the generation of reactive species, that can cause the activation of several proteins involved in apoptotic cell death, as occurs with pancreatic β-cells (Allen *et al*., 2005; Robertson & Harmon, 2006). In order to determine the effects of SFT treatment at the level of diabetes-induced apoptosis of intestinal epithelial cells, a piece of jejunum from these animals was collected and subjected to the TUNEL assay.

The TUNEL assay was carried out using the *In Situ* Cell Death Detection Kit, POD (Roche Diagnostics GmbH, Penzberg, Germany), following manufacturer instructions.

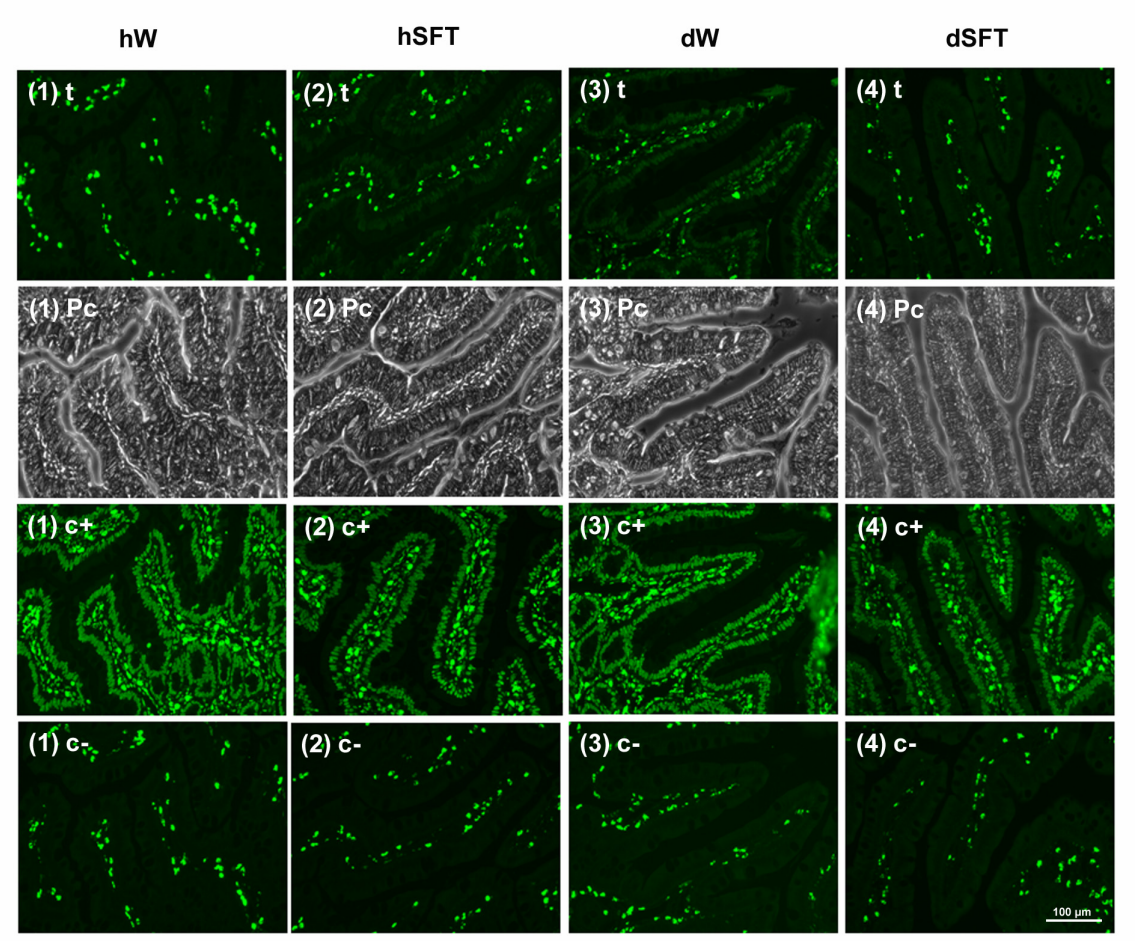

Fig. 3 Effects of *S. fruticosa* tea (SFT) treatment on the apoptosis of intestinal epithelial cells. Representative images of TUNEL assay in rat jejunum of healthy and STZ-induced diabetic rats. (1) – Healthy water group (hW); (2) – healthy SFT group (hSFT); (3) – diabetic water group (dW); (4) – diabetic SFT group (dSFT). Pc – Phase contrast; c^+ - positive control (with DNAse) and c^- - negative control.

As shown in **Fig. 3**, a slight signal of apoptosis in healthy SFT treated animals was obtained, although without any specific region. A similar result was observed in diabetic water animals. However, in diabetic SFT treated animals, no apoptosis signal was visible, which seems to indicate that sage tea is not affecting intestinal epithelial cells from apoptosis.

In conclusion, SFT treatment does not interfere with pancreatic β-cell mass regeneration. In addition, SFT treatment seems to slightly decrease intestinal cell proliferation while not having effects on apoptosis.

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Chapter 3

Salvia fruticosa tea – effective under diet manipulation

3.1. Chapter overview

In the previous chapter, using STZ-induced diabetic rats we showed that *S. fruticosa* tea treatment for 14 days stabilised plasma glucose levels only in diabetic rats and significantly decreased the diabetes associated increase in Na^+/glucose cotransporter 1 (SGLT1) expression in the brush-border membrane (BBM) of enterocytes, suggesting that this sage tea may be beneficial in the reduction of postprandial hyperglycaemia, which is particularly helpful in the control of T2DM progression.

In the present study, we intended to verify the effects of *S. fruticosa* tea on the adaptive increase of SGLT1 expression on the intestinal epithelium using a different model of SGLT1 induction. Thus, the effects of this sage tea and those of rosmarinic acid (RA; the main *S. fruticosa* tea phenolic compound in tea) on the diet-induced adaptive increase of SGLT1 expression in enterocytes were studied.

The administration of low-carbohydrate diet (LC) for 7 days was effective in down-regulating the expression of SGLT1 on the BBM. Rats fed again with a high-carbohydrate (HC) diet but only for 4 days (different groups drinking water or sage tea or RA) were used to test sage's ability to modulate the recovery of the SGLT1 expression on BBM. As expected, SGLT1 expression was almost totally recovered 4 days after the reintroduction of HC diet. Both *Salvia fruticosa* tea drinking and more significantly RA inhibited this adaptive increase in SGLT1 expression in BBM. Additionally, only RA significantly decreased plasma glucose concentration after 4 days of treatment, which means that this seems to be the active principle of this herbal tea. No effects of sage tea and RA on the basolateral expression levels of Na^+/K^+-ATPase and GLUT2 were observed on jejunal mucosa whole cell homogenates.

In order to shed some light on the molecular mechanisms behind the effects of *S. fruticosa* and RA, the expression of the heat shock protein 70 (Hsp70), a protein involved in the regulation of the expression and activity of SGLT1 on BBM, and protein kinase C (kinase involved in the regulation of endocytosis and trafficking of various recycling molecules) was measured in jejunal mucosa whole cell homogenates.

The effects of *S. fruticosa* tea and RA on Hsp70 expression on the BBM were similar to those obtained for SGLT1. In what concerns PKC, only RA significantly decreased the expression of this kinase in whole cell homogenates. Although these results can give some information and orientation about the molecular mechanisms behind *S. fruticosa* and RA effects on SGLT1 expression on BBM, it is necessary to clarify the involvement and relationship of both Hsp70 and PKC on this result, and therefore further research will be needed.

In conclusion, our results identify the active compound in water extract responsible for the beneficial effects of sage tea drinking in a condition where the intestinal glucose transport capacity increases with diet or disease progression. These data agree with the previous reports on the antidiabetic effects of *S. fruticosa* and reinforce the possible use of this plant in primary prevention of diseases, such as diabetes and obesity, through dietary interventions.

3.2. Manuscript

This chapter comprises the following manuscript:

Azevedo MF, Almeida MJ, Fernandes-Ferreira M & Pereira-Wilson C. *Salvia fruticosa* tea drinking modulates nutritional regulation of the intestinal Na^+/glucose cotransporter 1 – involvement of rosmarinic acid. Submitted to *British Journal of Nutrition*.

Salvia fruticosa tea drinking modulates nutritional regulation of the intestinal Na^+/glucose cotransporter 1 – involvement of rosmarinic acid

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Abstract

Salvia fruticosa (Greek sage) is used as an antidiabetic medicinal plant in the Mediterranean region. This plant's water extract has been shown to be antihyperglycaemic in normal and diabetic animals, after an oral glucose tolerance test (GTT) but not after an intravenous GTT, indicating possible postprandial effects. In order to clarify its antidiabetic potential, we studied the effects of *S. fruticosa* tea and its main phenolic compound – rosmarinic acid (RA) on the expression of the sodium dependent glucose transporter 1 (SGLT1) on the enterocyte's brush-border membrane (BBM) of rat small intestine, in response to changes in carbohydrate composition of the diet. To down-regulate the expression of SGLT1 on the BBM, rats were fed with a low carbohydrate (LC) diet for one week after which they returned to the high carbohydrate (HC) diet for 4 days. In these last 4 days, water was replaced with sage tea or RA in treatment groups to test their ability to modulate the recovery of the SGLT1 expression on BBM. As expected, SGLT1 expression quantified by Western blot significantly decreased after the LC diet and was almost totally recovered after 4 days of the reintroduction of HC diet. *Salvia fruticosa* tea drinking significantly inhibited this adaptive increase in SGLT1 expression in BBM and RA showed a strong inhibition. A similar effect was obtained for Hsp70 expression on the BBM. No effects of sage tea and RA on the basolateral expression levels of Na^+/K^+-ATPase and GLUT2 were observed on jejunal whole cell homogenates. After 4 days of treatment, only RA significantly decreased the plasma glucose concentration of rats. Our results identify the active compound responsible for the beneficial effects of sage tea drinking in conditions where the intestinal glucose transport capacity increases (diet and diabetes

progression). This work may present a novel possibility for therapeutical interventions, in which RA may be useful in antihyperglycaemic and weight control strategies.

Keywords: Salvia fruticosa Miller; rosmarinic acid; diet carbohydrate manipulation; SGLT1 expression; intestinal glucose absorption.

1. Introduction

The intestine has the ability to adapt functionally as well as morphologically to both internal and external stimuli such as diet composition (Drozdowski & Thomson, 2006*a*). Ingesting diets rich in digestible carbohydrates increases available luminal glucose and an adaptive increase in the abundance of enterocyte hexose transporters takes place to allow a higher rate of sugar absorption (Diamond *et al*., 1984). The main intestinal sugar transporters responsible for transporting glucose from the lumen into the blood are the Na^+/glucose cotransporter (SGLT1) and the facilitative glucose transporter 2 (GLUT2). SGLT1 is located to the brush-border or apical membrane (BBM) of the enterocyte and transports glucose and galactose, using the inward Na^+ gradient, from the intestinal lumen into the cytosol. Enterocyte basolateral Na^+/K^+-ATPase is responsible for the Na^+ gradient needed for glucose uptake. GLUT2 is mostly basolateral, transporting glucose, galactose and fructose from the cytosol to the blood (Wright, 1998). SGLT1 in BBM and GLUT2 in the BLM have been shown to be elevated in animals fed a high carbohydrate diet and found to correlate with an increase in glucose absorption (Cheeseman & Harley, 1991). Recently, it was shown that GLUT2 can also be found in BBM when the luminal sugar concentrations are high. In fact, high levels of glucose in the lumen promote the rapid recruitment of GLUT2 to the BBM, a phenomenon that is dependent on SGLT1 activity and occurs via protein kinase C βII and mitogen activated protein kinase-dependent signal transduction pathways (Helliwell *et al*., 2000*a*; Helliwell *et al*., 2000*b*; Kellett & Helliwell, 2000).

Luminal sugars are therefore involved in the regulation of sugar transporters expression and activity. It was demonstrated by Karasov *et al.* (1983) that changing diet composition from a high-carbohydrate (HC) to a no-carbohydrate, for several days, decreases glucose transport by the intestinal epithelium of rats. Switching again to HC diet, a recovery of glucose transport capacity is achieved. The down-regulation of sugar transport requires several days (it starts 2-3 days after switch to no-carbohydrate diet), whereas the up-regulation after reintroduction of HC diet is achieved within 1 day (Karasov *et al*., 1983). Changes in glucose transport induced by alterations in dietary carbohydrate are usually correlated with changes in transporter protein abundance (in SGLT1 levels) (Dyer *et al*., 1997), and according with Miyamoto *et*

al. (1993) this is also correlated with changes in mRNA levels. However, this is a controversial matter since other authors demonstrate that changes in SGLT1 levels can be dissociated from changes in mRNA abundance, suggesting a translational or post-translational regulation by luminal sugars (Lescale-Matys *et al*., 1993). Also internal factors, such as those present in diabetes, lead to increases in intestinal glucose absorption due to an increase in glucose transporter levels or intrinsic activity. In diabetic patients the glucose transport capacity is 3 to 4 times higher than that of healthy individuals due mainly to an up-regulation of SGLT1 expression in BBM of the enterocytes. This increase in glucose transport capacity exacerbates hyperglycaemia, in particular postprandially, which represents an undesirable consequence (Dyer *et al*., 2002; Drozdowski & Thomson, 2006*a*).

Compounds supplied in the diet with inhibitory effects on carbohydrate digestion and/or intestinal absorption may provide strategies to improve glycaemic control and reduce diabetic complications. Therapeutic interventions aiming at the inhibition of digestive enzymes (e.g. with acarbose) are already in use (Josse *et al*., 2003). A number of species of the genus *Salvia*, in particular *Salvia officinalis* and *Salvia fruticosa,* are among the medicinal plants suggested to have antidiabetic effects (Baricevic & Bartol, 2000). These plants may represent a source of novel therapeutical drugs. *S. fruticosa* showed to be hypoglycaemic in normal and diabetic animals, after an oral glucose tolerance test (GTT) but not after an intravenous GTT, indicating possible postprandial effects (Perfumi *et al*., 1991). We recently showed that *S. fruticosa* tea drinking significantly diminished the STZ-induced increase of SGLT1 expression (Azevedo *et al*., submitted *b*), providing therefore a possible explanation for the antidiabetic effects of Greek sage.

Recently, *S. fruticosa* tea and its phenolic compound rosmarinic acid (RA) did not show effects on the inhibition of the activity of α-amylase (*in vitro*), which indicate no effects at this level of carbohydrate digestion (Azevedo *et al*., submitted *a*). Therefore, in the present study we aim to assess the effects of *S. fruticosa* tea and RA (used in the same concentration as found in *S. fruticosa* tea) on the level of intestinal expression of glucose transporters (SGLT1 and GLUT2), and Na^+/K^+-ATPase in response to dietary carbohydrate. In view of the Hsp70 and PKC involvement on the SGLT1 activity or trafficking to the BBM, their expression levels were also determined.

2. Materials and methods

2.1. Plant material, preparation of S. fruticosa infusion (water extract)

Salvia fruticosa plants were cultivated in an experimental farm located in Merelim, Braga, Portugal, and were collected in June 2004. The aerial parts of plants were air-dried and

kept at -20ºC. Voucher specimen is kept in an active bank under the responsibility of the DRAEDM (Direcção Regional de Agricultura de Entre Douro e Minho) from the Portuguese Ministry of Agricultural.

Considering that sage is traditionally used as a tea, an infusion of *S. fruticosa* was routinely prepared as previously described by Azevedo *et al.* (submitted *a*). The preparation produced a 2.8 ± 0.1 mg of extract dry weight per ml of infusion, being rosmarinic acid (577.29μg/ml), 6-hydroxyluteolin-7-glucoside (104.78 μg/ml) and a heteroside of an unidentified flavone (99.13 μg/ml) the most representative phenolic compounds, and 1,8-cineole (61.74 μg/ml), camphor (25.22 μg/ml) and α-terpineol (5.77 μg/ml) the major volatile compounds (Azevedo *et al*., submitted *a*).

2.2. Animals

Male Wistar rats (5 weeks) were purchased from Charles River Laboratories (Barcelona, Spain) and acclimated to the Bioterium animal facilities of the Life and Health Science Research Institute (ICVS), University of Minho, for at least one week before the start of the experiments. The animals were maintained under controlled temperature (20 ± 2ºC), and humidity (55 ± 10%) with a 12 h light: 12 h dark cycle and given food and tap water *ad libitum*. The animals used in the experiment were kept and handled in accordance with our university regulations, which follow the *Principles of Laboratory Animal Care* (National Institutes of Health, 1985).

2.3. Experimental design

Thirty rats were used and divided into five groups of six animals each, where (**Fig. 1**): group 1 – rats were fed with water and food (normal rat chow – referred as high carbohydrate diet – HC) *ad libitum* for 14 days; group 2 – rats were fed with water and food (HC) *ad libitum* for 7 days, and afterwards fed for 7 days with a soybean diet (low carbohydrate diet – LC) replacing the normal rat chow *ad libitum*; group 3 – rats were treated as in group 2, and afterwards fed for more 4 days with water and HC diet *ad libitum*; group 4 – rats were treated as in group 2, and afterwards fed for more 4 days with daily fresh *S. fruticosa* tea (replacing the water drinking) and HC diet *ad libitum*; group 5 – rats were treated as in group 4, with daily fresh RA solution replacing the *S. fruticosa* tea drinking. The RA solution was prepared in tap water diluted in the same concentration found in the *S. fruticosa* tea (577 μg/ml; Azevedo *et al*., submitted *a*).

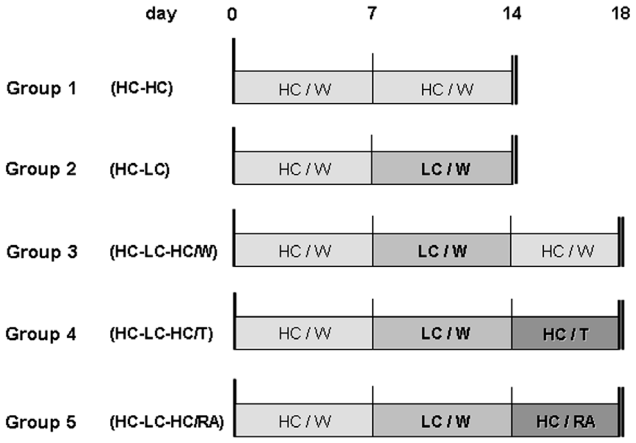

Fig. 1. Schematic representation of the experimental design. Thirty rats were divided into 5 groups with 6 animals each. Animals were fed either high-carbohydrate (HC) or low-carbohydrate (LC) diets and given either water (W), *S. fruticosa tea* (T), or rosmarinic acid (RA) to drink according to the schematic representation. Double vertical bar at the right end of each group indicate terminal sampling.

The composition of the two isocaloric diets given to the rats is presented in the **Table 1**. The normal rat chow (UAR-A04 chow diet, Reus, Spain) with 60.3% carbohydrate will be referred to throughout the text as HC diet and the soybean diet with about 28.0% of carbohydrates referred to as LC diet (Soybean meal 47.5, Cargill S.A.C.I., Buenos Aires, Argentina, kindly supplied by NANTA, Fábricas de Moagem do Marco S.A., Marco de Canaveses, Portugal).

Table 1 – Analytical constituents of the different diets (approximately values, in %).

	HC diet	LC diet
Carbohydrate	60.3	28.0
Protein	15.4	47.5
Fibre	4.1	4.2
Fat	2.9	2.0
Ash	5.3	6.3
Water	12	12

Animals were sacrificed by decapitation and the intestinal mucosa (40 cm of jejunum) was scraped off on ice with a glass microscope slide, after washing with PBS pH 7.4 (with 40 mM PMSF in ethanol added fresh). The intestinal mucosa was immediately frozen in liquid nitrogen and stored at -80ºC until use.

2.4. Plasma glucose measurement

The amount of glucose in rat plasma was measured using a colorimetric enzymatic method – Glucofix (A. Menarini Diagnostics, Firenze, Italy) – following manufacturer specifications.

2.5. Preparation of Brush-Border Membrane Vesicles

Brush-border membrane vesicles (BBMV) were prepared from frozen jejunal mucosa scrapings using a combination of cation precipitation and differential centrifugation as reported by Shirazi-Beechey *et al.* (1990) with few modifications as previously described (Azevedo *et al*., submitted *b*).

The final purified BBMV were suspended in a buffer containing 300 mM mannitol, 20 mM HEPES, pH 7.5 with 2M-Tris, and 0.1 mM $MgSO_4$ added after pH adjustment. BBMV were then frozen in liquid nitrogen and stored at -80ºC until used to quantify the SGLT1 expression in BBM by Western blotting. Protein content was measured with the Bradford Reagent (Sigma-Aldrich, St. Louis, MO, USA) using bovine serum albumin as a standard. The enrichment of the BBM was obtained by measuring the activity of alkaline phosphatase) and was about 10 times the mucosa whole cell homogenate.

2.6. Immunodetection of SGLT1, GLUT2, Na^+/K^+-ATPase and Hsp70

The abundance of SGLT1 and Hsp70 protein was measured in rat jejunal BBM by Western blotting as previously described (Azevedo *et al*., submitted *b*). The primary antibodies used were rabbit polyclonal to SGLT1 (Abcam, Cambridge, UK) diluted 1:2000, and a mouse monoclonal antibody against Hsp70 (Sigma-Aldrich, St. Louis, MO, USA) diluted 1:4,000. The same procedure was used to quantify the abundance of GLUT2, Na^+/K^+-ATPase and PKC in whole cell homogenates of jejunal mucosa, using a rabbit polyclonal antibody (Chemicon International, Temecula, CA, USA) diluted 1:2,500, a α5 mouse monoclonal antibody (Takeyasu *et al.*, 1988) diluted 1:500, and a rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:500, overnight at 4ºC, respectively. The intensity of the immunoreactive bands detected in the BBM (75 kDa for SGLT1 and 70 kDa for Hsp70) and intestinal mucosa whole cell homogenates (52 kDa for GLUT2, 110 kDa for Na^+/K^+-ATPase and about 80 kDa for PKC) were measured from digitised images, calibrated with a Kodak grey scale using Sigma Scan Pro (v5) software (SPSS Chicago, IL, USA). Results are presented as percentage of band area intensity of controls.

2.7. Statistical Analysis

Data are expressed as means with standard errors of the means (SEM), unless otherwise stated. For statistical analysis of the data, Student t-tests were used to compare differences within the different carbohydrate groups, namely HC-HC, HC-LC and HC-LC-HC/W groups. Then, to compare the effects of different drinking regimes (water-W; *S. fruticosa* tea-T; and, rosmarinic acid-RA) within the HC-LC-HC carbohydrate groups, a one-way ANOVA followed by the Newman-Keuls multiple comparison test were employed. P values < 0.05 were considered statistically significant.

3. Results

3.1. Effects of S. fruticosa tea and rosmarinic acid on plasma glucose levels

Since we were studying the effects of Greek sage and their main phenolic compound RA on the expression of glucose transporters in the small intestine, and in order to correlate that with intestinal glucose absorption plasma glucose levels (without a fasting period) was measured at the end of experiment for all groups. As shown in **Fig. 2**, the feeding regime of LC diet for 7 days produced a significant 15% reduction of plasma glucose levels, when compared with HC-HC group. Then, after 4 days of the reintroduction of the HC diet, plasma glucose returns to normal levels. However, in the group where RA replaced water, the return of plasma glucose to normal levels was completely inhibited (**Fig. 2**).

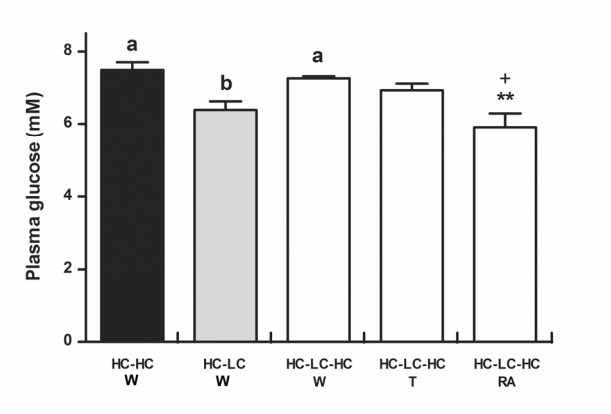

Fig. 2. Plasma glucose concentration of water drinking (W) animals maintained on a high carbohydrate diet (HC-HC; black bar), changed from a HC to low carbohydrate (LC) diet (HC-LC; grey bar), and finally returned to a HC diet following LC diet (HC-LC-HC; white bars). In addition to water drinking, HC-LC-HC animals were also given either sage tea (T) or rosmarinic acid (RA) *ad libitum* for the final 4 days on HC. Values were means ± SEM, n= 6. Effect of carbohydrate diet – groups with the same letter are not significantly different from each other ($P > 0.05$). Effect of drinking (white bars) – $^{**}P \leq 0.01$ when compared with the HC-LC-HC/W group and $^{+}P \leq 0.05$ when compared with HC-LC-HC/T group.

3.2. Effects of S. fruticosa tea and rosmarinic acid on SGLT1, GLUT2 and Na^+/K^+-ATPase expression

One way by which diabetes can be modulated is through the regulation of SGLT1 expression on BBM of the intestinal epithelium. Previously it has been suggested that the antidiabetic effects of *S. fruticosa* may be attained by the regulation of the SGLT1 expression in intestine, decreasing intestinal glucose absorption. Therefore, in this study we decided to investigate the effects of the Greek sage tea and its main phenolic compound RA on the expression of SGLT1 in rat BBM in an experimental design where SGLT1 levels were regulated by carbohydrate content in the diet. For that, the SGLT1 expression was decreased by 85% by feeding the animals with a LC diet (**Fig. 3**) for 7 days (HC-LC group). Then, the levels of this protein returned near normal levels by reintroduction of the HC diet (**Fig. 3**) to observe possible inhibition effects of sage tea and RA on the expression of this protein on the BBM of rat enterocytes. As shown in **Fig. 3**, the replacement of water by *S. fruticosa* tea in the reintroduction of the HC diet, a significant inhibition of about 32% on the adaptive increase of SGLT1 expression was observed. Rosmarinic acid, given to the rats instead of water for 4 days, strongly diminished (by 50%) the adaptive SGLT1 increase observed by reintroduction of the HC diet. Although water-drinking rats achieved normal SGLT1 expression levels, RA and sage tea drinking rats did not.

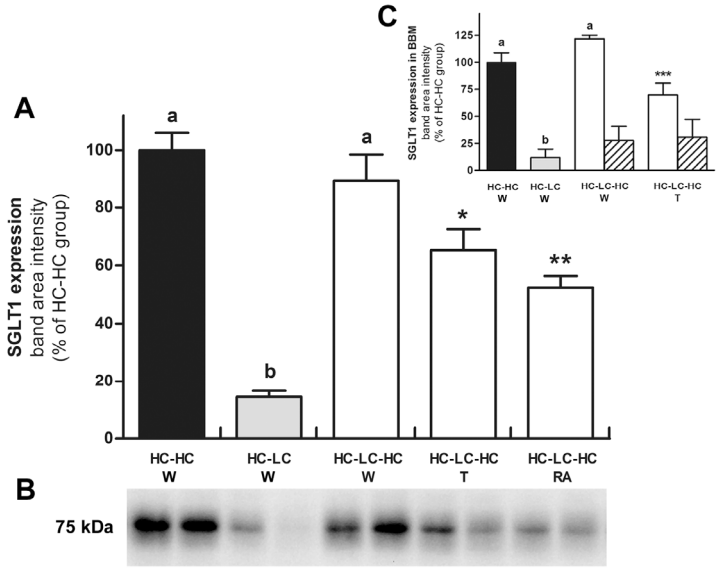

Fig. 3. Expression levels of SGLT1 protein in jejunum BBM from rats of the different treatment groups determined by western blotting. (**A**) – Western blot analysis of SGLT1 expression in BBM of rats fed either their regular high carbohydrate (HC-HC; black bar), changed from a HC to low carbohydrate (LC) diet (HC-LC; grey bar) or returned to a HC diet following LC diet (HC-LC-HC; white bar). In this latter feeding regime animals were given either water (W), sage tea (T) or rosmarinic acid (RA) to drink *ad libitum* for 4 days (□) or 2 days (▨ - W or T only, shown in the inset **C**). The results presented in the insert concern a preliminary experiment, performed to adjust experimental conditions. Representative

blots of samples from a pair of animals from each treatment group (**B**). Values are means ± SEM, n= 6. Effect of carbohydrate diet – groups with the same letter are not significantly different from each other (P > 0.05). Effect of drinking (white bars) – $^{*}P \leq 0.05$ and $^{**}P \leq 0.01$ when compared with the HC-LC-HC/W group.

Expression of Na^+/K^+-ATPase protein and of the facilitative glucose transporter GLUT2 in jejunal whole cell homogenates was also measured in this study. It is known that Na^+/K^+-ATPase protein is important in maintaining the Na^+ gradient for glucose uptake through SGLT1, being therefore related with this glucose transporter activity and possibly with their expression (Wright, 1998). On the other hand, it is also important to measure GLUT2 expression since it is the other intestinal glucose transporter responsible for the facilitative transport of a considerable amount of glucose to the blood, particularly in the basolateral membrane of enterocytes (Wright, 1998). As shown in **Fig. 4A**, GLUT2 expression was not changed by LC diet or *S. fruticosa* tea drinking, as well as by rosmarinic acid (P>0.05). Moreover, Na^+/K^+-ATPase expression significantly decreased with LC diet and after the reintroduction of HC diet a tendency to achieve the normal levels of this protein was observed for all the treatment groups, with less evidence in RA treated group (**Fig. 4B**).

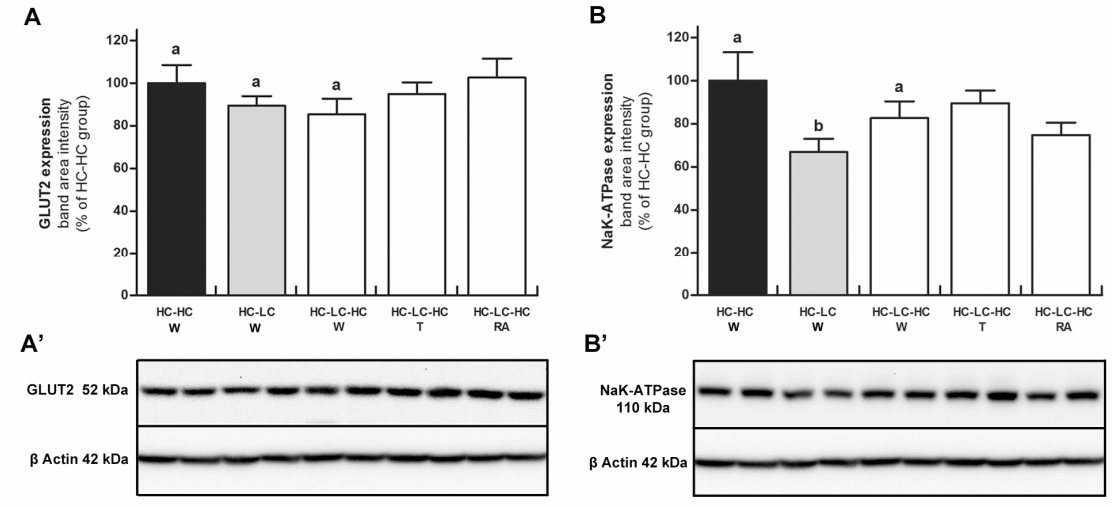

Fig. 4. Western blot analysis of (**A**) GLUT2 and (**B**) Na^+/K^+-ATPase expression in small intestine whole cell homogenates of rats with representative immunoblots and corresponding loading control (actin) from a pair of animals form each treatment (**A'** and **B'**). Water drinking rats were fed either their regular high carbohydrate (HC-HC; black bar) diet, changed to low carbohydrate (LC) diet (HC-LC; grey bar), or returned to a HC diet following LC diet (HC-LC-HC; white bar). In this latter feeding regime animals were also given either sage tea (T) or rosmarinic acid (RA) to drink for 4 days. Values are means ± SEM, n 6. Effect of carbohydrate diet – groups with the same letter are not significantly different from each other (P > 0.05). Effect of drinking (white bars) – no statistical significances were obtained by one-way ANOVA.

3.3. Effects of S. fruticosa tea and rosmarinic acid on Hsp70 and PKC expression

Since it is known an involvement of Hsp70 on the translocation of SGLT1 to the BBM due to an increase in TGF-β1 secretion as result of stress (Ikari, 2002) the expression of this protein was also measured on the BBM in order to verify if there is a correlation between SGLT1 and Hsp70 expression levels. As shown in **Fig. 5**, a similar pattern as SGLT1 was obtained by Western blotting for Hsp70 expression, although less pronounced. A significant decrease of Hsp70 expression was obtained by feeding the animals with a LC diet, which returned again to normal levels after 4 days of the HC diet reintroduction in water drinking rats (**Fig. 5**). When *S. fruticosa* tea or RA was given for 4 days to the animals replacing water, with the reintroduction of the HC diet, an inhibition on Hsp70 expression levels in BBM was observed in both groups, although not statistically significant (**Fig. 5**).

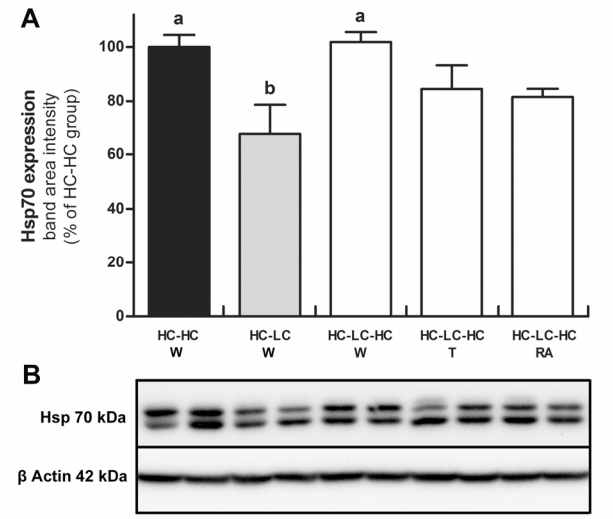

Fig. 5. Expression levels of Hsp70 expression in jejunum BBM from rats of the different treatment groups determined by western blotting. Western blot analysis of Hsp70 expression in BBM of rats fed either their regular high carbohydrate (HC-HC; black bar), changed from a HC to low carbohydrate (LC) diet (HC-LC; grey bar) or returned to a HC diet following LC diet (HC-LC-HC; white bar). In this latter feeding regime animals were given either water (W), sage tea (T) or rosmarinic acid (RA) to drink *ad libitum* for 4 days (**A**). Representative blots of samples and corresponding loading control (actin) from a pair of animals form each treatment group (**B**). Values are means ± SEM, $n = 6$. Effect of carbohydrate diet – groups with the same letter are not significantly different from each other ($P > 0.05$). Effect of drinking (white bars) – no statistical significances were obtained by one-way ANOVA. The two bands obtained were analysed together.

Additionally, it is known that there is an involvement of protein kinases, such as PKC in vesicle trafficking of SGLT1 to BBM (Wright *et al*., 1997), and therefore we decided to verify if there was a correlation between SGLT1 in BBM and PKC levels in intestinal whole cell homogenates.

As shown in **Fig. 6** no effect on PKC expression was observed when LC diet was given to the animals. A decrease on the expression of PKC was obtained when *S. fruticosa* tea or RA was replacing water after reintroduction of HC diet, although only significantly different in RA treated animals (**Fig. 6**).

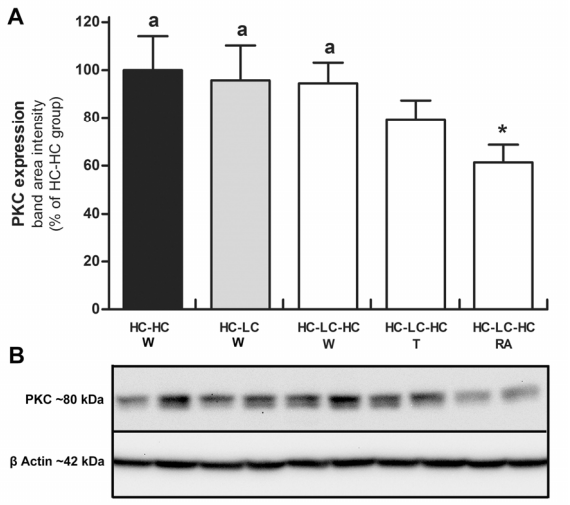

Fig. 6. Expression levels of PKC expression in small intestine whole cell homogenates from rats of the different treatment groups determined by western blotting. Western blot analysis of PKC expression in small intestine whole cell homogenates from rats fed either their regular high carbohydrate (HC-HC; black bar), changed from a HC to low carbohydrate (LC) diet (HC-LC; grey bar) or returned to a HC diet following LC diet (HC-LC-HC; white bar). In this latter feeding regime animals were given either water (W), sage tea (T) or rosmarinic acid (RA) to drink *ad libitum* for 4 days (**A**). Representative blots of samples and corresponding loading control (actin) from a pair of animals form each treatment group (**B**). Values are means ± SEM, n= 6. Effect of carbohydrate diet – groups with the same letter are not significantly different from each other ($P > 0.05$). Effect of drinking (white bars) – $^{*}P \leq 0.05$ when compared with the HC-LC-HC/W group.

4. Discussion and conclusions

Salvia fruticosa is one of the plants with antidiabetic reputation and previous experiments have confirmed that. In fact, *S. fruticosa* tea treatment for 14 days significantly decreased the expression of SGLT1 in the BBM induced by the diabetic state (Azevedo *et al*., submitted *b*). An improvement in the regulation of blood glucose levels of those diabetic animals was also observed during the experimental period. In view of these results, in the

present study we aimed to assess the effects of *S. fruticosa* tea and RA (its main phenolic compound, corresponding to 72% of the total phenolics) on the expression of enterocyte glucose transporters in response to dietary manipulation. A down-regulation of SGLT1 expression in BBM by replacing the normal HC diet by a LC diet was induced. This was attained 7 days after the introduction of the LC diet and an 85% reduction was observed (Fig. 3). The reintroduction of the HC diet for 4 days was able to completely restore the normal levels of SGLT1 (contrarily to what was obtained with 2 days). During this period, we studied the effects of sage and RA on this induced up-regulation of SGLT1, replacing the drinking water with the extract or a solution of the isolated compound RA. We observed that replacement of drinking water with sage tea or RA inhibited the restoration of SGLT1 levels in the BBM by about 32% and 50%, respectively. None of the effects of *S. fruticosa* tea treatment on SGLT1 expression were observed in healthy animals not subjected to previous down-regulation of SGLT1 (Azevedo *et al*., submitted *b*).

The previous results with diabetic rats, together with those of the present study indicate that this sage tea seems only to be effective on mechanisms of adaptive increase of SGLT1 expression present in diabetes and assimilation of a carbohydrate rich diet. In these conditions, where intestinal SGLT1 expression is increased and leads to higher glucose uptake, RA and *S. fruticosa* modulate SGLT1 expression in the BBM, therefore helping to control postprandial glucose increases. Moreover, the present study identifies RA as the active compound present in the water extract, responsible for this effect.

Taking into account that the small intestine is in constant state of turnover, with cells proliferating at the crypt, differentiating into enterocytes (among other cell types) as they migrate (and maturate) toward the villus tip (Ferraris, 2001; Gordon, 1989; de Santa Barbara *et al*., 2003), effects at 2 days after reintroduction of HC diet were also studied, in order to understand the time dependency of this response. In the present study, and in agreement with previous publications (Cheeseman & Harley, 1991; Ferraris & Diamond, 1992), a decrease in SGLT1 expression induced by LC diet was observed and an up-regulation of this transporter in BBM started to be produced 2 days after HC diet reintroduction, although so far than the normal levels. It is known that the cells in the crypts perceive the signal, reprogram transporters expression, and the newly formed cells need time to migrate and maturate along the villus, which according to Ferraris and Diamond (1992) takes 2-3 days. According to these results we may consider a long-term effect of sage tea, which suggests sage effects to be dependent on mechanisms such as crypt cell turnover rate and enterocyte migration/maturation rate, associated with the reprogramming of nutrient transporter expression in the crypts.

Using this model of reprogramming SGLT1 expression on BBM, we were able to show the inhibitory effects of *S. fruticosa* and RA on the diet-induced increase of SGLT1 levels. To our knowledge, this was the first report that shows an effect on SGLT1 expression by *S. fruticosa* and to identify RA as its active principle. In addition to its effect on SGLT1, RA also

significantly decreased (about 20%) plasma glucose levels. Previously, also luteolin-7-glucoside (a flavonoid also present in sage water extracts) and ursolic acid (a triterpene present in sage plants, but poorly represented or absent in water extracts) tested *in vivo* through orally administration in normal rats (Azevedo *et al*., submitted *a*) decreased plasma glucose levels, although no effect on SGLT1 or GLUT2 expression in intestinal epithelium was observed.

Also GLUT2 expression has been shown to increase in response to a carbohydrate rich diet (Drozdowski & Thomson, 2006*a*), in response to the hormone glucagon-like peptide 2 (Au *et al*., 2002) and also diabetes (Cheeseman & O'Neill, 1998). However, in the present study neither dietary manipulation nor sage tea on RA changed GLUT2 expression in intestinal epithelial cells. Since the LC diet did not decrease the expression of GLUT2, no effects of sage tea or RA on the inhibition of the adaptive increase of GLUT2 expression induced by HC reintroduction were expected. Cheeseman & Harley (1991) reported that the GLUT2 activity decreased within 3 days by a diet with lower carbohydrate content. However in their study activity levels and not protein expression were measured, which prevents direct comparison with our study. Recently, a mechanism involving a transient translocation of GLUT2 to the BBM has been proposed. Kellet & Brot-Laroche (2005) and Corpe *et al*. (1996) have detected GLUT2 in the apical membrane during assimilation of a carbohydrate-rich meal and in diabetes, which they claim contributes to an increased sugar uptake from the lumen. In the present study GLUT2 results correspond to whole mucosal cell homogenates.

Since the activity of SGLT1 in transporting glucose depends on the Na^+-gradient across the plasma membrane (Wright *et al*., 2007), we also measured effects on the expression levels of Na^+/K^+-ATPase in the small intestine. This protein is localised in the BLM of enterocytes and is responsible for maintaining the Na^+ gradient in the enterocytes (Drozdowski & Thomson 2006*b*; Wright *et al*., 2007). We observed that, Na^+/K^+-ATPase expression was affected significantly by LC diet and returned back to near control levels after the reintroduction of the HC diet. Neither *S. fruticosa* nor RA interfered significantly with this process. The similar behaviour of Na^+/K^+-ATPase and SGLT1 expression observed in this study could be explained by common mechanisms of regulation between the two proteins. However, several other transporters notably amino acids transporters also use the Na^+ gradient generated by this ATPase and the protein rich diet (LC) may have provided excess amino acids and down-regulated Na^+/K^+-ATPase and the sodium gradient.

Salvia fruticosa tea and RA interfere with SGLT1 expression in the BBM through mechanisms that seem to include crypt glucose sensing mechanisms and cell migration/maturation rates, as well as posttranslational mechanisms and trafficking of SGLT1 from cytosolic pools to the BBM of enterocytes. However, more research is needed in order to clarify the regulation of these processes by sage tea.

In order to shed some light on the molecular mechanisms behind the effects of *S. fruticosa* and RA some first efforts were made. Heat shock protein 70 (Hsp70), an important protein related with cellular antioxidant defences, has been also reported to be involved in the regulation of the expression and activity of the main intestinal glucose transporter – SGLT1. Recently, Hsp70 was identified in the apical membrane of porcine renal LLC-PK (1) cells. Ikari and colleagues (2002, 2003) showed in these cells a formation of Hsp70/SGLT1 complexes and that Hsp70 increased SGLT1 translocation to the BBM and activity. Our results show that Hsp70 expression profile obtained in BBM was similar to that of SGLT1. This indicates that Hsp70 may be associated with SGLT1, and its translocation and stabilisation in the BBM. *S. fruticosa* tea and RA are affecting SGLT1 expression, but may also be affecting its activity through the inactivation of the signalling pathway that leads to the formation of Hsp70-SGLT1 complexes and its translocation to the BBM.

Protein kinase C (PKC) has also been shown to be implicated in the regulation of translocation of vesicles containing glucose transporters such as GLUT4 and GLUT2 (Alvi *et al*., 2007; Chang *et al*., 2004; Helliwell *et al*., 2000*b*). It is well established that insulin through its signalling pathway (insulin receptor/insulin receptor substrate/phosphatidylinositol 3-kinase) leads to the activation of atypical PKC and/or Akt, resulting in the translocation of GLUT4 from intracellular pools to the plasma membrane of myocytes or adipocytes (Chang *et al*., 2004). Inhibition of atypical PKCs could lead to insulin-resistant diabetes by affecting GLUT4 trafficking in skeletal muscle and adipose tissue (Farese *et al*., 2005; Alvi *et al*., 2007). At the enterocyte level, the involvement of PKCβII in the transient translocation of GLUT2 to the apical membrane of enterocytes has been reported, when luminal sugar levels rise (Helliwell *et al*., 2000*b*). This effect is also mediated by molecules of the insulin-signalling cascade, suggesting an involvement of insulin in the regulation of intestinal sugar absorption. Helliwell *et al*. (2003) suggested that as sugar absorption increases (particularly due to activity of SGLT1), plasma glucose levels rise, which stimulates insulin release from pancreatic β-cells, activating phosphatidylinositol 3-kinase and PKCβII. Thus, the dynamic control of intestinal sugar absorption may be achieved by the rapid turnover of PKCβII. This pattern of trafficking involving PKCβII activation was not observed for SGLT1 (Helliwell *et al*., 2000*b*), although the present study suggests a dependency of BBM expression of SGLT1 on PKC levels. Wright and colleagues (1997) showed in *Xenopus laevis* oocytes that activation of PKC, may also regulate SGLT1 activity although this effect seems to depend on the sequence of the cotransporter since results were different between human and rabbit or rat SGLT1. Our results show that although sage tea only slightly affected PKC expression in enterocytes, RA significantly decreased the expression of this kinase. This effect on PKC resulted in a lower expression of SGLT1, although more research is needed to clarify this mechanism. These results suggest that RA may be interfering with the insulin signalling pathway (via PKC), although through a mechanism

opposite to those observed in other tissues, which result in GLUT4 and GLUT2 translocation to the plasma membrane. RA by decreasing PKC levels may be contributing to SGLT1 internalisation. The insulin-signalling pathway has been shown to be involved with the regulation of enterocyte SGLT1 activity and expression (Kurokava *et al*., 1995) where its effects seem to be opposite to those observed in other cell types. These authors showed in diabetic rat with increased BBM SGLT1 expression that treatment with subcutaneous insulin significantly decreased SGLT1 levels without changes in mRNA, suggesting a mechanism involving vesicle trafficking. A significant amount of SGLT1 transporters reside intracellularly in microtubule-associated vesicular structures, sufficient to respond rapidly and efficiently to mechanisms of vesicle trafficking (Kipp *et al*., 2003). However, further research will be needed to clarify the molecular mechanism(s) behind *S. fruticosa* and RA effects on SGLT1 expression on BBM, and the involvement and relationship of both Hsp70 and PKC in this process.

In conclusion, although *S. fruticosa* tea did not have effects on plasma insulin secretion of healthy and diabetic rats (Azevedo *et al*., submitted *b*), nor on carbohydrate digestion (as well as RA, Azevedo *et al*., submitted *a*) the present study shows that *S. fruticosa* tea and RA treatment inhibits the adaptive increase in SGLT1 expression in BBM of rat enterocytes after stimulation with HC diet. Although we did not directly measure the effects on glucose absorption, in view of the RA effect on plasma glucose levels and on the decreased SGLT1 and Hsp70 expression in the BBM and PKC in jejunal mucosa whole cell homogenates, a contribution to clarify the mechanisms of action of *S. fruticosa* tea and a demonstration of its potential to reduce glucose absorption by the intestine was made. These data agree with previous reports on the antidiabetic effects of *S. fruticosa* and reinforce the possible use of this plant in primary prevention of diseases such as diabetes and obesity, through dietary interventions. Lifestyle changes through implementation of healthy eating have been considered important in the prevention of age-associated diseases (Harris & Zinman, 2000), and the use of medicinal plants, such as *S. fruticosa*, could provide a good "tool" in this regard.

Acknowledgements

We thank NANTA, Fábrica de Moagem do Marco S.A., Portugal for offering the Soybean meal 47.5 (LC diet). We also thank Dr. Hermann Koepsell, University of Würzburg for kindly supplying a SGLT1 antibody, used in previous assays, and Alice Ramos, University of Minho for their help with animal handling. We thank Cristóvão Lima for the fruitful discussions.
M. F. A. was supported by the Foundation for Science and Technology, Portugal, grant SFRH/BD/12527/2003. This work was supported by the Foundation for Science and Technology, Portugal, research grant POCI/AGR/62040/2004.

Abbreviations

LC: low carbohydrate diet; HC: high carbohydrate diet; SGLT1: Na (+)-glucose cotransporter 1; GLUT2: facilitative glucose transporter 2; BBM: brush-border membrane; BLM: basolateral membrane; BBMV: brush-border membrane vesicles; TRIS: Trizma base; Mw: Molecular weight; Hsp70: heat shock protein 70; PKA: protein kinase A; PKC: protein kinase C.

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Chapter

4

Antidiabetic potential of major constituents of sage plants

4.1. Chapter overview

Several species of sage are used by popular medicine due to their antidiabetic properties (Baricevic & Bartol, 2000)[1]. However, few studies have been performed to confirm the veracity of this belief.

In the work presented in this chapter three *Salvia* species (*Salvia officinalis* L. (a common sage), *Salvia fruticosa* Mill. (greek sage) and *Salvia lavandulifolia* Vahl. (spanish sage), of the Lamiaceae family), namely their water extracts (WEs) and some individual compounds were used to confirm antidiabetic properties that have been attributed to these plants

Phytochemical characterisation of WEs of *S. fruticosa* and *S. lavandulifolia* was made, and effects of *S. officinalis*, *S. fruticosa* and *S. lavandulifolia* WEs and some individual compounds on the activity of α-amylase were determined. The results show that of the sage constituents only luteolin-7-glucoside (L7G) has some inhibitory effect on the activity of α-amylase. Other individual compounds and the three sage WEs, rather than inhibiting, increased α-amylase activity though not significantly.

The control of plasma glucose is a central feature of an antidiabetic strategy. Previously we have shown that *in vivo* treatment with *S. officinalis* and *S. fruticosa* WEs decrease fasting plasma glucose (Lima *et al.*, 2006; Azevedo *et al.*, submitted) [2,3], although not affecting other relevant parameters such as liver glycogen (unpublished data). The *in vivo* effects of L7G and ursolic acid (UA), two compounds abundant in these plants (Lima *et al.*, 2005; Janicsak *et al.*, 2006)[4,5] on liver glycogen, plasma glucose and lipids (total cholesterol, HDL and LDL) were assessed. From this *in vivo* experiment we verified that UA and L7G significantly decreased plasma glucose concentration. Ursolic acid also significantly increased liver glycogen, whereas total plasma cholesterol and low-density lipoprotein cholesterol levels were significantly lowered by both compounds. Additionally, high-density lipoprotein levels were significantly increased by UA.

Our results show that UA acts on glycogen deposition (and therefore on glucose clearance mechanisms maybe by activating insulin receptor (IR) and the phosphatidylinositol 3-kinase (PI3K) signalling pathway, thus increasing liver insulin sensitivity (Lee & Kim, 2007)[6], whereas L7G may act on carbohydrate digestion and possibly also on gluconeogenesis.

Therefore, UA seem to have a potential for use in the treatment of T2DM, because on the one hand it increases liver glycogen deposition and on the other hand contributes positively to the control of dyslipidaemia (increasing HDL cholesterol and at the same time decreasing LDL cholesterol levels).

These sage species enjoy the antidiabetic reputation, although their potential is not at the level of inhibition of α-amylase. Additionally, *S. officinalis* and *S. fruticosa* WEs decrease

plasma glucose *in vivo*, although did not reveal effects at the level of liver glycogen deposition (data not shown). However, UA and L7G, major constituents of this genus, showed that they may be useful in the amelioration of the metabolic deregulation associated with diabetes, since they showed to be effective in the control of hyperglycaemia and dyslipidaemia.

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4.2. Manuscript

This chapter comprises the following manuscript:

Azevedo MF, Camsari Ç, Braga PSC, Valentao PCR, Andrade PB, Seabra RM, Fernandes-Ferreira M & Pereira-Wilson C. Sage plants as potential sources of antidiabetic compounds. Submitted to *Journal of Agricultural and Food Chemistry*.

Sage plants as potential sources of antidiabetic compounds

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Abstract

In order to study the involvement of sage constituents on plasma glucose regulation, water extracts of *Salvia fruticosa* and *S. lavandulifolia* were characterised and their effects and those of some major constituents on the capacity to inhibit α-amylase *in vitro* were determined. Also effects on plasma glucose and lipid profile of ursolic acid (UA) and luteolin-7-glucoside (L7G) were assessed *in vivo*. Our results show that, of the water extracts and individual constituents, only L7G inhibited α-amylase. However, both UA and L7G significantly decreased plasma glucose concentration *in vivo*. Ursolic acid also significantly increased liver glycogen and plasma high-density lipoprotein levels. Total plasma cholesterol and low-density lipoprotein levels were significantly lowered by both compounds. Taken together, our results confirm the antidiabetic potential attributed to these plants and suggest that, particularly UA, may contribute to improve the health status of diabetic patients and to reduce the risk of cardiovascular complications.

Keywords: Salvia fruticosa Mill.; *Salvia officinalis* L.; *Salvia lavandulifolia* Vahl.; Lamiaceae; ursolic acid; luteolin-7-glucoside; blood cholesterol; antidiabetic; phytopharmaceuticals; functional foods.

1. Introduction

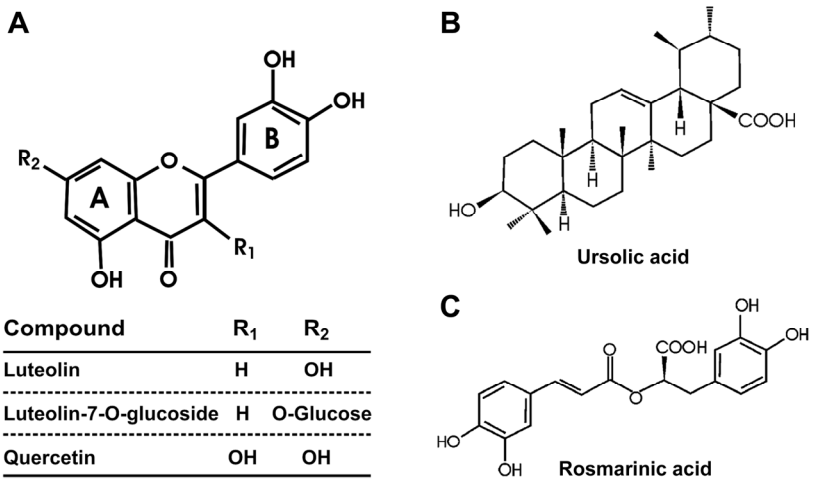
Glucose is a major source of energy for humans and its concentration in the blood is tightly regulated by the pancreatic hormone insulin. Diabetes mellitus is a metabolic disorder characterised by chronically elevated blood glucose associated with the impairment of insulin

secretion and/or a deficient action on peripheral tissues (Klover & Mooney, 2004). Once absorbed after a meal, rising plasma glucose stimulates insulin release and, in tissues such as liver and skeletal muscle, glycogen synthesis is promoted which constitutes an important mechanism of glycaemic control. In diabetic patients this mechanism of plasma glucose clearance is less efficient, contributing to hyperglycaemia (Postic *et al.*, 2004). Diabetic hyperglycaemia is aggravated postprandially as a consequence of intestinal glucose absorption. In the gastrointestinal tract, dietary carbohydrates are digested by pancreatic α-amylase and intestinal brush-border disaccharidases to D-glucose, D-galactose and D-fructose which are then absorbed to the blood by intestinal sugar transporters. Acarbose, an inhibitor of α-amylase, is used as a pharmaceutical oral antidiabetic agent that by decreasing carbohydrate digestion reduces glucose available for absorption and contributes to the control of plasma glucose (Bischoff, 1995).

In addition to hyperglycaemia, diabetic patients show an abnormal plasma lipid profile with high levels of low-density lipoprotein (LDL) cholesterol and lower than desirable levels of high-density lipoprotein (HDL) cholesterol, a combination implicated in the development of cardiovascular complications (Kastelein, 2005). By lowering plasma glucose and LDL cholesterol on one hand, and increasing HDL cholesterol levels on the other, an improvement of diabetic health status may be achieved that would also help prevent cardiovascular complications (Kastelein, 2005; Boden & Pearson, 2000).

The fact that the number of cases of diabetes is increasing worldwide and the realisation that the therapeutic drugs currently in use are not a hundred percent efficient motivates the search for new active principles among natural compounds from medicinal plants. In this study, we used water extracts (WEs) of three species of the genus *Salvia* (Lamiaceae family) for which antidiabetic properties have been suggested (Baricevic & Bartol, 2000) – *S. officinalis* L. (common sage), *S. fruticosa* Mill (Greek sage) and *S. lavandulifolia* Vahl. (Spanish sage) – in *in vitro* evaluation of effects on α-amylase. Effects of some individual constituents were evaluated as well in this assay.

The *in vivo* effects of luteolin-7-glucoside (L7G) and ursolic acid (UA), two abundant compounds in plants of this genus (Lima *et al.*, 2005; Janicsak *et al.*, 2006) (**Fig. 1**) on liver glycogen, plasma glucose and lipid profile (total cholesterol, HDL and LDL) were also measured in rats.



Compound	R_1	R_2
Luteolin	H	OH
Luteolin-7-O-glucoside	H	O-Glucose
Quercetin	OH	OH

Fig. 1. Chemical structures of the compounds used in this study. (**A**) – Flavonoids, (**B**) – pentacyclic triterpenoid (ursolic acid) and (**C**) – phenolic acid (rosmarinic acid).

2. Materials and methods

2.1. Chemicals

Porcine pancreatic α-amylase (EC 3.2.1.1), quercetin, rosmarinic acid and ursolic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Luteolin and luteolin-7-*O*-glucoside were purchase from Extrasynthese (Genay, France). Acarbose was kindly provided by Bayer Portugal SA (Carnaxide, Portugal). Glucofix was acquired from A. Menarini Diagnostics (Firenze, Italy). Commercial kits to measure total cholesterol, LDL cholesterol and HDL cholesterol were acquired from Spinreact (Girona, Spain). All others reagents were of analytical grade.

2.2. Plant material, preparation of S. fruticosa and S. lavandulifolia water extracts and analysis of its phenolic and volatile compounds

Salvia fruticosa and *S. lavandulifolia* were cultivated at an experimental farm located in Braga, Portugal, and were collected in June 2004 and July 2006, respectively.

The aerial parts of plants were air-dried and kept a -20 ºC. Voucher specimen is kept in an active bank under the responsibility of the DRAEDM (Direcção Regional de Agricultura de Entre Douro e Minho) from the Portuguese Ministry of Agricultural.

Salvia fruticosa and *S. lavandulifolia* WEs were routinely prepared as previously described for *S. officinalis* (Lima *et al.*, 2005) by pouring 150 ml of boiling water onto 2 g of the dried plant material and allowing to steep for 5 min. This preparation produced a WE of 2.8 ± 0.1 mg and

2.3 ± 0.1 mg of extract dry weight per ml of WE (0.28%, w/v and 0.23%, w/v) and a yield of 19.1% (w/w) and 17.0% (w/w) in terms of initial crude plant material for *S. fruticosa* and *S. lavandulifolia*, respectively.

Phenolic compounds were analysed by HPLC/DAD as previously described (Santos-Gomes *et al.*, 2002). Sub samples of freeze-dried extract (0.01 g) were redissolved in 1 ml of ultrapure Milli Q water and aliquots of 20 µl were injected into the HPLC/DAD system. The volatile constituents of the WE (100 ml) were extracted twice, at room temperature, with 5 ml of *n*-pentane containing 5-α-cholestane (0.5 mg/ml) for 15 and 45 min, respectively. The resulting extracts were gathered and analysed by GC and GC-MS. GC analytical conditions were the previously described by Lima *et al*. (2004). GC-MS analyses were performed with a Thermo Trace GC Ultra gas chromatograph equipped with a fused silica TR-5 (5% phenyl methylpolysiloxane) connected to a Thermo-Finnigan Polaris Q ion trap detector, operating in EI FullScan mode in the range 40-400 m/z. Injector, interface, and ion source temperatures were 300, 300, and 250 °C, respectively. The oven temperature program included a ramp from 60 (0.00 min) to 285 °C at 3 °C min^{-1}. Helium (He) was used as carrier gas, with an even flow rate of 1.5 $ml.min^{-1}$. The injections of the samples (0.5 µl) were made in splitless mode with the split valve opening at the end of 0.1 second after the injection. The identification and quantification of the compounds was performed following the methodology previously described (Santos-Gomes & Fernandes-Ferreira, 2001).

2.3. Inhibition assay for alpha-amylase activity

The ability of a compound or extract to inhibit α-amylase was determined by measuring this enzyme activity by determination of the reducing groups arising from hydrolysis of soluble starch as described elsewhere (Vogel, 2002).

In brief, the enzyme activity was assayed at 30º C for 7 min with various concentrations of test compounds/WEs dissolved in dimethyl sulfoxide (5% final concentration) or phosphate buffer, pH 6.9 (controls received vehicle only), in a reaction mixture consisting of 0.2% (w/v) starch, 0.02% (w/v) bovine serum albumin and 0.05 U/ml α-amylase in phosphate buffer (10 mM Na_2HPO_4, 10 mM KH_2PO_4, pH 6.9) in a total volume of 2 ml. The reaction was stopped by addition of 1 ml of DNS solution (1% (w/v) dinitrosalicylic acid, 12% (w/v) Na-K tartrate and 0.4 M NaOH). The mixture was heated for 10 min at 100 ºC and absorbance was then measured at 540 nm. The IC50 value was defined as the concentration of tested compound/WE that inhibits by 50% the α-amylase activity under the assay conditions.

2.4. Animals

Male Wistar rats (6 weeks) were purchased from Charles River Laboratories (Barcelona, Spain) and acclimated to our laboratory animal facilities for at least one week before the start of the experiment. During the experimental period, animals were maintained on a natural light/dark cycle at 20 ± 2 ºC and given food and tap water *ad libitum*. The animals used in the experiment were kept and handled in accordance with our university regulations, which follow the *Principles of Laboratory Animal Care* (National Institutes of Health, 1985).

2.5. Experimental design

Fifteen male Wistar rats were used and divided into three groups: control; UA-supplemented diet and L7G-supplemented diet. To all groups of animals the compound was administered orally by hand, once a day, for 7 consecutive days, well mixed in a small piece of food (control group received vehicle only) in a dose of 2 mg of compound per kg animal body weight. The dose of the compound administered was based in the work of Hertog *et al.* (1993). During the experiment, standard laboratory diet and water were given *ad libitum* to the animals. The administration of each compound did not change food and beverage consumption, as well as animal body weights when compared to the control. At the end of the treatment, animals were sacrificed by decapitation. Blood samples were collected for the measurement of glucose and lipid parameters (total cholesterol, LDL cholesterol, HDL cholesterol). Liver samples were also collected, frozen in liquid nitrogen and kept at -80 ºC for further glycogen measurement.

2.6. Measurement of plasma glucose, total cholesterol, low-density lipoprotein and high-density lipoprotein cholesterol levels

The amount of glucose in rat plasma was measured using a colorimetric enzymatic method - Glucofix - following manufacturer's specifications.

The plasma total cholesterol, LDL cholesterol and HDL cholesterol levels were measured in rat plasma using spectrophotometric commercial kits from Spinreact (Girona, Spain), following manufacturer's specifications.

2.7. Glycogen measurement

The liver glycogen content was quantified with amyloglucosidase as described previously (Keppler & Decker, 1974) with minor modifications. Briefly, a piece of tissue was homogenised individually in the proportion of 1:6 with perchloric acid 0.6 N. Then, 1 part of $NaHCO_3$ 1 N

was added to two parts of the previous homogenate for neutralisation. After that, 1 ml of amyloglucosidase 100U/ml (prepared in acetate buffer; controls of each sample received acetate buffer only) was added to 100µl of the neutralised homogenate, and the mixture incubated at 40 ºC for 2 h with shaking. The reaction was stopped by addition of 300 µl of perchloric acid 0.6 N, and after centrifugation, the amount of glucose was measured spectrofotometrically using the colorimetric enzymatic method - Glucofix - following manufacturer specifications. Dilutions of the liver homogenate were used to ensure that the determination was done within the linear phase. The glycogen content was expressed in µmol glucose per g of liver.

2.8. Statistical Analysis

Results are expressed as the mean ± standard error of the mean (SEM) for the number of animals in the group. Statistical significance between groups was determined using the one-way ANOVA followed by the Newman-Keuls multiple comparison test. A P value < 0.05 was considered statistically significant.

3. Results and Discussion

The three tested sage species are medicinal plants to which antidiabetic properties have been attributed. The objectives of the present study were to verify the antidiabetic potential of the sage WEs and to identify active principles among the plants' constituents. Because most commonly these plants are used to prepare teas (herein referred as WE), we characterised their WEs and studied their effects on carbohydrate digestion, namely on the activity of the enzyme α-amylase. The effects of L7G and UA, two major sage constituents, on plasma glucose, cholesterol and liver glycogen were also determined *in vivo*.

The phytochemical characterisation of WEs of *S. fruticosa* and *S. lavandulifolia* is shown in **Table 1**. The main phenolic compounds of *S. fruticosa* WE are rosmarinic acid (577.29 µg/ml), 6-hydroxyluteolin-7-glucoside (104.78 µg/ml) and a heteroside of an unidentified flavone (99.13 µg/ml). Of the 26 identified volatile compounds the most abundant were 1,8-cineole (61.74 µg/ml), camphor (25.22 µg/ml) and α-terpineol (5.77 µg/ml) (**Table 1**). In *S. lavandulifolia* WE the main phenolic compounds are rosmarinic acid (146.43 µg/ml), ferulic acid (6.46 µg/ml) and luteolin-7-glucoside (29.63 µg/ml). Thirty five volatile compounds were also identified of which 1,8-cineole (74.76 µg/ml), camphor (17.06 µg/ml) and borneol (3.29 µg/ml) are major constituents (**Table 1**). The total of 26 and 35 volatile compounds correspond to more than 99% of the total constituents in the *n*-pentane extracts from the *S. fruticosa* and *S. lavandulifolia* infusions, respectively. Characterisation of the phenolic and volatile composition

of *S. officinalis* WE identified rosmarinic acid (362 μg/ml) and L7G (115.3 μg/ml) as the most abundant phenolic compounds, and *cis*-thujone (1.7 μg/ml), 1,8-cineole (0.9 μg/ml), and borneol (0.7 μg/ml) as the most representative volatile compounds of a total of 25 identified as previously reported (Lima *et al.*, 2005).

Table 1 – Phenolic and volatile compounds of *Salvia fruticosa* and *Salvia lavandulifolia* water extracts.

Component	***S. fruticosa*** (μg/ml WE)	***S. lavandulifolia*** (μg/ml WE)
Phenolic compounds		
Phenolic acids		
Rosmarinic acid	577.29	146.43
Caffeic acid	8.69	-
Ferulic acid	3.49	6.46
3-Caffeoylquinic acid	tr	-
5-Caffeoylquinic acid	tr	-
Flavonoids		
6-Hydroxyluteolin-7-glucoside	104.78	-
Not identified flavone*	99.13	-
Apigenin-7-glucoside	6.68	-
Luteolin-7-glucoside	tr	29.63
Volatile compounds		
1,8-cineole	61.74	74.76
camphor	25.22	17.06
α-terpineol	5.77	0.32
δ-terpineol	1.73	0.50
cis-thujone	0.75	-
terpinen-4-ol	0.70	1.31
borneol	0.64	3.29
Others	3.39	3.17

WE – water extract; tr – trace amounts; * - flavone heteroside with a spectrum characteristic of apigenin glycoside, quantified as apigenin-7-glycoside.

The inhibition of the polysaccharide digestion enzyme α-amylase is a strategy used by the antidiabetic therapeutic agent acarbose which reduces the amount of free glucose available for absorption and therefore limits the postprandial elevation of plasma glucose. The possibility that sage WEs and individual compounds act by inhibiting the activity of this enzyme was tested *in vitro*. As shown in **Table 2**, *S. officinalis*, *S. fruticosa* and *S. lavandulifolia* WEs at 2.5 mg/ml increased (rather than decreased) the activity of α-amylase by 21%, 33% and 30%, respectively.

An increase of 21.9% was also observed for the main phenolic constituent – rosmarinic acid (500 μM).

Table 2 – Inhibition capacity of alpha-amylase activity of some sage constituents.

Compound	Concentration Tested[a]	Inhibition (%)	IC_{50}
Acarbose	25 μg/ml	71.8 ± 1.4	8.89 ± 0.59 μg/ml
S. officinalis water extract	2,5 mg/ml	-21.2 ± 3.0	-
S. fruticosa water extract	2,5 mg/ml	-32.7 ± 2.2	-
S. lavandulifolia water extract	2,5 mg/ml	-29.6 ± 4.1	-
Luteolin	500 μM	61.2 ± 0.5	357 ± 7 μM
	250 μM	38.5 ± 0.5	
	100 μM	13.2 ± 1.0	
Luteolin 7-*O*-glucoside	500 μM	25.2 ± 0.3	-
	250 μM	19.3 ± 1.7	
	100 μM	8.9 ± 1.4	
Quercetin	500 μM	1.6 ± 4.5	-
	250 μM	9.0 ± 2.5	
Rosmarinic Acid	500 μM	-21.9 ± 2.6	-
	250 μM	-6.5 ± 3.6	
Ursolic Acid	250 μM	-0.3 ± 1.2	-
	100 μM	3.4 ± 1.7	

Values (means ± s.e.) were obtained from four independent experiments.
[a] – for some compounds was the highest concentration that remains totally dissolved in the incubation mixture containing 5% of DMSO.

UA, an abundant triterpenoid in sage plants, did not change significantly the activity of this enzyme. Both acarbose (positive control) and luteolin significantly inhibited α-amylase activity with IC_{50}'s of 8.9 mg/ml and 357 μM, respectively, whereas quercetin (also used for being the correspondent flavonol of luteolin, **Fig. 1**) inhibited α-amylase but only by 9% at 250 μM. L7G (one of the most abundant flavonoids in sage WEs) inhibited α-amylase by 25% at 500 μM (at lower concentrations this effect was smaller). Both L7G and luteolin (the correspondent aglycone, **Fig. 1**) have been shown to inhibit α-amylase and α-glucosidase activities *in vitro* (Kim *et al.*, 2000). However, there are conflicting reports in the literature concerning effects of many natural compounds on α-amylase activity (Kusano *et al*., 1998; McCue & Shetty, 2004). This is probably due to the use of different methodologies as evidenced in the study done by Ali *et al.* (2006). In the present study, the methodology used is the one suggested in "Drug discovery and evaluation – pharmacological assays" (Vogel, 2002)

and we feel that the use of standardised methodologies to the identification of new active compound is necessary for results of this research to be meaningful. The present study show that the antidiabetic effects attributed to the teas of these sage species do not seem to be due to effects on carbohydrate digestion through α-amylase inhibition, although individually L7G showed moderate inhibitory effects on the activity of this enzyme.

The high blood glucose concentrations that characterise diabetes is responsible for the production of an excess of reactive oxygen species leading to oxidative damage (glucose toxicity) in several tissues, particularly in pancreatic β-cells and blood vessels. Pancreatic β-cell dysfunction and consequent decrease insulin secretion as well as micro- and macrovascular complications are consequences of this glucose toxicity (Robertson & Harmon, 2006; Jay *et al*., 2006). Thus, the control of plasma glucose is a central feature of an antidiabetic strategy. Previous studies have shown that *in vivo* treatment with *S. officinalis* and *S. fruticosa* WEs decrease plasma glucose (Lima *et al.*, 2006; Azevedo *et al.*, submitted) although not affecting other relevant parameters such as liver glycogen (unpublished data). In this study the individual compounds L7G and UA (among the major constituents of sage plants) were tested *in vivo* in Wistar rats in order to determine their effects on glycaemic control and lipid profile. After 7 days treatment (with 2mg kg^{-1} day^{-1} of UA or L7G in the diet), plasma glucose levels were significantly decreased (158.1 mg/dl (8.78 mM) and 157.6 mg/dl (8.76 mM), respectively) when compared to the control group (176.1 mg/dl (9.78 mM), **Fig. 2A**). The significantly lower plasma glucose in L7G treated animals may be due to effects on carbohydrate digestion or other aspects of glucose clearance although not to liver glycogen deposition. Effects on gluconeogenesis have been shown for *S. officinalis* WE where this compound is abundant (Lima *et al*., 2006), however this is not likely to be the dominant effect in the present case where the rats were not fasted overnight before sampling. Also UA treatment decreased plasma glucose significantly which was accompanied by an increase in liver glycogen content in contrast with L7G (**Fig. 2B**).

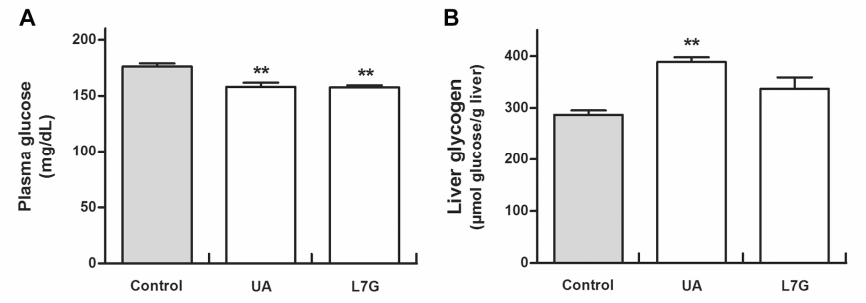


Fig. 2. Effect of 7 days diet supplied with the test compound (UA or L7G) on rat plasma glucose concentration (**A**), and on rat liver glycogen content (**B**). Values are expressed as mean ± SEM (n = 5). **, $P < 0.01$ when compared with the control group.

This suggests that UA acts on glycogen deposition (and therefore glucose clearance mechanisms) contrarily to what had been previously observed *in vivo* with *S. officinalis* and *S. fruticosa* WEs (unpublished data). Recently, *in vitro* studies performed by Jung *et al*. (2007), in adipocytes and CHO/IR cells showed that UA is an insulin mimetic/sensitiser, leading to an increase in insulin receptor (IR)*β* auto-phosphorylation and a subsequent activation of downstream phosphatidylinositol 3-kinase (PI3K) signalling pathway activation. The activation of this pathway would, according to Lee & Kim (2007), result in phosphorylation and inactivation of glycogen synthase kinase-3, leading to an increase glycogen synthase activation and glycogen synthesis. The increase of liver glycogen induced by UA observed in our study is in agreement with this and suggests that in the rat this compound may be activating IR and the PI3K signalling pathway which, by increasing liver insulin sensitivity, results in increased liver glycogen synthesis. It seems, therefore, that UA could be useful in the treatment of T2DM, where patients show a decrease in IR and insulin receptor substrate-1-phosphorylation and decrease PI3K activity in response to insulin (Goodyear *et al*., 1995).

In addition to plasma glucose, lipids are important as predictors of T2DM progression and development of cardiovascular complications. In order to assess possible effects of the UA and L7G on lipid profile, levels of plasma lipids (total cholesterol, LDL and HDL) were measured.

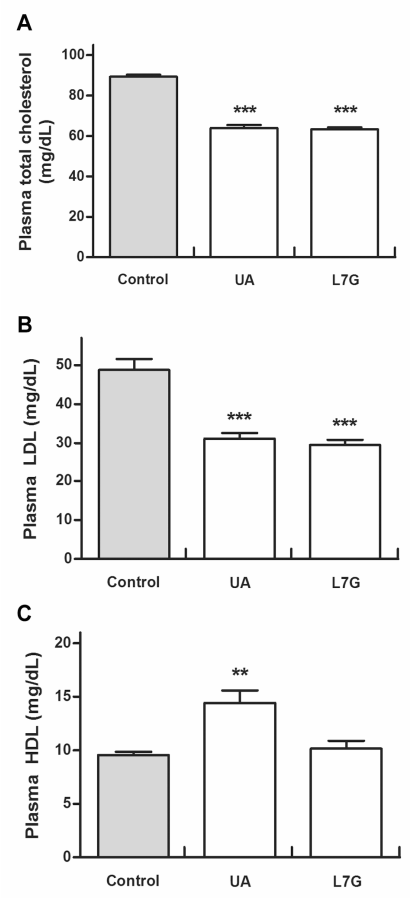


Fig. 3. Effects of 7 days diet supplied with the test compound (UA or L7G) on rat plasma total cholesterol (**A**), LDL cholesterol (**B**) and HDL cholesterol (**C**). Values are expressed as mean ± SEM (n = 5). ***, $P < 0.001$ and **, $P < 0.01$ when compared with the control group.

As shown in **Fig. 3A**, total plasma cholesterol levels were significantly reduced by both treatments (by 28.5% with UA and by 29.2% with L7G), which was accompanied by a consistent reduction of plasma LDL levels (by 36.2% and 39.5%, respectively, **Fig. 3B**). Simultaneously, UA increased by 51.1% plasma levels of HDL while L7G was not active on this parameter (**Fig. 3C**). Thus, both compounds showed relevant effects on the control of plasma lipids, by diminishing total cholesterol and LDL while UA additionally increased HDL levels. Statins are pharmaceutical drugs currently used in the treatment of diabetic dyslipidaemia and act mainly by reducing LDL cholesterol levels (Boden & Pearson, 2000). However, a therapy that in addition to a reduction of LDL cholesterol increases HDL cholesterol has been shown to be more effective in diminishing the risk of cardiovascular diseases and associated morbidity and mortality (Boden & Pearson, 2000). Combination therapy of statins and niacin is used and leads to a reduction of LDL associated with an increase of HDL levels, although contraindications are reported for both drugs (Toth, 2005). In this context, it is important to identify compounds that alone or in combination produce these results without side effects. UA administered alone produced this double effect in the rat, with an HDL increase of 51% apparently more effective than the drugs in current use (statins cause a HDL increase of 5-15% and niacin of 15-35%, (Toth, 2005). The effects of UA and L7G, in particular those of UA, suggest that this compound could contribute positively to the control of both hyperglycaemia and dyslipidaemia observed in T2DM. In addition, these beneficial effects of UA and L7G may be assisted by their improvement of cellular antioxidant defences (Lima *et al*., 2006; Yin & Chan, 2007; Ramos *et al*., 2008) known to be overwhelmed by the inherent excess of reactive oxygen species production observed in diabetes.

Since we have recently demonstrated that sage tea treatment affects intestinal glucose transporter expression (Azevedo *et al*., submitted), effects of UA and L7G on jejunal sodium glucose cotransporter 1 (SGLT1) and facilitative glucose transporter 2 (GLUT2) protein levels were also assessed. Phlorizin (PHZ) was also used in this study because it is a natural chalcone known to be a competitive inhibitor of SGLT1 *in vitro* (Panayotova-Heiermann *et al*., 1994). In our *in vivo* experiment, it had no effect on SGLT1 expression probably because it is degraded by digestive enzymes, such as intestinal lactase-phlorizin hydrolase (Ehrenkranz *et al*., 2005). Additionally, PHZ had no effects on plasma glucose or liver glycogen although it improved lipid profile (data not shown). UA and L7G were also without effect on the expression of intestinal glucose transporters (SGLT1 and GLUT2; data not shown). It is not known if their lack of effect is due to the compounds being degraded or to their not being effective at this level.

In conclusion, the antidiabetic properties of sage WEs do not seem to be due to inhibition of α-amylase activity in spite of the slight inhibitory effect of L7G on the activity of this digestive enzyme. L7G also showed effects *in vivo* on plasma glucose and lipid profile whereas UA in

addition to these was also effective in increasing liver glycogen deposition and HDL levels. The antidiabetic reputation enjoyed by these sage species may, at least in part, be the result of the effects of these two constituents. UA and L7G are present in the human diet (e.g. in berries, apples, prunes and herbs such as basil, rosemary and sage (Aggarwal & Shishodia, 2006; Lima *et al*., 2005), and are easily accessible to the population that, when informed, may make conscious choices of particular foods. In addition, both compounds should be considered for the production of enriched added value functional foods and included in dietary strategies for prevention of T2DM and health improvement of diabetic patients.

Abbreviations used

WE, water extract; UA, ursolic acid; L7G, luteolin-7-*O*-glucoside; LDL, low-density lipoprotein; HDL, high-density lipoprotein; IR, insulin receptor; PI3K, phosphatidylinositol 3-kinase; T2DM, type 2 diabetes mellitus ; PHZ, phlorizin ; SGLT1, Na+/glucose cotransporter 1 ; GLUT2, facilitative glucose transporter 2; GC-MS, gas-chromatography-mass spectrometry; HPLC/DAD, high-performance liquid chromatography diode array detection.

Acknowledgments

MFA is supported by the Foundation for Science and Technology, Portugal, grant - SFRH/BD/12527/2003. This work was supported by the Foundation for Science and Technology, Portugal, research grant POCI/AGR/62040/2004. We thank Cristóvão Lima for the fruitful discussions.

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Yin MC & Chan KC (2007) Nonenzymatic antioxidative and antiglycative effects of oleanolic acid and ursolic acid. *J Agric Food Chem* **55**, 7177-7181.

4.3. Supplementary data

The decrease of postprandial hyperglycaemia is one of the therapeutical approaches to treat diabetes, which could be reached both trough effects on carbohydrate digestion and on monosaccharide absorption. Therefore, the absorption of glucose could be diminished by inhibition of the activity of carbohydrate-hydrolyzing enzymes, such as α-amylase, in the digestive tract. Glucose absorption can also be diminished by reducing glucose transporters expression in intestinal epithelial cells or by decreasing their intrinsic activity. Water extracts from several medicinal plants, rich in flavonoids and other phenolic compounds have been shown to have hypoglycaemic effects by different mechanisms including inhibition of intestinal glucose absorption.

In the study reported in the manuscript enclosed in this chapter, both ursolic acid (UA) and luteolin-7-glucoside (L7G) (two major sage compounds) significantly decreased plasma glucose concentration *in vivo*. UA also significantly increased liver glycogen which seems to act on glucose clearance mechanisms, such as liver glycogen deposition. Since we did not obtain strong effects of these natural products at the digestive level (as observed by the results obtained from the *in vitro* assay of α-amylase activity), effects on intestinal glucose absorption, namely on the expression of intestinal glucose transporters sodium glucose cotransporter 1 (SGLT1) and facilitative glucose transporter 2 (GLUT2) were also assessed by Western blotting (see experimental procedure in the manuscript of chapter 2).

SGLT1 is located to the brush-border or apical membrane (BBM) of the enterocyte and transports glucose and galactose using the inward Na^+ gradient, from the intestinal lumen into the cytosol. Enterocyte basolateral Na^+/K^+-ATPase is important in maintaining the Na^+ gradient for glucose uptake. GLUT2 is mainly localised in the basolateral membrane of the enterocyte and transports glucose, galactose and fructose from the cytosol to the blood (Wright, 1998)[1].

As it was mentioned in the enclosed manuscript phlorizin (PHZ) was also used in this study because it is a natural chalcone known to be a competitive inhibitor of SGLT1 *in vitro*. Their effects on the expression of intestinal SGLT1 and GLUT2 were assessed, in addition to the effects on plasma glucose, lipid profile and liver glycogen.

Both UA and L7G did not affect the expression of the intestinal SGLT1 in BBM (**Fig. 1A**) and GLUT2 on the basolateral membrane of rat enterocytes (**Fig. 1B**).

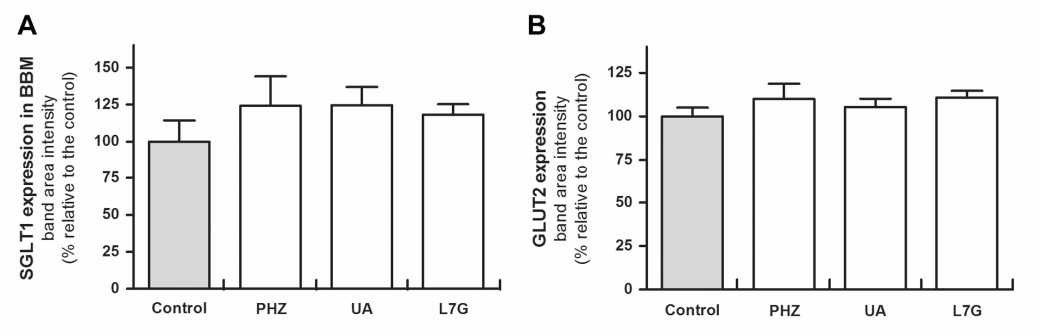

Fig. 1. Western blot analysis of SGLT1 and GLUT2 expression in jejunal brush-border membrane (BBM) of rats submitted to the treatment for 7 days with diet supplied with the test compound (PHZ, UA or L7G). Values are expressed as mean ± SEM (n = 4, 5).

PHZ had also no effect on SGLT1 expression (**Fig. 1A**). Probably, this compound affects only the activity of this glucose transporter, in view of its known competitive inhibition of SGLT1 and not its expression.

Additionally, in our *in vivo* experiment, PHZ had no effects on plasma glucose (**Fig. 2A**) and on liver glycogen (**Fig. 2B**), although it improved the lipid profile (**Fig. 3**).

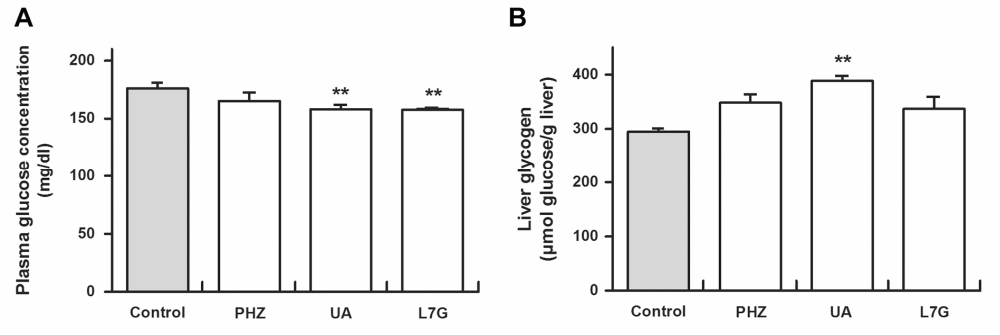

Fig. 2. Effect of test compound (PHZ, UA or L7G) supplied in the diet for 7 days on rat plasma glucose concentration (A) and liver glycogen content (B). Values are expressed as mean ± SEM (n = 4, 5). **, $P < 0.01$ when compared with the control group.

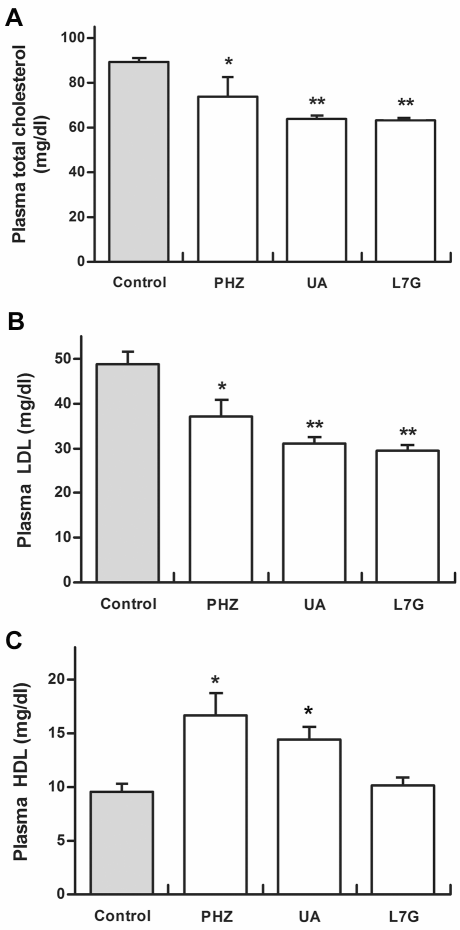

Fig. 3. Effects of test compound (PHZ, UA or L7G) supplied in the diet for 7 days on rat plasma total cholesterol (A), LDL cholesterol (B) and HDL cholesterol (C). Values are expressed as mean ± SEM (n = 4, 5). *, $P < 0.05$ and **, $P < 0.01$ when compared with the control group.

Note: Statistical Analysis was made using the One Way-ANOVA, followed by the Newman-Keuls multiple comparison test. Changes in the statistical significance of some groups, when compared with the ones in the previous manuscript, were due to the inclusion of PHZ group in the figure and in the one-way-ANOVA statistical test.

To conclude, despite the effects observed for UA and L7G reported in the manuscript of this chapter, these additionally results lead us to verify that these two sage compounds did not interfere with SGLT1 and GLUT2 expression and probably with intestinal glucose absorption capacity.

[1]Wright EM (1998) Glucose galactose malabsorption. *Am J Physiol* **275**, G879-G882.

Chapter

5

Antidiabetic effects of *Salvia officinalis*

5.1. Chapter overview

Apart from *S. fruticosa*, another sage species enjoys the reputation of antidiabetic – *S. officinalis* (common sage).

The first part of this chapter includes results of a study that was performed with the aim of verifying the antidiabetic effects of *S. officinalis* water extract (tea) *in vivo* in streptozotocin (STZ)-induced diabetic Wistar rats. Since hepatic glucose production is considered one of the main mechanisms of glucose homeostasis (regulated by two pancreatic hormones – insulin and glucagon), the modulation of hepatic glucose production in response to these pancreatic hormones and the modulation by *S. officinalis* essential oil (EO) was assessed using primary cultures of hepatocytes isolated from these diabetic rats. Metformin, a pharmaceutical drug currently used in the treatment and prevention of T2DM, particularly due to its capacity to inhibit hepatic gluconeogenesis, was used in this study as positive control for gluconeogenesis inhibition. The replacement of water by *S. officinalis* tea for 14 days lowered the fasting plasma glucose level in mice but had no significant effect on glucose clearance in response to an intraperitoneal glucose tolerance test, which suggested effects on hepatic gluconeogenesis (Lima *et al*., 2006)[1]. When primary cultures of hepatocytes from healthy sage tea drinking rats were stimulated by glucagon, they showed reduced gluconeogenesis compared to the response of cells from water drinking animals. Sage EO also inhibited gluconeogenesis and increased hepatocyte sensitivity to insulin. These were similar effects to those obtained with metformin. However, in the study with hepatocytes from STZ-diabetic rats no effects of sage tea treatment on insulin stimulation glucose consumption were observed in cells placed in a high glucose concentration medium. Additionally, EO did not inhibit hepatocyte glucose production, contrarily to what had been observed in healthy rat hepatocytes (Lima *et al*., 2006)[1]. Only metformin reduced glucose production by hepatocytes from diabetic water and sage tea treated rats. Although sage tea dinking did not show hepatic effects in a diabetic situation, its effects on fasting plasma glucose levels in healthy rats, and its metformin-like effects on hepatocytes from these healthy animals suggest that sage may be useful as a food supplement in the prevention of T2DM.

The second part of this chapter includes the results of a second study with *S. officinalis* tea, namely a pilot trial where the beneficial effects of sage tea drinking were assessed in healthy female volunteers aged 40-50, an age group of risk for T2DM. From this pilot trial, we verified that drinking *S. officinalis* tea is safe, since no significant increases in the levels of plasma aminotransferases were obtained, which indicate that hepatotoxicity was not present. In addition, no adverse effects on blood pressure, heart rate at rest and body weight were observed. By gradually decreasing plasma total cholesterol and LDL levels, while increasing HDL levels

in the plasma, *S. officinalis* tea drinking showed to be effective in improving lipid profile. An improvement in the erythrocyte antioxidant status by increasing SOD and CAT activities was also observed. There were no effects on blood glucose levels (fasting and postprandial), which indicates no effects on glucose clearance in healthy subjects, but also excludes the risk of hypoglycaemia associated with sage tea drinking

These results support the popular believe that sage tea drinking is safe and can contribute for an improvement of diabetic patient health condition.

[1] Lima CF, Azevedo MF, Araujo R, Fernandes-Ferreira M & Pereira-Wilson C (2006) Metformin-like effect of *Salvia officinalis* (common sage): is it useful in diabetes prevention? *British Journal of Nutrition* **96**, 326-333.

5.2. Metformin-like effect of *S. officinalis* tea

The first part of the data presented in this chapter was published in the *British Journal of Nutrition* together with data from other experiments. The publication is the following:
Lima CF, Azevedo MF, Araujo R, Fernandes-Ferreira M & Pereira-Wilson C (2006) Metformin-like effect of *Salvia officinalis* (common sage): is it useful in diabetes prevention? *British Journal of Nutrition* **96(2)**, 326-333.

5.2.1 Introduction

Changes in dietary habits and sedentary behaviour can reduce by 50-60% the progression from impaired glucose tolerance to T2DM, contributing to decrease the epidemic proportions of this disease (Chiasson *et al*., 2002; Simpson *et al*., 2003). Since pharmacological interventions in asymptomatic populations usually raises ethical considerations, in addition to practical and economical issues, dietary supplements with glucose-lowering properties could be a viable alternative. Plants have been used for centuries in popular medicine. Among them *Salvia officinalis* L. (common sage) is claimed to be beneficial to diabetic patients (Baricevic & Bartol, 2000), and previous studies have suggested that some of its extracts have hypoglycaemic effects in normal and diabetic animals (Alarcon-Aguilar *et al*., 2002; Eidi *et al*., 2005). Alarcon-Aguilar *et al*. (2002) showed that a water ethanol extract from *S. officinalis* injected intraperitoneally had hypoglycaemic effects in fasted normoglycaemic mice and in fasted mildly alloxan-induced diabetic mice. Additionally, Eidi *et al*. (2005) showed that sage methanolic extract given intraperitoneally significantly reduced serum glucose level in fasted streptozotocin (STZ)-induced diabetic rats without changes in insulin level.

In the study presented in the publication, we aimed to verify the antidiabetic effects of an infusion (tea) of common sage, which is the most common form of this plant consumed. Analysing the results obtained from my colleagues Lima CF and Araújo R, we verified that replacing water with sage tea for 14 days lowered the fasting plasma glucose levels in mice but had no effect on glucose clearance in response to an intraperitoneal glucose tolerance test. This indicated effects on hepatic gluconeogenesis and/or glycogenolysis. Primary cultures of hepatocytes from healthy (hereafter referred as normal) water or sage-tea-drinking rats where then utilised to further study this effect. After stimulation with insulin or glucagon they showed a high glucose uptake capacity and decreased gluconeogenesis, respectively. Essential oil from sage further increased hepatocyte sensitivity to insulin and inhibited gluconeogenesis. Overall, these effects resemble those of the

pharmaceutical drug metformin, a known inhibitor of gluconeogenesis used in the treatment and prevention of type 2 diabetes mellitus. My contribution to this work was the study of the responses to insulin and glucagon of primary cultures of hepatocytes isolated from STZ-induced diabetic rats. A series of *in vitro* studies with primary cultures of hepatocytes from these diabetic animals (previously treated for 14 days with sage tea or water) were performed in order to evaluate their response (hepatic glucose production/consumption) to the pancreatic hormones – insulin and glucagon, and modulation by *S. officinalis* essential oil (EO). Similarly to what was done with hepatocytes from normal animals, in this experiment the pharmaceutical drug – metformin – was also used as positive control.

5.2.2. Methods

Plant material, preparation of S. officinalis tea, isolation of essential oil and analysis of its constituents

Salvia officinalis L. plants were grown in an experimental farm located in Arouca, Portugal, and were collected in April, 2001. The aerial parts of plants were lyophilised and kept at – 20ºC.

The sage tea was routinely prepared as in a previous study by pouring 150 ml boiling water onto 2 g dried plant material and allowing it to steep for 5 min (Lima *et al*., 2005). This preparation produced a 3.5 ± 0.1 mg extract dry weight per ml infusion, with rosmarinic acid (362 mg/ml infusion) and luteolin 7-glucoside (115.3mg/ml infusion) as major phenolic compounds, and 1,8-cineole, *cis*-thujone, *trans*-thujone, camphor and borneol as the major volatile components (4.8 mg/ml infusion; Lima *et al*., 2005). The EO was obtained by hydrodistillation, and the compounds were identified by GC and GC–MS in a previous work (Lima *et al*., 2004). The EO included approximately sixty compounds, the most abundant being *cis*-thujone (17.4 %), alpha-humulene (13.3 %), 1,8-cineole (12.7 %), E-caryophyllene (8.5 %) and borneol (8.3 %; Lima *et al*., 2004).

Animals

Male Wistar rats (150-200g) were purchased from Charles River Laboratories (Barcelona, Spain) and during the experimental period were maintained under controlled temperature (20 ± 2ºC), and humidity (55 ± 10%) with a 12 h light:12 h dark cycle and given food and tap water *ad libitum*. The animals used in the experiment were kept and handled in accordance with our university regulations, which follow the *Principles of Laboratory Animal Care* (National Institutes of Health, 1985).

Induction of experimental diabetes

Diabetes was induced in rats by the intraperitoneal injection of a freshly prepared streptozotocin (STZ, Sigma-Aldrich, St. Louis, MO, USA) solution (50 mg/ kg in 0.1M citrate buffer, pH 4.5). Experiments with diabetic rats were carried out 1 week after STZ injection. During this period, diabetes was well established, with polydipsia, polyuria and non-fasting blood glucose levels of over 250 mg/dl. The animals were used in four different experiments.

Experimental design

In this experiment primary cultures of hepatocytes from STZ-induced diabetic rats were used in media with low and high concentrations of glucose (both containing the gluconeogenic substrate lactate) to evaluate effects of sage-tea drinking on cell glucose production. Eight STZ-induced diabetic rats (male Wistar) were randomly divided into two groups and given food *ad libitum* with either tap water or sage tea *ad libitum* for 14 days (the beverage being renewed daily). Because the diabetic rats were polydipsic, sage-tea drinking animals were given diluted sage tea, in order to ensure a similar intake of tea dry weight to that of the normal rats involved in the other experiments reported in Lima *et al*. (2006).

Hepatocyte isolation from diabetic animals was performed as described in Lima *et al*. (2006) for normal animals. Briefly, hepatocyte isolation was performed between 10.00 and 11.00 hours by collagenase perfusion, as previously described by Moldeus *et al*. (1978) with few modifications (Lima *et al*., 2004), from overnight-fasted rats. Cell viability was over 85 %, as estimated by a trypan blue exclusion test.

Cells were suspended in Dulbecco's modified Eagle's medium containing either 5.6 mM or 22 mM glucose, supplemented with 10 mM lactate, 100 ml FBS/l, 10^{-9} M insulin and 10^{-9} M dexamethasone, and seeded onto six-well culture plates at a density of 1×10^6 cells/well. The culture plates were incubated at 37ºC in a humidified incubator gassed with 50 ml/l CO_2/air. After plating, culture medium was replaced with Dulbecco's modified Eagle's medium supplemented with 10 mM lactate, 100 ml FBS/l and none, one or more of the following compounds: glucose (to a final concentration of 22 mM), 10^{-7} M insulin, 10^{-7} M glucagon, 10^{-3} M metformin and/or 4 nl sage EO/ml. After 24 h incubation, the medium was recovered for glucose quantification. Metformin was used as a positive control.

In an attempt to preserve the altered physiological conditions, introduced by both the fasting and the STZ-induced diabetes, cells were plated for 3 h for attachment (although plating periods of 24 h were used in cell cultures from normal animals) before exposure to the different test conditions.

In all experiments with rat hepatocytes, lactate dehydrogenase activity was measured in the media to ensure no toxicity of the treatment to the cells.

Biochemical analysis

The concentrations of glucose in the rat plasma and culture media were measured using a colorimetric enzymatic method (Glucofix) following the manufacturer's specifications.

The lactate dehydrogenase activity of the culture media was used as an indicator of hepatocyte plasma membrane integrity. The activity of the enzyme was measured at 30ºC by quantifying NADH consumption by continuous spectrophotometry on a plate reader (Spectra Max 340pc; Molecular Devices, Sunnyvale, CA, USA; as described in Lima *et al.*, 2005).

Protein content was measured with the Bradford reagent purchased from Sigma-Aldrich using bovine serum albumin as a standard.

Statistical analysis

Data are expressed as means ± standard errors of the means (SEM). In the experiment, a two-way ANOVA followed by the Student–Newman–Keuls *post hoc* test (SigmaStat, version 2.03; SPSS Inc., San Rafael, CA, USA) was employed to compare the effects of the *in vivo* beverage (water *v*. sage tea) and the *in vitro* treatments (insulin/glucagon; EO; metformin). For the latter, when a significant effect was obtained, a paired student's *t* test was employed to find the differences between each treatment. *P* values ≤ 0.05 were considered statistically significant.

5.2.3. Results

Contrarily the situation of cells from normal animals, when hepatocytes from STZ-diabetic rats were plated with medium containing 22 mM-glucose (and 10 mM-lactate), glucose production (and not consumption) was observed. In addition, there was no stimulation of glucose consumption by insulin (**Fig. 1**). Sage-tea-drinking did not modify this situation. Also EO did not inhibit hepatocyte glucose production. Only metformin was able to reduce the glucose production of hepatocytes isolated from diabetic water and sage-tea-drinking rats (**Fig. 1**).

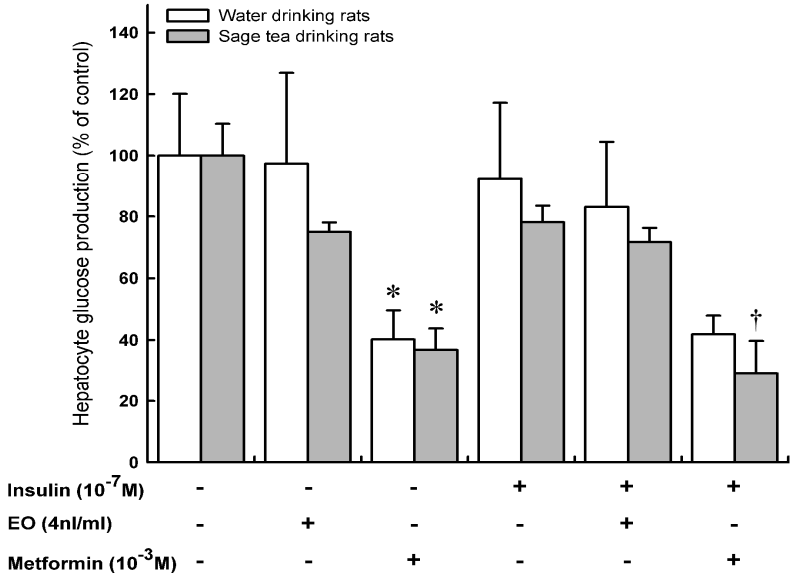

Fig. 1. Hepatocyte glucose production (24 h) by primary cultures of rat hepatocytes (isolated from water drinking streptozotocin-diabetic animals; □), and the effects of previous *in vivo* treatment with sage tea (for 14 days; ▩) on hepatocyte responses to insulin (10^{-7} M), essential oil (EO; 4 nl/ml) and metformin (10^{-3} M). The initial glucose concentrations of the medium were 22 mM, and experiments were performed 3 h after plating. Values are means with standard errors of the means shown by vertical bars, n=4. Water-drinking rats: 100% = 8.4 ± 1.7 μmol glucose/mg protein, sage-tea-drinking rats: 100% = 8.7 ± 0.9 μmol glucose/mg protein. Mean values are significantly different compared with the respective control group: *$P \leq 0.05$. Mean values are significantly different compared with the respective insulin group: †$P \leq 0.05$.

When hepatocytes from STZ-induced diabetic rats were plated with 5.6 mM glucose (and 10 mM lactate) containing medium, glucose production was similar in cells isolated from both water and sage-tea-drinking rats (**Fig. 2**). Glucagon did not further stimulate glucose production (**Fig. 2**). As above, no effect was observed for EO. Once again, metformin significantly reduced (by about 60%) hepatocyte glucose production in cells from both water and sage-tea-drinking rats (**Fig. 2**).

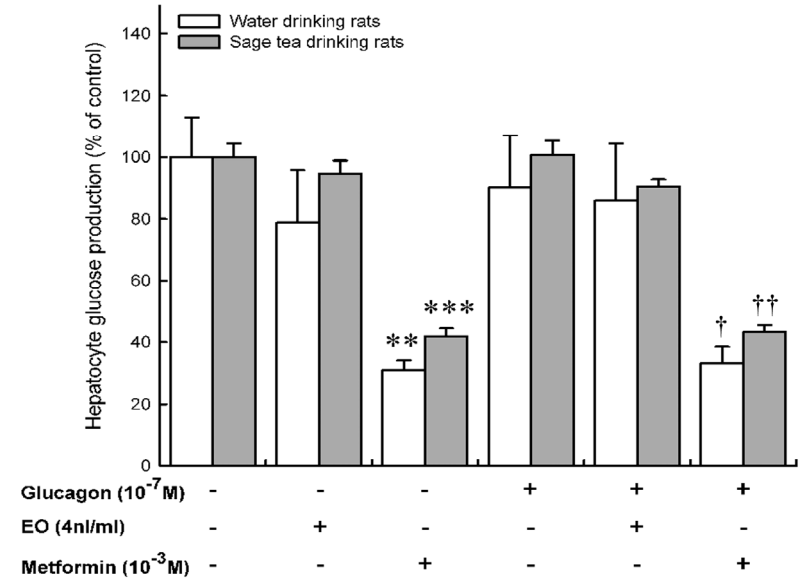

Fig. 2. Hepatocyte glucose production (24 h) by primary cultures of rat hepatocytes (isolated from water drinking streptozotocin-diabetic rats; □), and the effects of previous *in vivo* treatment with sage tea (for 14 days; ▩) on hepatocyte responses to glucagon (10^{-7} M), essential oil (EO; 4 nl/ml) and metformin (10^{-3} M). The initial glucose concentration of the

medium was 5.6 mM, and experiments were performed 3 h after plating. Values are means with standard errors of the means shown by vertical bars, n=4. Water-drinking rats: 100% = 7.9 ± 1.0 μmol glucose/mg protein, sage-tea-drinking rats: 100% = 7.6 ± 0.3 μmol glucose/mg protein. Mean values are significantly different compared with the respective control group: $^{**}P \leq 0.01$, $^{***}P \leq 0.001$. Mean values are significantly different compared with the respective glucagon group: †$P \leq 0.05$, ††$P \leq 0.001$.

All the treatments in the primary cultures did not induce lactate dehydrogenase release to the medium, an indicator that there was no cell toxicity in any of the *in vitro* treatments.

5.2.4. Discussion and conclusions

The abnormal glucose metabolism observed in both pre-diabetic and T2DM humans results in part from a deregulation of glucose production by the liver (mainly caused by an excessive glucagon stimulation of gluconeogenesis). In these cases, gluconeogenesis is active even when plasma glucose concentrations are elevated, which further aggravates hyperglycaemia (Roden & Bernroider, 2003). Sage tea drinking significantly reduced fasting plasma glucose levels in mice, suggesting an inhibition of gluconeogenesis and/or glycogenolysis (Lima *et al*., 2006). In agreement with this, sage tea drinking increased rat hepatocyte glucose consumption, decreased fasting gluconeogenesis and inhibited the stimulation of hepatic glucose production by glucagon. However, in spite of decreasing plasma glucose, sage tea did not improve glucose clearance (after an *in vivo* intraperitoneal glucose tolerance test). Thus, sage tea does not seem to increase the insulin response *in vivo*, although co-incubations of hepatocytes with sage EO result in an improvement of the *in vitro* effect of insulin, which suggests a role for EO in the increase in sensitivity to insulin.

In hepatocytes of sage tea drinking STZ-diabetic animals, *in vitro* EO incubation did not lead to any significant improvement in the response to insulin. The liver (and hepatocytes in culture) usually suppresses glucose release in response to insulin (Klover & Mooney, 2004). In STZ rat hepatocytes, insulin administration failed to suppress glucose production. Previous studies have also indicated that insulin is incapable of stimulating glucose utilisation *in vitro* by hepatocytes from STZ-induced diabetic rats (Salhanick *et al*., 1983; Amatruda *et al*., 1984; Hussin & Skett, 1988). The "insulin resistance" imposed by STZ treatment was not reversed by sage tea and/or EO. The lack of effect of sage tea/EO on STZ hepatocytes seems to indicate that sage requires an intact insulin signalling pathway to produce its effects. Contrarily to normal animal results, in the STZ diabetic rat, stimulation of the hepatocytes with glucagon did not enhance gluconeogenesis. Others have also failed to significantly stimulate gluconeogenesis *in vitro* in hepatocytes from STZ-induced diabetic rats (Dunbar *et al*., 1989).

One possibility for the lack of glucagon stimulation of gluconeogenesis in diabetic hepatocytes is that gluconeogenesis, *in vivo*, had been maximally stimulated. Cells are however,

metabolically competent and respond to metformin with a decrease in glucose production. In STZ-treated rats, insulin deficiency increases gluconeogenesis through enhanced lactate and pyruvate uptake and flux through the enzyme phosphoenolpyruvate carboxykinase (PEPCK) (Large & Beylot, 1999). Metformin has been shown to reduce substrate flux through this enzyme (Large & Beylot, 1999) and to inhibit PPCK gene expression (Cheng *et al*., 2001; Yuan *et al*., 2002), thereby decreasing gluconeogenesis. This inhibition of gene expression seems to occur mainly through an insulin-independent pathway (Yuan *et al*., 2002). It was recently demonstrated that the mechanism of action of metformin involves the AMP-activated protein kinase (AMPK), a kinase that was initially described as sensor of energy status of the cell, activated by exercise (Zhou *et al*., 2001; Towler & Hardie, 2007). Activation of AMPK results in enhanced glucose uptake and lipid catabolism by skeletal muscle, although at the level of the liver decreases hepatic glucose production and increases glucose utilisation (Zhou *et al*., 2001). It was shown that activation of AMPK results in the repression of both PEPCK and glucose-6-phosphatase gene expression (Towler & Hardie, 2007), an action similar to those of insulin in the liver.

This agrees with the possibility that sage tea and/or sage EO requires an intact insulin signalling pathway to produce its effects, which were observed only in normal rats (Lima *et al*., 2006).

In conclusion, by analogy with the effects of the drug metformin, used in the prevention and treatment of diabetes, the observed decrease in hepatocyte glucose production of normal sage tea drinking animals could be favourable, by preventing the liver's contribution to hyperglycaemia in groups at risk. The reduction in fasting plasma glucose shown in normal animals indicates a potential for sage extracts to help prevent T2DM, and considering the increasing prevalence of this disease in addition to the high costs involved in its treatment, the primary prevention is an important issue (Jermendy, 2005). In fact, T2DM is considered a preventable disease through changes in lifestyle that include, among others, dietary factors (Jermendy, 2005). Sage products can easily be used in food supplements that could have beneficial impact in low-cost-prevention strategies for diabetes, and in addition they are rich in antioxidants, which provide them with further health enhancing properties. Sage tea although not effective in diabetics, did not change the effect of metformin which exclude undesirable interactions with the commonly used metformin therapy. Taking these results and considerations into account, a pilot study with human volunteers treated with *S. officinalis* tea was then performed in order to evaluate the effects of this plant on the control of glycaemia and other diabetes relevant parameters in humans.

5.3. Effects of the regular intake of *S. officinalis* tea by humans – a pilot study

The second part of the data presented in this chapter corresponds to a pilot trial with healthy female volunteers carried out in our laboratory. The results which will be discussed here represent part of a manuscript in preparation.

5.3.1. Introduction

Type 2 diabetes mellitus (T2DM) accounts for the majority of diabetic cases (about 90%) and will become more prevalent over the coming decades due to the increasing rates of obesity in youth and adulthood and due to the increased sedentary lifestyles (Williams & Pickup, 2004). Hyperglycaemia exist in T2DM not only postprandially where it reveals the inability of insulin to stimulate peripheral glucose uptake, but elevated blood glucose levels persist even during fasting owing to increased hepatic gluconeogenesis (Roden & Bernroider, 2003; Williams & Pickup, 2004). Before the establishment of T2DM, individuals at high risk to develop it usually show the first signs of abnormal glucose metabolism, such as impaired glucose tolerance and/or impaired fasting glucose (Simpson *et al*., 2003). This provides an asymptomatic period at the beginning of T2DM progression, during which preventive interventions can be applied.

In fact, this type of diabetes is considered preventable (Gruber *et al*., 2006) and alternatives to pharmacological interventions, through dietary strategies including herbal teas with glucose-lowering properties, seem to have high acceptance particularly among the elders more at risk and be beneficial.

It has long been known that black and green tea (*Camellia sinensis*) drinking brings numerous health benefits, particularly because of their high content of antioxidant compounds. Tea intake has been associated with weight reduction and lowering of blood pressure and blood glucose levels, thus reducing the risk of cardiovascular diseases, T2DM and obesity (Polychronopoulos *et al*., 2007 and references therein).

Salvia officinalis (common sage) extracts and constituents are known not only for their antioxidant and anti-inflammatory properties (Baricevic & Bartol, 2000) but also for their beneficial effects on glycaemic control (Alarcon-Aguilar *et al*., 2002).

Recent results obtained in our laboratory revealed a metformin-like effect at the rat liver level (Lima *et al*., 2006), and these evidences that indicate an antidiabetic potential to this medicinal plant, a pilot trial was performed in our laboratory in order to confirm its effects in humans.

The pilot trial was performed with six healthy female volunteers during eight weeks. The trial was divided in different phases (baseline, sage tea treatment and wash-out), as shown in the experimental outline of **Fig. 1**. Effects of *S. officinalis* tea drinking on several parameters, such as liver toxicity, glycaemia (fasting and postprandial blood glucose levels), lipid profile and antioxidative defences, were assessed.

Although all the volunteers were non-diabetic, they belong to an age group at risk, and as mentioned it is important to detect the first signs of abnormal glucose metabolism for steps to be taken that prevent the progression of the disease. Blood glucose measurements were performed in three different situations, namely after an overnight fast, postprandially and after an oral glucose tolerance test – OGTT.

In a fasting sate, blood glucose levels tend to decrease because there is no intestinal absorption and hepatic gluconeogenesis is stimulated by the pancreatic hormone glucagon in order to increase glucose production, re-establishing plasma glucose to the physiological range. Disturbances at this level can be diagnosed by measuring fasting blood glucose concentration, allowing to identify people with impaired fasting glucose (IFG) (Ravel, 1989).

Impaired glucose tolerance (IGT) results when insulin secretion and action are not sufficient to activate the mechanisms of glucose clearance, contributing to increase insulin resistance and therefore creating postprandial hyperglycaemia (Chiasson *et al*., 2002). However, when insulin deficiency is small, fasting and postprandially blood glucose measurements can not identify efficiently individuals with IFG and IGT. Therefore, the OGTT is considered the most efficient method currently used in clinical practice. By loading a significant amount of glucose to the body, the relevant homeostatic mechanisms are challenged, and the plasma glucose profile in the subsequent 2 hours allow to identify healthy, diabetic or intolerant people by evaluating the individual's capacity to metabolise and consequently regulate blood glucose back to basal levels (Ravel, 1998).

Associated with T2DM characterised by insulin resistance are abdominal obesity, hypertension and dyslipidaemia, which in turn is characterised by high levels of triglycerides, associated with low levels of high-density lipoprotein - HDL, with or without a raise in low-density lipoprotein - LDL (Moller 2001; Saxena *et al*., 2005). Metabolic dyslipidaemia is one of the most important risk factors for the development of cardiovascular complications and is common not only in patients with T2DM, but also in subjects with impairment glucose tolerance (Toth, 2005; Veiraiah, 2005). Ideally, the management of dyslipidaemia and metabolic syndrome should result in both reduction of LDL and increase in HDL plasma levels (Boden & Pearson, 2000; Kastelein, 2005). Statins, niacin and fibrates (alone or in combination) are pharmacologic agents currently used in order to achieve this usually called "dual strategy", although contraindications have been reported for these drugs (Toth, 2005). In this context, it is important to identify alternative compounds that alone or in combination produce these results without

side effects. Moreover, oxidative stress has been demonstrated to be present in T2DM patients and to be a contributor to the progression of the disease and accelerated cardiovascular complications associated with it. Oxidative stress of cells caused by hyperglycaemia overwhelms the body's oxidative defences (enzymatic and non-enzymatic systems) and impairs physiological processes (Finkel & Holbrook, 2000). Therefore, it is important to diminish this diabetes related increased oxidative stress and its associated cardiovascular complications (Jay *et al*., 2006). Since plants, and particularly *S. officinalis*, are rich in antioxidant compounds, the beneficial effect of sage tea drinking on the improvement of antioxidant defences should be also exploited. Thus, in this study parameters such as erythrocyte antioxidant enzymes activities (superoxide dismutase – SOD and catalase – CAT) and plasma total cholesterol, LDL and HDL were also determined. Assessment of safety and clarification of possible contraindications is important if natural products and medicinal plants are going to be recommended as health promoting agents.

5.3.2. Methods

Subjects and study design

Six female volunteers (ages 40-50) participated in this trial. They were informed about the aims and methods of the study and signed an informed consent. The volunteers were non-diabetic, non-smokers and none was taking oral contraceptives or other medication (criteria on which the volunteers were selected). All six completed the study.

This trial was carried out in three phases: two weeks of baseline, four weeks of sage tea treatment (4 g dry leaves in 300ml boiled water taken twice a day) and two weeks of wash-out (**Fig. 1**). In order to obtain control values for all the volunteers, a baseline phase during two weeks was included. During this phase, all the parameters were measured and values treated as controls (basal levels). A treatment phase followed with sage tea drinking during four weeks. In this phase samples were collected twice (at the end of 2^{nd} week and 4^{th} week sage treatment). The two sampling times were planned in order to assess "short" or "long" term effects of sage tea treatment. After that a two week wash-out phase was included (without sage tea drinking) with the aim to assess the duration of sage tea effects beyond the treatment period.

During the study phases (baseline, treatment 2 (T2), treatment 4 (T4) and at the end of wash-out, as indicated by the arrows in **Fig. 1**) blood samples were collected, and then fasting and postprandial glucose as well as blood glucose concentration after an oral glucose tolerance test (OGTT) were measured. Activities of plasma aminotransferases ALT and AST (hepatotoxicity enzymes markers), levels of lipoproteins LDL and HDL, and total cholesterol

were also measured. Erythrocyte's antioxidant enzymes activities (SOD and CAT) were determined from haemolysed samples. Effects on body weight, blood pressure, heart rate at rest, perceived negative events and concomitant medication were recorded at the end of every week.

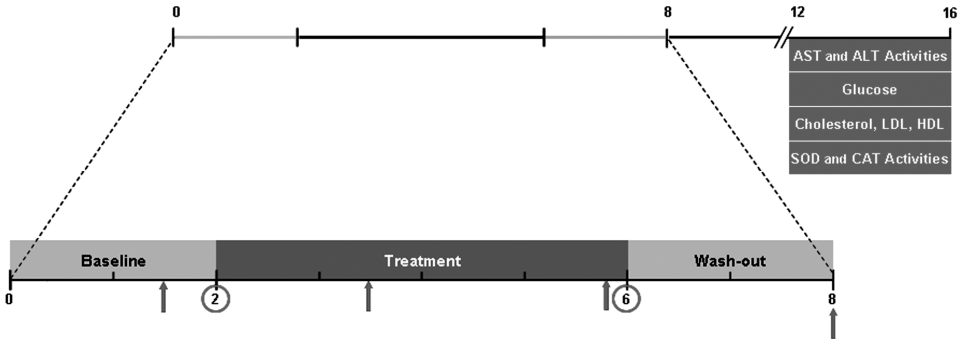

Fig.1. Schematic representation of experimental outline of the pilot study. The study comprises three different phases (two weeks baseline, four weeks of sage tea treatment and two weeks wash-out). Blood samples were taken at the times indicated by the arrows. Oral glucose tolerance tests were performed at the times indicated by the circles.

Plant material and preparation of S. officinalis tea

Salvia officinalis L. plants were grown in an experimental farm located in Arouca, Portugal, and were collected in April, 2001. The aerial parts of plants were lyophilised and kept at -20ºC. The sage tea was routinely prepared as in previous studies performed with rats. Briefly, 300 ml of boiling water were poured onto 4 g dried plant material and allowed it to steep for 5 min (Lima *et al*., 2005). This preparation usually produces a 3.5 ± 0.1 mg extract dry weight per ml infusion, with rosmarinic acid (362 mg/ml infusion) and luteolin 7-glucoside (115.3mg/ml infusion) as major phenolic compounds, and 1,8-cineole, *cis*-thujone, *trans*-thujone, camphor and borneol as the major volatile components (4.8 mg/ml infusion; Lima *et al*., 2005).

Oral glucose tolerance test

Oral glucose tolerance tests (OGTTs) were performed by measuring the concentration of blood glucose before and at 45 and 165 min after an oral glucose solution administration. The test dose used was 1 g of glucose monohydrate per Kg body weight of volunteer. The dose was given in up to 300 ml of warm water. These OGTTs were carried out after an overnight fast at the baseline phase and the fourth week of sage tea treatment. The glucose solution was consumed by the volunteers within 5 min of start. The OGGT started when the subjects began to drink.

Preparation of blood samples and erytrocyte's hemolysates for the different parameters measurement

Venous blood samples were postprandial collected in EDTA vacutainers. Immediately, the blood was centrifuged at 200 × g (KUBOTA 2100, Tokyo, Japan) for 10 min to separate the plasma. Then, the plasma was stored at -80ºC in aliquots until use and the remaining erytrocyte's fraction was washed three times in cold saline solution (NaCl 0.9%). Then, 2 ml of ice-cold distilled water was added (1:4) to produce hemolysates, which were then frozen at -80ºC for later analysis.

Blood and plasma glucose measurement

Blood glucose levels were measured with the Accutrend® GCT device (Roche diagnostics GmbH, Mannheim, Germany) using Accutrend® test strips for glucose (Roche diagnostics GmbH, Mannheim, Germany), in the baseline and treatment study phases.

The amount of glucose in the plasma was measured in all study phases using a colorimetric enzymatic method – Glucofix (A. Menarini Diagnostics, Firenze, Italy) – following manufacturer's specifications.

Plasma aminotransferases activities

The alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were measured spectrophotometrically in plasma following the NADH oxidation method (at 30ºC) at 340 nm on a plate reader (Spectra Max 340pc, Molecular Devices, Sunnyvale, CA, USA), as previously described by Lima *et al.* (2005).

Plasma total cholesterol, low-density lipoprotein and high-density lipoprotein cholesterol levels

Total plasma cholesterol, LDL cholesterol and HDL cholesterol levels were measured in plasma using spectrophotometric commercial kits from Spinreact (Girona, Spain), according to manufacturer's specifications. The commercial kits used were the following: total cholesterol (Cat. #41021), LDL cholesterol (Cat. #41023) and HDL cholesterol (Cat. #1001096).

Superoxide dismutase and catalase activities in haemolysed erythrocytes

The determination of superoxide dismutase (SOD) activity in haemolysed erythrocytes was performed using the kit Ransod (Randox, Crumlin, UK) following manufacturer's specifications. The method uses xanthine and xanthine oxidase to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye. The activity of superoxide dismutase in our sample was measured by

the degree of inhibition of this reaction. SOD activity was expressed as U/ml (1U being 50% of inhibition of INT reduction under assay conditions).

The same haemolysates were used to measure catalase (CAT) activity as described elsewhere (Aebi, 1984). In brief, the decomposition of H_2O_2 was followed at 240 nm in a spectrophotometer (Cary IE, UV-Visible Spectrophotometer Varian, Australia) and the activity expressed as U/ml (U being μmol of H_2O_2 decomposed per minute) using the molar extinction coefficient of 0.0394 $ml.\mu mol^{-1}.cm^{-1}$.

Statistical Analysis

Data are expressed as means ± standard errors of the means (SEM). For statistical analysis of OGTT results, a two-way ANOVA was employed followed by the *Bonferroni test* (GraphPad Prism, version 4.03; GraphPad Software Inc., San Diego, CA, USA). The remaining parameters were analysed by repeated one-way ANOVA measurements followed by the Student-Newman-Keuls. *P* values ≤ 0.05 were considered statistically significant.

5.3.3. Results

In order to evaluate the safety of *S. officinalis* tea drinking in humans (no hepatotoxic effects of this tea were observed in mice, Lima *et al*., 2005), plasma aminotransferase activities (AST and ALT) were determined. These two enzymes are present in cells of several organs, particularly in hepatocytes (liver). When the liver is injured, the plasma levels of these two enzymes increase, which indicates hepatotoxicity. As shown in **Table 1**, at 4th week of treatment (T4) with sage tea, an increase in plasma activity of both enzymes was observed. However, this increase was only significant for AST enzyme. Despite these results, toxicity did not occur as shown by the results well below the reference values (**Table 1**).

Table 1 – Enzymatic activities of ALT and AST in the plasma, quantified throughout the different phases of the assay.

Enzimatic activities (IU/L)	Baseline	Treatment 2 (T2)	Treatment 4 (T4)	Wash-out	Reference values (IU/L)
ALT	7.3 ± 1.0	6.8 ± 1.4	8.4 ± 1.6	7.6 ± 1.5	< 40[a]
AST	8.1 ± 1.1	10.0 ± 2.0	10.6 ± 1.8*	9.8 ± 1.2	< 40[a]

Values are mean ± SEM (n=6). Statistical differences were assessed by one-way ANOVA followed by a Student-Newman-Keuls test. *$P \leq 0.05$ when compared with baseline values. [a]Upper Limit of the Reference Range (ULRR).

The antidiabetic properties of *S. officinalis* tea were evaluated by analysing blood and plasma glucose concentrations.

Fasting glycaemia increased on the 2nd week of treatment (T2), although on the 4th week of treatment glycaemic values were smaller than those obtained in baseline (**Table 2**).

During the course of the trial, postprandial blood and plasma glucose levels were similar. A slight decrease on the T2 followed by an increase on the T4 was observed. The glycaemic values observed in wash-out were smaller than those of baseline (**Table 2**). No significant differences in glycaemia were observed between the different phases of the study.

Table 2 – Fasting and postprandial glucose levels in blood and plasma quantified throughout the different phases of the study.

Glucose levels (mg/dL)	Baseline	Treatment 2 (T2)	Treatment 4 (T4)	Wash-out	Reference values (mg/dL)*
Fasting[a]	77.5 ± 21.2	82.8 ± 16.5	75.8 ± 27.7	---	70 - 110
Postprandial[a]	96.0 ± 29.5	78.3 ± 9.6	87.8 ± 16.9	82.5 ± 16.2	< 120
Postprandial[b]	120.6 ± 41.2	105.3 ± 2.9	118.5 ± 25.4	111.3 ± 23.8	< 140

Values are mean ± SEM (n=6). Statistical differences were assessed by one-way ANOVA followed by a Student-Newman-Keuls test. [a]Blood glucose concentration; [b]Plasma glucose concentrations. *According to Ravel (1998).

Performing OGTTs it is possible to assess the predisposition of the volunteers for the development of diabetes, namely if their organism quickly restores the basal level of glucose in the blood or if they show glucose intolerance. According to the results presented in **Fig. 2**, the volunteers had no glucose intolerance and no changes in glucose clearance were observed in the OGTTs at the end of sage treatment period.

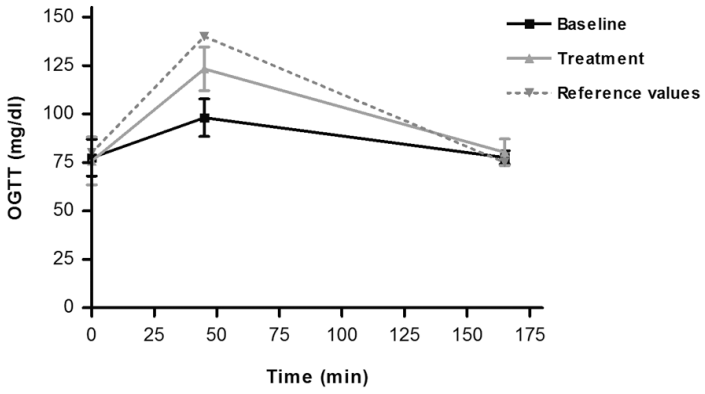

Fig. 2. Blood glucose concentration in response to an OGTT. The dash line corresponds to the typical response of a non-diabetic individual to the standard OGTT (75 g glucose/300ml water). Values are mean ± SEM (n=5). A two-way ANOVA was employed and no significant differences were obtained.

A wide range of cardiovascular complications are associated with diabetes. In order to prevent the development of these complications it is important not only to manage the plasma glucose concentration but also a good control on the lipid profile, namely of cholesterol levels. In this study the levels of lipid parameters – total cholesterol, LDL and HDL cholesterol – were determined in the plasma of the volunteers.

As shown in **Fig. 3A** sage tea treatment reduced plasma total cholesterol levels (by 22.9% in phase T2 and by 5.3% in phase T4). These effects still present two weeks after sage treatment (16%) lower than at baseline. The less effective reduction of the cholesterol level in the phase T4 was probably due to variations in diet composition, which could have interfered with these values. Despite this variation, the results obtained in wash-out period indicate a beneficial effect that was supported by the lipoproteins levels, where a gradual reduction of LDL (of 12.4% at the end of the treatment and 19.6% in the wash-out; **Fig. 3B**) and a gradual increase of HDL levels in plasma were observed (of 35.5% at T2 phase, 50.6% at the end of the treatment (T4 phase) and 37.6% in the wash-out; **Fig. 3C**).

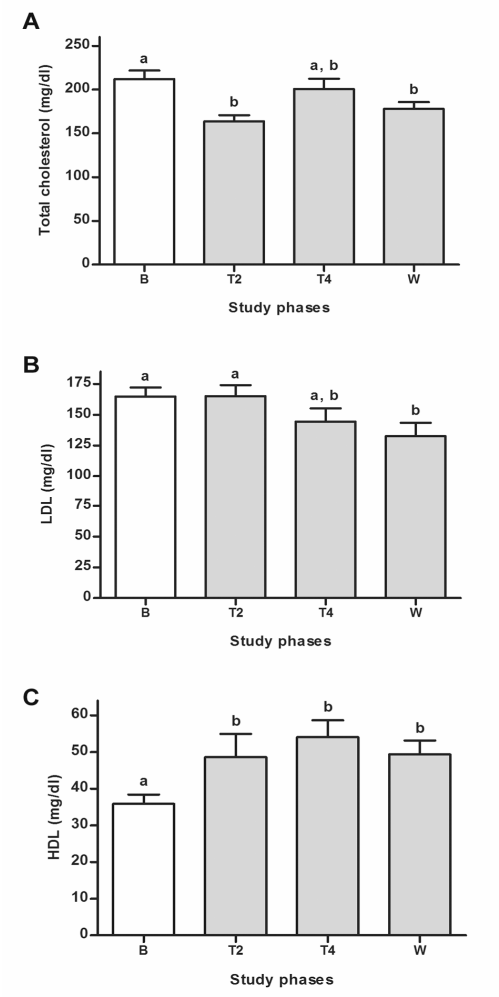

Fig. 3. Total cholesterol [A], LDL cholesterol [B] and HDL cholesterol [C] levels in plasma measured at different points during the study: baseline (B), second (T2) and fourth week of treatment (T4) and wash-out (W). Results are expressed as mean ± SEM (n=6). Statistical differences were assessed by One-way ANOVA followed by a Student-Newman-Keuls. Groups with the same letter notation are not significantly different from each other ($P > 0.05$).

Effects on antioxidant enzyme activity are also important to explore the full antidiabetic potential of plants and/or natural compounds. Recent reports have emphasised the involvement of oxidative stress in the development of cardiovascular complications associated with diabetes. Therefore, the activities of superoxide dismutase and catalase were measured, and as shown in **Fig. 4**, both SOD and CAT activities significantly increased after two weeks of sage treatment, decreasing afterwards to normal values.

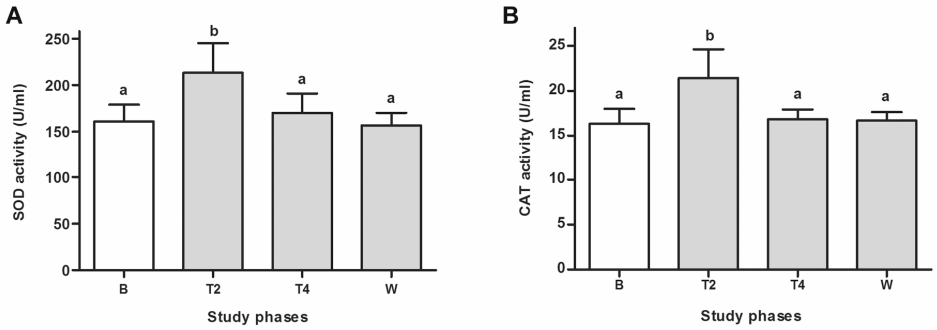

Fig. 4. Antioxidant activities of SOD [A] and CAT [B] measured in haemolysed erythrocytes. Samples were taken at different time points during the study: baseline (B), second (T2) and fourth week of treatment (T4) and wash-out (W). The results are expressed as mean ± SEM (n=5). Statistical differences were assessed by One-way ANOVA followed by a Student-Newman-Keuls. Groups with the same letter notation are not significantly different from each other ($P > 0.05$).

5.3.4 Discussion and conclusions

Beneficial effects of sage tea drinking on parameters relevant to diabetes and its associated cardiovascular complications were assessed in the present study, with volunteers in an age group at risk. The results show that drinking *S. officinalis* tea did not induce hepatotoxicity or other adverse effects, including on blood pressure, heart rate at rest and body weight. Considering that blood glucose levels were not affected, sage tea treatment does not seem to change the normal homeostatic mechanisms of glucose regulation, and hypoglycaemias are not likely, which supports the idea that sage tea drinking is safe. In addition to these results, sage tea was effective in the improvement of lipid profile and the antioxidant defences of the volunteers, which in the long term may be responsible for the general health improving effects of sage.

Two OGTT were performed, one before the sage tea treatment phase and the other at the end (four weeks after the first one), in order to assess if sage tea improved the mechanisms of glucose clearance. Although the volunteers in this trial represented a group of risk for the development of T2DM, the OGTT results show that they are not diabetics or glucose intolerant. No effects of the sage tea were detected on glucose metabolism.

The presence of plasma aminotransferases ALT and AST is considered indicator of liver injury (Sacher & McPherson, 2002). ALT is a cytosolic enzyme particularly abundant in the liver,

whereas AST can be found in the cytosol or mitochondria of cells from several organs (Green & Flamm, 2002). Despite slight variations of plasma aminotransferases activities through the trial, the values remained well below of the indicative level of hepatotoxicity (upper limit of the reference range: 40IU/L; Kim *et al*., 2004; Jamal *et al*., 1999). Thus, these results show that drinking sage tea does not lead to hepatotoxicity, which is in agreement with studies performed in our laboratory with rats (Lima *et al*., 2005).

High levels of low-density lipoproteins (LDL) and low levels of high-density lipoproteins (HDL) cholesterol are the major risk factors for the development of cardiovascular diseases. A variety of approaches have been developed in order to achieve both decrease LDL and rise HDL levels, with the aim to reduce the risk for cardiovascular diseases (Toth, 2005). Despite the available therapies based on statins, niacin and fibrates (pharmacological agents used to lower plasma LDL and increase HDL levels although cause several side effects), alternative compounds from natural plants have been searched. Several natural compounds have been shown to act through cholesterol metabolism (by reducing its absorption or its synthesis), such as phytosterols and catechins (Plana *et al*., 2008; Raederstorff *et al*., 2003). In this context, we also determined if sage tea could improve cholesterol levels free of side effects (such as hepatotoxicity and hyperglycaemia). Our results showed a gradual and significant HDL increase (of 50.6% at the end of the treatment and of 37.6% after two weeks wash-out) which seems more efficient than the drugs in current use (e.g., statins cause a HDL increase of 5-15% and niacin of 15-35%; Toth, 2005). A gradual LDL decrease (by 12.4% at the end of the treatment and 19.6% in the wash-out) was also observed. These results suggest that *S. officinalis* is accountable for an improvement of the lipid profile and therefore can contribute positively to the control of both hyperglycaemia and dyslipidaemia observed in T2DM.

Additional health benefits from the use of plant extracts may be related to the fact that they are good sources of antioxidants. According to Lamaison and collaborators (1990), some species of the genus *Salvia* (namely *S. officinalis*, *S. fruticosa*, *S. lavandulifolia* and *S. sclarea*) showed a significant antioxidant activity of hydroalcohol extracts on 1,1- diphenyl-2-picrylhydrazyl (DPPH). In particular with regard to the liver, in addition to not being toxic *S. officinalis* tea improved liver glutathione levels (Lima *et al*., 2005). Among other effects, this seems to indirectly improve the liver-mediated insulin response *in vivo* (Guarino *et al*., 2003). The enzymes SOD and CAT are important in protecting the cells against oxidative damage (Matés *et al*., 2000). Celik & Isik (2008) showed that the drinking of *S. officinalis* water infusion (tea) was efficient in reducing the oxidative damage induced by trichloroacetic acid in the liver, kidney and brain tissues of rats, which agreed with the improved antioxidant defences as demonstrated by the activities of SOD and CAT in these tissues. In the present study, *S. officinalis* tea drinking showed to improve human erythrocyte antioxidant status by increasing the activities of these two antioxidant enzymes. Some sage species have been shown to be effective in the prevention of

cardiovascular diseases, and a possible mechanism may be their effort on the prevention of LDL oxidation, thus inhibiting atherosclerosis (Chen *et al*., 2001).

In conclusion, our results support the popular believe that *S. officinalis* tea is safe and can contribute for an improvement of the individual health condition, also useful to diabetic patients although no effect on glucose clearance were observed. This pilot trial showed no adverse effects associated with sage tea drinking. The effects on antioxidant defences as well as on plasma lipid profile are beneficial in diabetes management and may have broader applications.

Acknowledgements

I would like to thank the people that were involved with the pilot study, including all the volunteers and Carla Sá for her contribution to the experimental work.

I also would like to thank Prof. Olga Coutinho for her help with the study design and the informed consent application, the nurse Rui Silva for assisting with blood sample collection and Prof. Manuel Fernandes-Ferreira for kindly providing the plant material used in this study.

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Chapter 6

Final considerations

6.1. General discussion and conclusions

Type 2 diabetes mellitus is a metabolic disease attaining epidemic proportions mainly due to the modern lifestyle and increasing incidence of obesity, which constitutes a main threat to human health in the 21st century. There is a growing interest in strategies based in natural products that can be both effective free of side effects and applicable to prevention strategies.

In preliminary *in vivo* studies using healthy mice and rats, the effects of *S. officinalis* and *S. fruticosa* tea on glucose clearance were determined by intraperitoneal glucose tolerance tests (GTT). The results showed that *S. officinalis* tea was not effective on the mechanisms of glucose clearance, although it lowered the fasting plasma glucose levels in mice, suggesting effects on liver gluconeogenesis (Lima *et al*., 2006). Similarly, *S. fruticosa* tea was not directly effective on glucose clearance. However, since the intraperitoneal GTT bypasses intestinal absorption, it does not reveal effects at intestinal level, which may still be present. Taking this into account, the major studies reported in this thesis focused on the antidiabetic effects of sage extracts on the small intestine (although in some studies pancreas and liver were also used). Since sage plants are mostly consumed orally, prepared in the same way as teas, studies at the level of the gastrointestinal tract make sense.

Firstly, antidiabetic activities of *S. fruticosa* tea at the small intestinal level were assessed in healthy and streptozotocin (STZ)-induced diabetic rats (chapter 2). *S. fruticosa* tea drinking for 14 days showed to be beneficial in the control of glycaemia since it stabilised fasting blood glucose levels in STZ-induced diabetic rats. This effect of sage tea was accompanied by a significant reduction of the diabetes associated increase in the expression of Na+/glucose cotransporter 1 (SGLT1) on the brush-border membrane (BBM) of rat enterocytes. No effects on insulin or islet regeneration were observed. The hormone GLP-1 is one of the incretins responsible for the glucose-induced insulin secretion by the pancreatic β-cells. In the experiment performed with diabetic animals (chapter 2) the number of enteroendocrine L-cells, the intensity of GLP-1 signal in these cells, as well as plasma insulin concentration were also determined. The results demonstrated that diabetic rats treated with sage tea showed smaller decrease in plasma insulin concentration, although no effects were observed on GLP-1 immunoreactive cells. Since sage plants are rich in antioxidant compounds, we suppose that this effect on plasma insulin could be due to an antioxidant protection from glucotoxicity in β-cells conferred by sage compounds.

The effect of *S. fruticosa* tea on SGLT1 expression in the BBM of enterocytes obtained in diabetic rats was corroborated by another *in vivo* study using healthy rats but where the adaptive increase of SGLT1 expression was manipulated by diet (chapter 3). In an *in vivo*

experiment, rats were subjected to low-carbohydrate (LC) diet to down-regulate the expression of SGLT1, and when the high-carbohydrate (HC) diet was reintroduced for 4 days an up-regulation on this sugar transporter took place. Treating the animals with *S. fruticosa* tea or rosmarinic acid (RA), instead of water, during the 4 days of HC diet reintroduction, a significant reduction on the adaptive increase of SGLT1 expression was observed. With this experiment we not only confirm that the effect of *S. fruticosa* tea is achieved when the expression of the glucose cotransporter SGLT1 is up-regulated (by diabetes or dietary regulation) but we also showed that RA is a compound present in this tea responsible for this effect.

Effects on GLUT2 expression were also assessed in both experiments (chapter 2 and 3) and we verified that induction of diabetes significantly increased GLUT2 expression in intestinal epithelial cells, although no effects were obtained with dietary manipulation. However, sage tea was not able to reduce the diabetes-induced GLUT2 expression, leading us to admit that at the level of intestinal glucose transporters, only effects on SGLT1 may have contributed for the stabilisation of blood glucose levels of diabetic animals by sage tea.

In order to shed some light on the molecular mechanisms behind the effects of *S. fruticosa* and RA we showed the likely involvement of the heat shock protein 70 (Hsp70) and protein kinase C (PKC) in the expression of SGLT1 in the BBM. The expression of Hsp70 was decreased by *S. fruticosa* tea treatment in a similar manner to the expression of SGLT1 in both experiments (with STZ-diabetic rats (chapter 2) and with healthy rats subjected to dietary manipulation; chapter 3). A similar but more significant result was obtained for RA than sage's tea effect, under dietary manipulation. In what concerns effects on PKC expression, although sage tea only slightly affected PKC expression in enterocytes, RA significantly decreased its expression (chapter 3). All of these results seem to indicate that *S. fruticosa* tea treatment and particularly RA may be producing their effects by interfering with the pathways of translocation of vesicles containing SGLT1 from intracellular stores to the BBM of the enterocytes. This possibility is supported by Ikari *et al*. (2002) and Wright *et al*. (1997) studies that show an involvement of these proteins with vesicle translocation and SGLT1 expression and activity in the apical membrane of renal LLC-PK(1) cells and *Xenopus laevis* oocytes. Sage tea does not seems to affect the transcriptional regulation of SGLT1 gene, since according to preliminary results no differences were obtained by RT-PCR in SGLT1 mRNA between water and sage tea treated animals.

The expression of the basolateral Na^+/K^+-ATPase protein, a pump that maintains the Na^+ gradient, was also measured in the experiments of both chapter 2 and 3. The results showed that a significant increase on Na^+/K^+-ATPase expression was obtained in the intestinal epithelium of diabetic animals (in agreement with the literature) and sage tea did not produce any effect at this level (chapter 2). This indicates that effects on Na^+/K^+-ATPase expression do not underlie the observed effects of sage tea on diabetic SGLT1 expression in the BBM and

glucose transport regulation. In chapter 3, Na^+/K^+-ATPase expression significantly decreased with LC diet and after the reintroduction of HC diet a tendency to achieve the normal levels of this protein was observed for all the treatment groups. Sage tea and RA did not affect significantly the expression of Na^+/K^+-ATPase, indicating that there is no relationship between this protein and the inhibitory effect of sage and RA on the diet-induced increase of SGLT1 in BBM.

Also at the level of the gastrointestinal tract, the effects of the different sage water extracts (teas) and some of their individual compounds on carbohydrate digestion were also assessed in order to ensure that the previous effects on SGLT1 expression were not due to smaller amounts of glucose in the lumen as a result of effects on carbohydrate digestion (chapter 4). The antidiabetic properties of sage seem not to be due to effects at this level, at least as shown by the *in vitro* results on alpha-amylase activity. With the aim to evaluate *in vivo* antidiabetic effects of two sage compounds (ursolic acid (UA) and luteolin-7-glucoside (L7G)) tested on alpha-amylase assay, a study was carried out with rats. This study revealed that both UA and L7G, major constituents of this plant genus, may be useful in the amelioration of the metabolic deregulation associated with diabetes (by decreasing plasma glucose levels, and particularly UA by increasing liver glycogen deposition) and dyslipidaemia (by increasing HDL cholesterol and at the same time decreasing LDL cholesterol levels). Moreover, this beneficial effect on the improvement of lipid profile obtained for both of these sage compounds in rats were corroborated by the results obtained in the pilot trial with humans (chapter 5). In fact, female volunteers showed lower levels of plasma total cholesterol and LDL and simultaneously higher levels of HDL, at the end of sage tea treatment period, which constitutes a beneficial effect on the prevention of cardiovascular diseases. In addition to these results, an improvement of antioxidant defences was observed in these healthy volunteers, as demonstrated by the levels of the antioxidant enzymes (SOD and CAT) activities. In this chapter it was also suggested that *S. officinalis* tea can be useful as a food supplement in the prevention of T2DM, particularly by its potential capacity to lower plasma glucose of individuals at risk. This preventive potential was supported by the results obtained from an *in vivo* study done with healthy and diabetic rats (treated with *S. officinalis* tea or water), also described and discussed in chapter 5. The results form this study, particularly those of healthy animals, showed that *S. officinalis* tea was effective in lowering fasting glucose levels and decreasing hepatic gluconeogenesis, like metformin effects – a common oral pharmaceutical agent used in the treatment of T2DM.

Both of these sage species and their water extracts (teas) are rich in antioxidant compounds that can contribute to the control of glycaemia and probably to the effects obtained on the improvement of lipid profile. Chronic hyperglycaemia leads to glucotoxicity that is associated with an increase in the production of free radicals and consequent oxidative stress. This process is responsible for cellular damages that may result in the development of vascular

complications and β-cell failure. The use of antioxidants has been shown to reduce oxidative cell damage, improving β-cell function and reducing the risk to develop T2DM or its associated complications.

In conclusion, the results presented in this thesis contributed to confirm and clarify the antidiabetic properties of these sage species. The studies performed emphasised the importance of the small intestine as a target for antidiabetic therapeutical interventions, and identified a novel mechanism for the control of hyperglycaemia by sage – modulation of expression of SGLT1. Moreover, these studies also confirm that sage plants and particularly some of their individual constituents have beneficial effect in the improvement of plasma lipid profile which may be particularly relevant in the prevention of cardiovascular complications.

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6.2. Future perspectives

The studies enclosed in this thesis were carried out in order to confirm the antidiabetic potential of two sage species, and identify mechanisms of action and active principles. Rosmarinic acid (RA) showed to be the active principle of this tea, responsible for *S. fruticosa* effects. These effects on SGLT1 expression seem to be through posttranscriptional mechanisms that involve Hsp70 as well as PKC. However, long-term effect may also involve regulation of intestinal epithelium cell proliferation.

The effects of sage tea treatment and RA on insulin signalling pathway should be clarified, through more specific studies on molecular targets, such as PI3K/Akt and on different PKC isoforms, using both *in vivo* and *in vitro* models (such as enterocyte differentiated Caco-2 and HT-29 cell lines). Using these cell line models, insulin and glucagon roles on SGLT1 expression will be characterised in addition to sage extracts and their individual constituents. The use of tools such as activators or inhibitors of PI3K, PKC, etc. should be exploited in the near future. Also the involvement of Hsp70 in translocation of SGLT1 containing vesicles should be assessed in the same cells.

Should an insulin-sensitizing mechanism be confirmed at the enterocyte level, studies on other cell types will be desirable to further reveal the full antidiabetic potential of these sage compounds.

Despite preliminary results that showed that the produced effect of sage tea on SGLT1 does not seem to be a result of effects on mRNA levels (gene transcription), we can not exclude completely this possibility, and this question should be answered in the near future by analysing the effects of *S. fruticosa* tea and RA treatment in the amount of mRNA by RT-PCR, in the referred intestinal cell lines and in tissue samples from *in vivo* experiments.

Also characterisation of the long-term effects of sage constituents on intestinal epithelium proliferation (PCNA expression) and apoptosis (TUNEL assay) should be carried out using proliferation (e.g. PCNA, Ki67), differentiation (e.g. DCT1, p21 and c-MYC) and migration markers (e.g. ephrin) by immunohistochemistry techniques. The involvement of GLP-2 should also be assessed and certainly will be a good complement to the results obtained with the other markers of enterocyte proliferation.

Additionally, studies at the level of the kidney are also important in order to understand effects of these plant extracts on the control of SGLT1 expression, due to its role on glucose reabsorption.

Finally, it is important to confirm the effects of individual compounds in animal models of insulin resistance (Zucker and Goto-Kakizaki rats).

The results presented in this thesis together with those provided by future work will certainly identify new compounds for use by the pharmaceutical industry as novel antidiabetic agents, as well as indicate preventive nutritional strategies that help contain the epidemic growth of diabetes.

Este trabalho foi co-financiado pelo “Programa Operacional Ciência e Inovação 2010” de Portugal e pelo “Fundo Social Europeu”.

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